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Comparative infectivity of *Plasmodium falciparum* to *Anopheles albimanus* and *Anopheles stephensi*

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"*Et in Arcadia ego.*"

- *Arcadia*, Tom Stoppard

"Lydgate was enamoured; he longed to demonstrate the more intimate relations of living structure and help to define men's thought more accurately after the true order."

- *Middlemarch*, George Eliot

"'...you're going to start getting closer and closer – that is, if you *want* to, and if you look for it and wait for it – to the kind of information that will be very, very dear to your heart. Among other things, you'll find that you're not the first person who was ever confused and frightened and even sickened by human behaviour. You're by no means alone on that score, you'll be excited and *stimulated* to know. Many, many men have been just as troubled morally and spiritually as you are right now. Happily, some of them kept records of their troubles. You'll learn from them – if you want to. Just as some day, if you have something to offer, someone will learn something from you. It's a beautiful reciprocal arrangement. And it isn't education. It's history. It's poetry.'"

- *The Catcher in the Rye*, J.D. Salinger.

Contents

TABLE OF FIGURES ..... **VII**

TABLE OF TABLES ..... **XI**

ACKNOWLEDGEMENTS ..... **XIII**

DECLARATION ..... **XIV**

ABSTRACT **1**

CHAPTER 1. GENERAL INTRODUCTION **2**

1. 1 INTRODUCTION 2
1. 2 OVERVIEW OF THE MALARIA PARASITE LIFE CYCLE 3
1. 3 GAMETOCYTOGENESIS: PREPARATION FOR TRANSMISSION TO THE MOSQUITO VECTOR 5
1. 4 MALARIA PARASITE DEVELOPMENT WITHIN THE MOSQUITO VECTOR 7
1.4.1 Gametogenesis *7*
1.4.2 Fertilisation & zygote formation *10*
1.4.3 Zygote differentiation into the ookinete *11*
1.4.4 Ookinete migration through the bloodmeal *14*
1.4.5 Ookinete penetration of the peritrophic matrix *16*
1.4.6 Ookinete migration through the microvilli-associated network *17*
1.4.7 Ookinete invasion of the midgut epithelium *18*
1.4.8 Sporogony: oocyst formation, growth & differentiation *23*
1.4.9 Sporozoite migration to the salivary glands *27*
1.4.10 Sporozoite invasion of the salivary glands *28*
1. 5 FACTORS AFFECTING MALARIA PARASITE INFECTION OF THE MOSQUITO VECTOR 32
1.5.1 Vertebrate host factors *32*
1.5.1.1 Components of the immune system 32
1.5.1.2 Asexual erythrocytic stage malaria parasites 35
1.5.2 Mosquito factors *36*
1.5.2.1 Gametogenesis and fertilization 36
1.5.2.2 Midgut digestive proteases 37
1.5.2.3 The peritrophic matrix 40
1.5.2.4 The midgut epithelium 40
1.5.2.5 Immune responses 41
1.5.3 Malaria parasite factors *45*
1. 6 SUMMARY 46

CHAPTER 2. COMPARATIVE INFECTIVITY OF DIFFERENT *P. FALCIPARUM* CLONES TO *AN. ALBIMANUS* AND *AN. STEPHENSI* **47**

2. 1 INTRODUCTION 47
2.1.1 Susceptibility of An. albimanus to Plasmodium infection *47*
2.1.1.1 Human malaria parasite species 47
2.1.1.2 Non-human primate malaria parasite species 52
2.1.1.3 Avian and rodent malaria parasite species 53
2.1.2 Susceptibility of An. stephensi to Plasmodium infection *54*
2.1.3 Comparative susceptibility of An. albimanus and An. stephensi to Plasmodium infection *55*
2.1.4 Summary *55*
2. 2 MATERIALS & METHODS 56
2.2.1 In vitro culture of P. falciparum *56*
2.2.1.1 P. falciparum clones 56
2.2.1.2 Long-term storage of parasite stocks 57
2.2.1.3 In vitro culture of asexual erythrocytic stage parasites 57
2.2.1.4 In vitro culture of sexual erythrocytic stage parasites 58
2.2.2 Maintenance of mosquito colonies *59*
2.2.2.1 Mosquito species 59
2.2.2.2 Insectary conditions and rearing of mosquitoes 59
2.2.3 Infection of mosquitoes with malaria parasites *60*
2.2.3.1 Collection of adult female mosquitoes 60
2.2.3.2 Membrane-feeding mosquitoes cultured gametocytes 60
2.2.3.3 Mosquito dissection & microscopic determination of oocyst infection 61
2.2.4 Statistical analysis *61*
2. 3 RESULTS 63
2.3.1 Comparison of the infectivty of the P. falciparum 3D7A clone to An. albimanus and An. stephensi *63*
2.3.2 Comparison of the infectivty of the P. falciparum HB3B-B2 clone to An. albimanus and An. stephensi *63*
2.3.3 Comparison of the infectivity of the P. falciparum 7G8 clone to An. albimanus and An. stephensi *66*
2. 4 DISCUSSION 66
2. 5 SUMMARY 69

CHAPTER 3. DIRECT IMMUNOFLUORESCENCE MICROSCOPY OF THE DEVELOPMENT OF THE *P. FALCIPARUM* 3D7A CLONE IN *AN. ALBIMANUS* AND *AN. STEPHENSI* **70**

3. 1 INTRODUCTION 70
3.1.1 Previous studies of Plasmodium development in An. albimanus *70*
3.1.2 Techniques for investigating Plasmodium development in the mosquito *74*
3. 2 MATERIALS & METHODS 76
3.2.1 Parasite culture & mosquito infection *76*
3.2.2 Direct immunofluorescence microscopy *76*

3.2.2.1 Malaria parasite development within the bloodmeal 77
3.2.2.2 Ookinete invasion of the midgut epithelium 79
3.2.2.3 Early oocyst formation 80
3.2.3 Estimation of total protein content per midgut 80
3.2.4 Estimation of malaria parasite survival between different life-cycle stages 81
3.2.5 Statistical analysis 81
3.2.5.1 Comparison of the levels of *P. falciparum* 3D7A infection 82
3.2.5.2 Comparison of total protein content per midgut between mosquito species 83
3.3 RESULTS 83
3.3.1 Comparison of P. falciparum 3D7A development in An. albimanus and An. stephensi 83
3.3.1.1 P. falciparum 3D7A development within the bloodmeal 83
3.3.1.2 P. falciparum 3D7A ookinete invasion of the midgut epithelium 95
3.3.1.3 P. falciparum 3D7A early oocyst formation 97
3.3.2 Population dynamics of P. falciparum 3D7A development 101
3.3.2.1 P. falciparum 3D7A stage-specific survival in *An. albimanus* 101
3.3.2.2 P. falciparum 3D7A stage-specific survival in *An. stephensi* 104
3.4 DISCUSSION 107
3.4.1 P. falciparum 3D7A development in An. albimanus 107
3.5 SUMMARY 110

CHAPTER 4. HISTOLOGICAL INVESTIGATION OF THE DEVELOPMENT OF THE *P. FALCIPARUM* 3D7A CLONE IN *AN. ALBIMANUS* AND *AN. STEPHENSI* 111

4.1 INTRODUCTION 111
4.2 MATERIALS & METHODS 115
4.2.1 Parasite culture and mosquito infection 115
4.2.2 Preparation and examination of histological sections 115
4.2.3 Statistical analysis 116
4.2.3.1 Comparison of the levels of *P. falciparum* 3D7A infection 116
4.2.3.2 Comparison of morphologically-abnormal midgut epithelial cells 116
4.3 RESULTS 117
4.3.1 Histological description of the mosquito midgut during bloodmeal digestion 118
4.3.1.1 Structure of the mosquito midgut 118
4.3.1.2 Comparison of the midguts of *An. albimanus* and *An. stephensi* 123
4.3.2 Comparison of P. falciparum 3D7A development in An. albimanus and An. stephensi 124
4.3.2.1 An. albimanus fed *P. falciparum* 3D7A gametocytes 124
4.3.2.2 An. stephensi fed *P. falciparum* 3D7A gametocytes 125
4.3.2.3 Quantitative comparison of the level of *P. falciparum* 3D7A infection in *An. albimanus* and *An. stephensi* 129
4.3.2.4 Population dynamics of *P. falciparum* 3D7A development within *An. albimanus* and *An. stephensi* 129
4.3.3 Detailed histological description of P. falciparum 3D7A infection of the An. stephensi midgut epithelium 135
4.3.3.1 Morphological description of *P. falciparum* 3D7A parasites 135

4.3.3.2 Ookinete penetration of the peritrophic matrix 137
4.3.3.3 Ookinete entry into the midgut epithelium 138
4.3.3.4 Ookinete migration through the midgut epithelium 140
4.3.3.5 Oocysts on the basal surface of the midgut epithelium 146
4.3.4 Detailed histological description of the morphologically-abnormal midgut epithelial cells 148
4.3.4.1 An. stephensi fed *P. falciparum* 3D7A gametocytes 148
4.3.4.2 An. stephensi fed uninfected or non-infectious *P. falciparum* 3D7A-infected blood 152
4.3.5 Temporal dynamics of morphologically-abnormal midgut epithelial cells 152
4.3.5.1 An. stephensi fed *P. falciparum* 3D7A gametocytes 152
4.3.5.2 An. stephensi fed uninfected or non-infectious *P. falciparum* 3D7A-infected blood 153
4.3.6 Quantitative relationships between malaria parasites invading the midgut epithelium and the occurrence of morphologically-abnormal midgut epithelial cells 153
4.3.6.1 An. stephensi fed *P. falciparum* 3D7A gametocytes 153
4.3.6.2 Comparison of *An. stephensi* fed uninfected and *P. falciparum* 3D7A-infected, non-infectious and infectious, blood 153
4. 4 DISCUSSION 155
4.4.1 P. falciparum 3D7A infection in An. albimanus 155
4.4.2 P. falciparum 3D7A infection in An. stephensi 156
4.4.2.1 The route of ookinete invasion across the midgut epithelium 156
4.4.2.2 Effect of ookinete invasion on midgut epithelial cells 161
4.4.3 Significance of morphologically-abnormal midgut epithelial cells 164
4. 5 SUMMARY 165

CHAPTER 5. PROLIFERATIVE REGENERATION OF THE *AN. STEPHENSI* MIDGUT EPITHELIUM FOLLOWING *P. FALCIPARUM* OOKINETE INVASION 216

5. 1 INTRODUCTION 216
5. 2 MATERIALS & METHODS 218
5.2.1 Parasite culture & mosquito infection 218
5.2.2 Preparation & examination of histological sections 218
5.2.3 Estimation of the amount of regenerative cell division/differentiation 218
5.2.4 Detection of cell proliferation using 5-Bromo-2'-deoxyuridine incorporation 218
5.2.5 Statistical analysis 220
5. 3 RESULTS 220
5.3.1 Histological evidence for division/differentiation of regenerative cells within the midgut epithelium of mature adult female An. stephensi mosquitoes 220
5.3.2 Quantitative relationships between regenerative cell division / differentiation, P. falciparum infection and abnormal midgut epithelial cells 228
5.3.3 Immunofluorescent detection of cell proliferation using BrdUrd incorporation 238
5. 4 DISCUSSION 238
5.4.1 A model for proliferative regeneration of the mosquito midgut epithelium 238

5.4.2 P. falciparum ookinete invasion indirectly triggers proliferative regeneration of the An. stephensi midgut epithelium *242*
5.4.3 Failure of BrdUrd incorporation to detect mitotically active cells within the midgut epithelium of mature adult female An. stephensi mosquitoes *245*
5. 5 SUMMARY 247

CHAPTER 6. GENERAL DISCUSSION 248

6. 1 INTRODUCTION 248
6. 2 COMPARATIVE SUSCEPTIBILITY OF *AN. ALBIMANUS* TO *P. FALCIPARUM* INFECTION 248
6.2.1 P. falciparum 3D7A development in An. albimanus *249*
6.2.2 The mechanism of An. albimanus refractoriness to P. falciparum 3D7A oocyst infection *250*
6.2.2.1 Bloodmeal digestion 250
6.2.2.2 Immune responses and co-infecting microorganisms 253
6.2.2.3 The peritrophic matrix and the midgut epithelium 254
6. 3 POPULATION BIOLOGY OF *P. FALCIPARUM* DEVELOPMENT IN *AN. ALBIMANUS* AND *AN. STEPHENSI* 255
6.3.1 Macrogametocyte to ookinete transition (k-1) *256*
6.3.1.1 Macrogametocyte to macrogamete transition (*k*-1a) 258
6.3.1.2 Macrogamete to ookinete transition (*k*-1b) 259
6.3.2 Ookinete to oocyst transition (k-2) *261*
6.3.2.1 Ookinete migration from the bloodmeal to the basal surface of the midgut epithelium 263
6.3.2.2 Oocyst survival in *An. stephensi* 267
6. 4 OOKINETE INVASION OF THE MOSQUITO MIDGUT EPITHELIUM 268
6.4.1 A unified model of ookinete invasion of the midgut epithelium *271*
6.4.1.1 Ookinete entry into the midgut epithelium 271
6.4.1.2 Ookinete exit from the midgut epithelium 279
6.4.2 Does a "cellular treadmill" explain the route of ookinete migration across the midgut epithelium? *280*
6.4.3 Is the unified model of ookinete invasion of the midgut epithelium generally applicable? *283*
6.4.4 Explaining the century of controversy surrounding the route of ookinete migration across the midgut epithelium *288*
6.4.4.1 Small sample sizes 288
6.4.4.2 Time of sampling 289
6.4.4.3 Method of sample preparation and examination 289
6.4.4.4 The complexity and specificity of the route of ookinete migration across the midgut epithelium 290
6.4.4.5 "Eyes without feeling, feeling without sight" (or conceptual blindness) 291
6.4.5 Summary *291*
6. 5 THE CELLULAR COMPOSITION OF THE MOSQUITO MIDGUT EPITHELIUM 291
6.5.1 Midgut epithelial cells *292*

6.5.2 Endocrine cells *292*
6.5.3 Regenerative cells *294*
6.5.4 Unusual midgut epithelial cell morphologies *295*
6. 6 CONCLUSIONS 300

APPENDIX 1 ..... **301**

APPENDIX 2 ..... **303**

REFERENCES **305**

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Table of Figures

Figure 2.1 Comparison of the distributions of mature oocyst infection of three *P. falciparum* clones at day 10 post-bloodfeeding in *An. albimanus* and *An. stephensi*.....62

Figure 3.1 Direct immunofluorescence microscopy of the different developmental stages of *P. falciparum* 3D7A within the bloodmeal of *An.albimanus*.....85

Figure 3.2 The prevalence of different *P. falciparum* 3D7A developmental stages within the bloodmeals of *An. albimanus* and *An. stephensi*.....86

Figure 3.3 Comparison of the prevalence of *P. falciparum* 3D7A malaria parasites within bloodmeals from *An. albimanus* and *An. stephensi*.....87

Figure 3.4 The intensity of different *P. falciparum* 3D7A developmental stages within the bloodmeals of *An. albimanus* and *An. stephensi*.....89

Figure 3.5 Comparison of the intensity of *P. falciparum* 3D7A malaria parasites within the bloodmeals from *An. albimanus* and *An. stephensi*.....90

Figure 3.6 Comparison of the proportion and total densities of different *P. falciparum* 3D7A developmental stages within the bloodmeals of *An. albimanus* and *An. stephensi*.....92

Figure 3.7 Comparison of the rate of bloodmeal digestion and the intensities of different *P. falciparum* 3D7A developmental stages within the bloodmeals of *An. albimanus* and *An. stephensi*.....94

Figure 3.8 Direct immunofluorescence microscopy of early *P. falciparum* 3D7A oocysts on the midgut of *An. stephensi*.....98

Figure 3.9 Comparison of the distributions of early and late *P. falciparum* 3D7A oocyst infections observed in *An. albimanus* and *An. stephensi*.....102

Figure 4.1 Diagrammatic representation of the structure of the mosquito midgut wall and midgut lumen.....167

Figure 4.2 The midgut epithelium of *An. stephensi*.....168

Figure 4.3 "Honeycomb" arrangement of the midgut epithelial cells within the mosquito midgut epithelium.....169

Figure 4.4 "Open" endocrine cells within the midgut epithelium of *An. stephensi*.....170

Figure 4.5 *P. falciparum* 3D7A ookinetes within the bloodmeal and peritrophic matrix of *An. albimanus*.....171

Figure 4.6 *P. falciparum* 3D7A ookinetes within the bloodmeal of *An. stephensi*.....172

Figure 4.7 *P. falciparum* 3D7A ookinetes penetrating the peritrophic matrix in the midgut lumen of *An. stephensi*.....174

Figure 4.8 *P. falciparum* 3D7A ookinetes within the ectoperitrophic space of the midgut lumen of *An. stephensi*.....176

Figure 4.9 *P. falciparum* 3D7A ookinetes within the microvillar brush border of the apical surface of the midgut epithelium of *An. stephensi*.....177

Figure 4.10 *P. falciparum* 3D7A ookinete entering the midgut epithelium of *An. stephensi*.....178

Figure 4.11 *P. falciparum* 3D7A ookinetes entering the midgut epithelium.....179

Figure 4.12 ***P. falciparum*** **3D7A ookinete entering the midgut epithelium of** ***An. stephensi*** **.....180**

Figure 4.13 ***P. falciparum*** **3D7A ookinete within a morphologically normal** ***An. stephensi*** **midgut epithelial cell.....181**

Figure 4.14 Intracellular ***P. falciparum*** **3D7A ookinetes within morphologically-normal midgut epithelial cells in** ***An. stephensi.*** **.....182**

Figure 4.15 Intracellular ***P. falciparum*** **3D7A ookinete within a microvillated** ***An. stephensi*** **midgut epithelial cell.....183**

Figure 4.16 ***P. falciparum*** **3D7A ookinetes within midgut epithelial cells from** ***An. stephensi*** **.....184**

Figure 4.17 Intracellular ***P. falciparum*** **3D7A ookinete within a lightly staining morphologically-abnormal midgut epithelial cell protruding from the midgut epithelium into the midgut lumen of** ***An. stephensi.*** **.....185**

Figure 4.18 ***P. falciparum*** **3D7A ookinetes within "lysing" midgut epithelial cells, exhibiting unusual blue staining, in** ***An. stephensi*** **.....186**

Figure 4.19 ***P. falciparum*** **3D7A ookinetes associated with multiple morphologically-abnormal midgut epithelial cells in** ***An. stephensi.*** **.....187**

Figure 4.20 Intracellular ***P. falciparum*** **3D7A ookinete associated with multiple morphologically-abnormal midgut epithelial cells in** ***An. stephensi.*** **.....188**

Figure 4.21 Intracellular ***P. falciparum*** **3D7A ookinete associated with a large cluster of morphologically-abnormal midgut epithelial cells protruding from the midgut epithelium into the midgut lumen of** ***An. stephensi*** **.....189**

Figure 4.22 ***P. falciparum*** **3D7A ookinete emerging from the basal surface of a pale and protruding** ***An. stephensi*** **midgut epithelial cell. 190**

Figure 4.23 ***P. falciparum*** **3D7A ookinetes emerging from the basal region of morphologically-abnormal midgut epithelial cells. 191**

Figure 4.24 ***P. falciparum*** **3D7A ookinete associated with multiple morphologically-abnormal midgut epithelial cells from** ***An. stephensi.*** **.....192**

Figure 4.25 ***P. falciparum*** **3D7A ookinete emerging from the basolateral surface of a "lysing" midgut epithelial cell located within the ectoperitrophic space of the midgut lumen of** ***An. stephensi*** **.....193**

Figure 4.26 ***P. falciparum*** **3D7A ookinetes and oocysts in intercellular locations within the** ***An. stephensi*** **midgut epithelium. 194**

Figure 4.27 Intercellular ***P. falciparum*** **3D7A ookinete within the basal region of the midgut epithelium of** ***An. stephensi.*** **.....195**

Figure 4.28 Extracellular ***P. falciparum*** **3D7A ookinete in the basal region of the midgut epithelium of** ***An. stephensi*** **associated with a dark staining and flaccid morphologically-abnormal midgut epithelial cell. 196**

Figure 4.29 Extracellular ***P. falciparum*** **3D7A ookinetes in the basal region of the** ***An. stephensi*** **midgut epithelium associated with abnormal midgut epithelial cells. 197**

Figure 4.30 "Stalk-form" ***P. falciparum*** **3D7A ookinetes within the midgut epithelium of** ***An. stephensi*** **.....198**

Figure 4.31 A "stalk-form" *P. falciparum* 3D7A ookinete within the midgut epithelium of *An. stephensi*.....199

Figure 4.32 Intracellular *P. falciparum* 3D7A ookinetes of dubious morphology within midgut epithelial cells from *An. stephensi*.....200

Figure 4.33 *P. falciparum* 3D7A ookinetes within morphologically-abnormal midgut epithelial cells located within the midgut lumen and completely separated from the midgut epithelium of *An. stephensi*.....201

Figure 4.34 *P. falciparum* 3D7A oocysts associated with morphologically-abnormal midgut epithelial cells in *An. stephensi*.....202

Figure 4.35 *P. falciparum* 3D7A ookinete/oocyst associated with a morphologically-abnormal midgut epithelial cell protruding into the midgut lumen in *An. stephensi*.....203

Figure 4.36 *P. falciparum* 3D7A oocysts surrounded by morphologically-normal midgut epithelium in *An. stephensi*.....204

Figure 4.37 *P. falciparum* 3D7A ookinetes and oocysts, located on the basal surface of the midgut epithelium, situated above invaginations of the apical surface of the midgut epithelium in *An. stephensi*.....206

Figure 4.38 *P. falciparum* 3D7A ookinetes and oocysts, in the basal region of the midgut epithelium, associated with junctions where multiple adjacent midgut epithelial cells converge in *An. stephensi*.....207

Figure 4.39 Normal and lightly staining "turgid" midgut epithelial cells exhibiting various degrees of extrusion from the midgut epithelium in *An. stephensi*.....208

Figure 4.40 Dark and pale blue staining morphologically-abnormal midgut epithelial cells in *An. stephensi*.....210

Figure 4.41 Multiple adjacent morphologically-abnormal midgut epithelial cells associated with invading *P. falciparum* 3D7A parasites in *An. stephensi*.....211

Figure 4.42 Morphologically-abnormal midgut epithelial cell protruding from the midgut epithelium in *An. stephensi*.....212

Figure 4.43 Extracellular *P. falciparum* 3D7A ookinetes within the midgut lumen associated with extruded midgut epithelial cells in *An. stephensi*.....213

Figure 4.44 Temporal changes in the location within the midgut epithelium of *P. falciparum* 3D7A parasites observed in histological sections from *An. stephensi*.....214

Figure 4.45 Relationship between the number of *P. falciparum* 3D7A parasites invading the midgut epithelium and the occurrence of morphologically-abnormal midgut epithelial cells observed in histological sections from *An. stephensi*.....215

Figure 5.1 Regenerative cells within the midgut epithelium of *An. stephensi*.....222

Figure 5.2 Unusual midgut epithelial cells possessing apical cavities lined by microvilli-like structures within *An. stephensi*.....223

Figure 5.3 Unusual midgut epithelial cells possessing apical cavities found immediately adjacent to regenerative cells in *An. stephensi*.....224

Figure 5.4 Unusual midgut epithelial cells possessing apical cavities extending through the midgut epithelium to the midgut lumen.....226

Figure 5.5 Unusual midgut epithelial cell within *An. stephensi* possessing an indistinct apical structure. 227
Figure 5.6 Unusual midgut epithelial cells possessing apical cavities from *An. stephensi* with atypical morphology. 229
Figure 5.7 Regenerative and unusual midgut epithelial cells possessing apical cavities associated with extruding midgut epithelial cells. 230
Figure 5.8 Temporal changes in the number of dividing and/or differentiating regenerative cells observed within the midgut epithelium of *An. stephensi*. 233
Figure 5.9 A model for division and differentiation of regenerative cells into columnar midgut epithelial cells within the adult mosquito midgut epithelium. 240
Figure 6.1 Comparison of the survival of different *P. falciparum* 3D7A developmental stages in *An. albimanus* and *An. stephensi*. 257
Figure 6.2 The various locations within the midgut epithelium that ookinetes have previously been observed in sectioned material examined by light or electron microscopy. 270
Figure 6.3 Ookinete entry into the midgut epithelium at "three-cell junctions". 272
Figure 6.4 Invagination of the midgut epithelium during ookinete invasion. 274
Figure 6.5 Ookinete invasion *via* a solely intracellular route. 276
Figure 6.6 Ookinete invasion *via* first intra- and then intercellular routes. 277
Figure 6.7 Ookinete invasion through multiple adjacent midgut epithelial cells. 278
Figure 6.8 *P. cathemerium* and *P. relictum* "zygotes" penetrating the midgut epithelium of *Cu. pipiens*. 287

Table of Tables

Table 1.1 Summary of previous studies on the route of ookinete migration across the mosquito midgut epithelium.....21

Table 2.1 Comparison of the levels of mature oocyst infection of the *P. falciparum* 3D7A clone at day 10 post-bloodfeeding in *An. albimanus* and *An. stephensi*.....64

Table 2.2 Comparison of the levels of mature oocyst infection of the *P. falciparum* HB3B-B2 clone at day 10 post-bloodfeeding in *An. albimanus* and *An. stephensi*.....65

Table 2.3 Comparison of the levels of mature oocyst infection of the *P. falciparum* 7G8 clone at day 10 post-bloodfeeding in *An. albimanus* and *An. stephensi*.....67

Table 3.1 Comparison of the levels of mature oocyst infection observed at day 10 post-bloodfeeding in the cohorts of *An. albimanus* and *An. stephensi* used to investigate *P. falciparum* 3D7A development within the bloodmeal and invasion of the midgut epithelium. 96

Table 3.2 Comparison of the prevalence of early and late *P. falciparum* 3D7A oocyst infection between *An. albimanus* and *An. stephensi*. 99

Table 3.3 Comparison of the intensity of early and late *P. falciparum* 3D7A oocyst infection between *An. albimanus* and *An. stephensi*. 100

Table 3.4 Estimated survival rates of different *P. falciparum* 3D7A developmental stages within *An. albimanus* and *An. stephensi*.....103

Table 3.5 Comparison of the prevalence of early and late *P. falciparum* 3D7A oocyst infection within *An. stephensi*. 105

Table 3.6 Comparison of the intensity of early and late *P. falciparum* 3D7A oocyst infection within *An. stephensi*. 106

Table 4.1 Summary of the histological sections from *An. albimanus* and *An. stephensi* midguts examined.....119

Table 4.2 Number of *P. falciparum* 3D7A parasites observed in histological sections from *An. albimanus* and *An. stephensi*.....126

Table 4.3 Levels of mature oocyst infection observed at day 10 post-bloodfeeding in the cohorts of *An. albimanus* and *An. stephensi* challenged with *P. falciparum* 3D7A and used to prepare the histological sections. 127

Table 4.4 Summary comparison of the prevalence of *P. falciparum* 3D7A parasites observed in histological sections in different locations within the midguts of *An. albimanus* and *An. stephensi*.....130

Table 4.5 Summary comparison of the intensity of *P. falciparum* 3D7A parasites observed in histological sections in different locations within the midguts of *An. albimanus* and *An. stephensi*.....131

Table 4.6 Comparison of the levels of *P. falciparum* 3D7A infection within *An. stephensi* at days 1 and 10 post-bloodfeeding.....133

Table 4.7 Estimated survival of *P. falciparum* 3D7A within *An. stephensi* between ookinete penetration of the peritrophic matrix and development into mature oocysts on the basal surface of the midgut epithelium. 134

Table 4.8 Comparison of the number of morphologically-abnormal midgut epithelial cells observed in histological sections from *An. stephensi* fed uninfected or *P. falciparum* 3D7A-infected blood. 154

Table 5.1 Comparison of the number of morphologically abnormal midgut epithelial cells observed in histological sections from *An. stephensi* fed uninfected or *P. falciparum* 3D7A-infected blood. 232

Table 5.2 Matrix of Pearson's (product-moment) correlation coefficients (r) describing the linear bivariate relationships between five variables in the midguts of *An. stephensi* mosquitoes 24 to 36 hours post-bloodfeeding (n, the number of midguts examined = 30). 235

Table 5.3 Details of the multiple linear regression model with the best fit to the relative number of dividing/differentiating regenerative cells observed within each *An. stephensi* mosquito midgut (n = 30). 236

Table 5.4 Hierarchical partitioning of the explained variation in the relative number of dividing/differentiating regenerative cells observed within each *An. stephensi* midgut between the four predictor variables included in the multiple linear regression analysis (n = 30). 237

Table 5.5 Comparison of the levels of oocyst infection in *An. stephensi* fed gametocytes of *P. falciparum* 3D7A with or without BrdUrd. 239

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Declaration

This work represents original work carried out by the author and has not been submitted in any form to any other University.

Signed:

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Abstract

The infectivity of three different clones of the human malaria parasite *Plasmodium falciparum* to two different natural vector mosquito species, *Anopheles albimanus* and *Anopheles stephensi* was investigated. Two of the *P. falciparum* clones studied (HB3B-B2 and 7G8) established relatively low levels of mature oocyst infection in both mosquito species. In contrast, the third *P. falciparum* clone investigated (3D7A) did not produce mature oocyst infections in *An. albimanus* but infected *An. stephensi* at a relatively high level. These observations demonstrate the existence of differences between the three malaria parasite clones in the ability to infect the mosquitoes, and the two mosquito species in their susceptibility to malaria parasite infection.

Direct immunofluorescence microscopy and examination of Giemsa-stained histological sections by light microscopy were used to investigate further the development of the *P. falciparum* 3D7A clone in *An. albimanus* and *An. stephensi*. The *P. falciparum* 3D7A clone formed mature ookinetes within the bloodmeals of both *An. albimanus* and *An. stephensi*. In *An. albimanus*, these malaria parasite stages subsequently failed to migrate from the bloodmeal and invade the surrounding midgut epithelium suggesting that ookinetes were destroyed within the endoperitrophic space of this mosquito species. The reasons for the disappearance of ookinetes within the endoperitrophic space of *An. albimanus* were uncertain but were possibly related to the faster time to completion of bloodmeal digestion in this mosquito species compared to *An. stephensi*. Simultaneous investigation of *P. falciparum* 3D7A development within *An. stephensi* revealed that in this mosquito species ookinetes entered the midgut epithelium *via* an intracellular route, causing destruction of invaded midgut epithelial cells, and subsequently exited the midgut epithelium by an intercellular route. A general model for the route of ookinete invasion across the midgut epithelium is proposed based upon these observations. Examination of the Giemsa-stained histological sections also provided evidence that regenerative cells within the midgut epithelium of *An. stephensi* undergo proliferation and differentiation into midgut epithelial cells following infection with *P. falciparum* 3D7A, presumably as a mechanism to replace the midgut epithelial cells destroyed as a consequence of ookinete invasion of the midgut epithelium.

Chapter 1. General Introduction

1. 1 Introduction

Malaria parasites are unicellular eukaryotes of the phylum Apicomplexa (Sporozoa) (Order Haemosporidia) that undergo a complex two-host life cycle alternating between distinct invasive and multiplicative developmental stages. There are many different malaria parasite species infecting birds, lizards and mammals that are transmitted between these vertebrate hosts by various invertebrate vectors (Garnham, 1966). Malaria parasite species are transmitted between vertebrate hosts by haematophagous flies (Order Diptera): mosquitoes (Family Culicidae) appear to be the most common invertebrate vectors, although sandflies (Family Psychodidae), biting midges (Family Ceratopogonidae) and louse flies (Family Hippoboscidae) are also known as vectors of malaria parasite species infecting various vertebrate hosts.

There are four species of malaria that are regarded as human pathogens: *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, although zoonotic infection with non-human primate malaria species is also known (e.g. Singh *et al.*, 2004). The most prevalent and severe form of human malaria is *P. falciparum* which is currently estimated to kill between 1 and 3 million people, and to cause between 300 and 660 million clinical cases of infection, every year (Hay *et al.*, 2004; Snow *et al.*, 2005). The majority of this mortality and morbidity occurs in children under five years of age in sub-Saharan Africa (Hay *et al.*, 2004; Snow *et al.*, 2005; Hay *et al.*, 2005), and is believed to be a significant hindrance to the socio-economic development of some of the poorest regions in the world (Sachs & Malaney, 2002; Malaney, Spielman & Sachs, 2004). Despite the World Health Organisation-sponsored plan to "Roll Back Malaria" and halve the number of malaria-related deaths by 2010, a number of complex biological, social, political and economic factors are currently contributing to an increase in the global burden of malaria (Greenwood & Mutabingwa, 2002; Attaran, 2004). Malaria control is primarily achieved through chemotherapy of infected individuals and insecticides against mosquito vectors (Greenwood *et al.*, 2005), although alternative interventions such as bed-nets are becoming increasingly used (Curtis, 2005). However, the global spread of drug-resistant malaria parasite strains (Wootton *et al.*, 2002; Roper *et al.*, 2004) and the evolution of insecticide-resistance in mosquito vectors (Hemingway *et al.*, 2004) has seriously reduced the arsenal of affordable tools in the battle against malaria (Greenwood *et al.*, 2005). Despite decades

of intensive research, no effective and practical vaccine against the disease-causing stages of human malaria is currently available (Targett, 2005).

In recent years, there has been a growing interest in developing so-called "transmission-blocking" interventions, which prevent malaria parasite infection of the mosquito vector (Carter, 2001; Sinden, 2002; Kaslow, 2002). One transmission-blocking strategy is the replacement of indigenous susceptible mosquito vector populations with genetically-modified mosquitoes refractory to malaria parasite infection (Alphey *et al.*, 2002; Christophides, 2005). *P. falciparum*, and other human malaria parasite species, are transmitted between people by mosquitoes of the genus *Anopheles*. There are many different species of *Anopheles* but only a small number of these are considered important vectors of human malaria (Gwadz & Collins, 1996). The ability of a given mosquito species to transmit malaria, known as *vectorial capacity*, is determined by a number of factors, including the density of mosquitoes, frequency of mosquito bloodfeeding, preferred vertebrate blood sources, and the longevity of adult female mosquitoes relative to the duration of malaria parasite development within the mosquito vector (MacDonald, 1957; Black & Moore, 1996; Beier & Vanderberg, 1998). The intrinsic susceptibility of mosquitoes to malaria parasite infection, known as *vector competence*, is also an important determinant of vectorial capacity, and great progress is being made in identifying the molecular determinants of mosquito variation in the susceptibility to malaria parasite infection (e.g. Blandin *et al.*, 2004). Ever since the discovery of mosquito-borne transmission of malaria at the end of the nineteenth century, malaria parasites have also been known to differ in their ability to infect mosquitoes (Harrison, 1978). However, relatively little attention has been given to the role of malaria parasite polymorphism in determining the outcome of malaria infection in the mosquito and no formal concept of *parasite competence*, defined here as the intrinsic ability of malaria parasites to infect mosquitoes, is recognised. With the advent of new molecular technologies and the promise of novel malaria control strategies aiming to prevent malaria parasite infection of the mosquito vector, understanding the role of malaria parasite polymorphism in determining the outcome of infection within the mosquito vector is of increasingly greater significance (Lambrechts *et al.*, 2005).

1. 2 Overview of the malaria parasite life cycle

Malaria parasite infection is initiated when an infectious adult female mosquito salivates, during bloodfeeding, into the skin of the vertebrate host and thus inoculates a

small number of haploid sporozoite stage malaria parasites into the dermis (Frischknecht *et al.*, 2004; Vanderberg & Frevert, 2004).[1] The sporozoites migrate through the dermal connective tissue and invade blood vessels within the skin. The sporozoites are subsequently carried passively within the bloodstream to the liver, where they attach to the endothelium of the liver sinusoids (Frevert *et al.*, 2005). The sporozoites invade resident macrophages, known as Kupffer cells, which line the endothelium of the liver sinusoids, cross the Space of Disse, and invade the liver parenchyma. The sporozoites rapidly migrate intracellularly through multiple hepatocytes, before initiating transformation into a schizont within the last invaded hepatocyte. The intracellular malaria parasite grows in size and undergoes multiple rounds of replication to produce a single hepatic schizont containing many thousand daughter merozoite stages (Frevert, 2004). The mature schizont eventually ruptures, releasing the merozoites into the bloodstream and thus initiating the asexual erythrocytic cycle of malaria parasite development (Bannister *et al.*, 2000; Bannister & Mitchell, 2003). Each merozoite rapidly invades an erythrocyte, transforms into an intracellular trophozoite stage that feeds upon the red blood cell haemoglobin and grows into another schizont stage that contains a small number of daughter merozoites. The mature erythrocytic schizont eventually ruptures releasing the merozoites into the bloodstream. These merozoites invade further erythrocytes and indefinitely repeat the asexual erythrocytic cycle of invasion, growth and multiplication that gives rise to the clinical symptoms of malaria infection. However, a small proportion of the merozoites terminally differentiate into non-replicative male and female sexual stages, known as micro- and macrogametocytes respectively, which circulate in the peripheral bloodstream and enable transmission of the malaria parasite to the mosquito vector. When ingested during mosquito bloodfeeding, the gametocytes rapidly transform into gametes and undergo fertilization to produce a diploid zygote within the midgut lumen of the mosquito. The sessile zygote subsequently transforms into a motile ookinete that migrates through the bolus of ingested blood, penetrates the surrounding peritrophic matrix and invades the mosquito midgut epithelium. The ookinete develops into a

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[1] The description of the malaria parasite life cycle within the *vertebrate* host given here only applies to mammalian malaria parasite species. Non-mammalian malaria parasites exhibit a number of differences in both the site and timing of development within the vertebrate host. In contrast, development of mammalian and non-mammalian malaria parasite species within the mosquito vector is remarkably conserved.

multiplicative oocyst stage on the outer surface of the midgut epithelium, internally differentiating to produce several thousand haploid daughter sporozoite malaria parasite stages. The mature oocyst eventually ruptures releasing the sporozoites into the mosquito haemolymph. The sporozoites migrate to the mosquito salivary glands and pass into the salivary gland duct ready for transmission to a new vertebrate host when the mosquito next takes a bloodmeal.

1. 3 Gametocytogenesis: preparation for transmission to the mosquito vector[2]

Transmission of malaria parasites to the mosquito vector begins inside the vertebrate host when a small minority of the haploid asexual erythrocytic stage malaria parasites commit to gametocytogenesis: the formation of gametocytes, sexually dimorphic and terminally differentiated intra-erythrocytic malaria parasite stages (Duncan *et al.*, 1959; Aikawa, Huff & Sprinz, 1969; Rudzinska, 1969; Ladda, 1969; Kass *et al.*, 1971; Sterling & Aikawa, 1973; Sinden *et al.*, 1978; Sinden, 1978; Jensen, 1979; Sinden, 1982; Sinden, 1983; Aikawa *et al.*, 1984; Ponnudurai *et al.*, 1986; Meszoely *et al.*, 1987; Kaidoh *et al.*, 1993). The triggers for sexual differentiation of malaria parasites are poorly understood (Talman *et al.*, 2004). Unlike related haemosporidians, *Plasmodium* species do not undergo a fixed number of asexual erythrocytic cycles prior to the onset of gametocytogenesis (Smith, Walliker & Ranford-Cartwright, 2002). High asexual parasitaemia, anaemia, antiparasitic immune responses, chemotherapeutic agents and possibly other environmental stresses probably contribute to the induction of gametocytogenesis (Talman *et al.*, 2004). Commitment to sexual differentiation occurs during the asexual erythrocytic cycle immediately preceding gametocyte formation, although some pre-erythrocytic merozoites derived from hepatic schizonts may also form gametocytes. Malaria parasites lack sex chromosomes and genetically-identical clonal asexual erythrocytic stage parasites transform into both micro- and macrogametocytes (Day, Hayward & Dyer, 1998). Consequently, malaria parasites are effectively simultaneous hermaphrodites (Talman *et al.*, 2004) capable of both self-fertilisation and cross-fertilisation in the presence of other malaria parasite clones (Walliker, Babiker & Ranford-Cartwright, 1998).

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[2] An animated version of the malaria parasite life cycle within the mosquito vector, including gametocytogenesis, is available in the online supplementary material to Baton & Ranford-Cartwright (2005b) at the following website: http://dx.doi.org/10.1016/j.pt.2005.09.012.

The location, duration and stages of gametocyte development vary between *Plasmodium* species (Day *et al.*, 1998). In *P. falciparum*, gametocytogenesis is unusually prolonged lasting approximately 8 days and passing through five morphologically distinct sub-stages (Day *et al.*, 1998; Talman *et al.*, 2004). In other *Plasmodium* species, gametocyte development is roughly proportional to the duration of the asexual erythrocytic cycle and lasts approximately 1 to 3 days. The morphology of mature gametocytes also differs markedly between *Plasmodium* species: *P. falciparum* gametocytes are crescent-shaped while other species are spherical. The reasons for the differences in the duration of gametocytogenesis and gametocyte morphology observed between malaria parasite species are unknown. Initially, gametocyte development is similar to the asexual erythrocytic cycle: sexually-committed merozoites escape host erythrocytes during schizont rupture, invade new erythrocytes *via* parasitophorous vacuole formation and grow into trophozoites (Gk. "feeding animal"), intracellular stages digesting haemoglobin to produce the insoluble waste-product haemozoin (Bannister & Mitchell, 2003). Rather than developing into schizonts, the sexually-committed trophozoites subsequently transform into gametocytes. Gametocyte development is first detectable through sexual stage-specific gene expression (Alano *et al.*, 1991; Bruce *et al.*, 1994; Dechering *et al.*, 1997). During early sexual differentiation, intranuclear microtubules appear within the malaria parasite suggesting mitotic replication (Sinden, 1983) while the nuclear DNA content approximately doubles (Janse *et al.*, 1986b). However, there is no nuclear division suggesting the occurrence of selective genome amplification. Simultaneously, two closely apposed inner membranes and associated microtubules form beneath the gametocyte plasma membrane. These structures are reminiscent of the pellicular structures of extracellular invasive stage apicomplexan parasites (Bannister & Mitchell, 2003; Frevert, 2004). The purpose of these structures in non-invasive intracellular gametocytes is unknown. In mature gametocytes, the microtubules are no longer present and only the double inner membranes remain, eventually disappearing during transformation within the mosquito (Sinden, 1983; Aikawa *et al.*, 1984). As sexual development proceeds, micro- and macrogametocytes become increasingly dimorphic. Microgametocytes possess little synthetic apparatus and few cytoplasmic organelles, while the large lobular nucleus lacks a nucleolus, consistent with pre-synthesis and storage of proteins required for subsequent gamete formation (Khan *et al.*, 2005). Osmiophilic bodies, discoid membrane-bound structures reminiscent of the secretory organelles of invasive stage

malaria parasites, are also occasionally found in the peripheral cytoplasm. In contrast to microgametocytes, macrogametocytes are packed with ribosomes, endoplasmic reticulum, mitochondria, and numerous osmiophilic bodies, while the nucleus is relatively small and compact (Sinden, 1983; Khan *et al.*, 2005). An intranuclear electron-dense body is present in mature macrogametocytes (Sinden, 1983; Aikawa *et al.*, 1984); formerly considered to be the nucleolus, this structure is now regarded as a transcription or replication factory (Shaw, Thompson & Sinden, 1996). Translationally repressed mRNA is also present within the macrogametocyte cytoplasm (Shaw *et al.*, 1996), enabling rapid stage-specific protein expression following gametogenesis and fertilization in the mosquito (Hall *et al.*, 2005). The sexual dimorphism of gametocytes reflects their different developmental roles: microgametocytes undergo rapid differentiation into ephemeral motile male gametes, whereas macrogametocytes progressively transform into persistent stages capable of establishing infection in the hostile environment of the mosquito midgut (Sinden, 1983). Once formed, mature gametocytes may persist within the peripheral bloodstream of the vertebrate host for many days, and sometimes weeks. However, unlike the cycling asexual erythrocytic stage malaria parasites adapted for maintaining and perpetuating infection in the vertebrate host (Bannister & Mitchell, 2003), mature gametocytes are specialised escapists, arrested in cell-cycle development. Unless ingestion by a suitable mosquito enables continued existence, gametocytes eventually die within the vertebrate host through senescence or immune clearance.

1. 4 Malaria parasite development within the mosquito vector

1.4.1 Gametogenesis

When an adult female mosquito feeds upon an infectious vertebrate host, gametocytes and asexual erythrocytic parasite stages circulating in the peripheral bloodstream are ingested along with all the other components of the vertebrate host's blood. The ingested blood passes directly into the posterior midgut lumen of the mosquito: the one-stop site of bloodmeal storage, digestion and absorption (Billingsley, 1990b; Lehane, 1991; Romoser, 1996). This transition from vertebrate to mosquito host initiates the process of gametogenesis: the rapid transformation within the bloodmeal of ingested gametocytes into extracellular micro- and macrogametes (Garnham, Bird & Baker, 1967; Sinden & Croll, 1975; Sinden, Canning & Spain, 1976; Sinden *et al.*, 1978; Sinden, 1978; Sinden, 1983; Aikawa *et al.*, 1984; Ono, Nakabayashi & Ohnishi,

1991). Gametogenesis is induced within minutes of malaria parasite ingestion by the mosquito and is typically completed within approximately 10 to 30 minutes. The two main triggers for gametogenesis *in vivo* are believed to be the falling temperature associated with the transition from a homeothermic vertebrate to a poikilothermic invertebrate host (Ogwan'g *et al.*, 1993b; Billker *et al.*, 1997) and mosquito-derived factors (Micks, de Caires & Franco, 1948; Yoeli & Upmanis, 1968; Nijhout, 1979; Garcia, Wirtz & Rosenberg, 1997). Xanthurenic acid, a by-product of tryptophan metabolism, has been identified as one of the triggers of gametogenesis (Billker *et al.*, 1998; Garcia *et al.*, 1998; Billker, Miller & Sinden, 2000). The exact origin of the xanthurenic acid triggering gametogenesis is unclear: it may derive from either the mosquito midgut and/or mosquito saliva ingested during bloodfeeding (Hirai *et al.*, 2001; Arai *et al.*, 2001). Falling temperature and xanthurenic acid trigger gametogenesis *via* calcium-, cyclic GMP- and phosphoinositide-dependent signalling pathways (Martin *et al.*, 1978; Kawamoto *et al.*, 1990; Kawamoto *et al.*, 1992; Kawamoto *et al.*, 1993; Ogwan'g *et al.*, 1993a; Martin, Jett & Schneider, 1994; Muhia *et al.*, 2001; Billker *et al.*, 2004). Consistent with the role of phosphoinositide in signalling gametocyte activation, several protein kinases have also been identified that are essential for the successful completion of gametogenesis (Billker *et al.*, 2004; Rangarajan *et al.*, 2005). Vertebrate host-derived factors (possibly also xanthurenic acid) may further contribute to gametocyte activation *in vivo* (Billker *et al.*, 1998; Arai *et al.*, 2001).

Following activation, *Plasmodium* species with non-spherical gametocytes 'round up' before emergence from host erythrocytes (Sinden, 1983; Aikawa *et al.*, 1984). The intracellular gametocytes then swell, increasing several-fold in volume, while the osmiophilic body contents are released *via* small ductules into the parasitophorous vacuole. These events probably cause disruption and disintegration of the parasitophorous vacuole and erythrocyte plasma membranes surrounding the intracellular gametocytes. A malaria parasite molecule present within the osmiophilic bodies, Pfg377, has been identified but its function is currently unknown (Alano *et al.*, 1995a; Severini *et al.*, 1999). The efficiency of malaria parasite escape from the host erythrocyte varies between the gametocyte sexes, and is possibly related to differences in the number of osmiophilic bodies found within micro- and macrogametocytes. Macrogametocytes emerge completely from their host erythrocyte producing a single extracellular and non-motile spherical macrogamete approximately 10 to 15 μm in

diameter, whereas microgametocytes frequently remain trapped within the host erythrocyte plasma membrane (Sinden, 1983; Aikawa *et al.*, 1984). However, microgametocytes subsequently undergo the spectacular process of exflagellation, which was famously first observed by the French Army surgeon Charles Louis Alphonse Laveran on 6th November 1880 in Constantine, Algeria, during examination of blood freshly drawn from a feverish soldier (Harrison, 1978; Sherman, 1998; Dobson, 1999). The spectacular transformation observed by Laveran marked the discovery of the causative agent of malaria. However, the full significance of exflagellation was not appreciated until 1896 when William George MacCallum, a physician working in the United States, observed fertilisation between male and female gametes of the malaria parasite (MacCallum, 1897; MacCallum, 1898). Exflagellation entails three rapid rounds of endomitotic replication, which produces eight daughter genomes within a single octoploid nucleus (Janse *et al.*, 1986a; Janse *et al.*, 1986b; Janse *et al.*, 1988). Simultaneously, axonemes are rapidly assembled within the microgametocyte cytoplasm from microtubule organising centres (MTOCs) located around the periphery of the nucleus (Sinden, 1983; Aikawa *et al.*, 1984; Billker *et al.*, 2002). The axonemes writhe within, and lash without, the transforming microgametocyte as the emergent microgametes struggle to separate from the central residual parasite body and remaining erythrocyte plasma membranes (Sinden, 1983). Up to eight haploid microgametes eventually break free leaving behind a residuum of parasite cytoplasm and haemozoin. Microgametes are effectively single membrane-bound nucleated flagella, approximately 0.2 to 0.4 by 15 to 25 μm, possessing little cytoplasm which lacks apicoplasts and mitochondria. Consequently, the apicoplast and mitochondrial organelles are inherited exclusively from the macrogamete (Creasey *et al.*, 1993; Vaidya *et al.*, 1993; Creasey *et al.*, 1994). A significant proportion of microgametes are apparently morphologically abnormal, some being anucleate and others possessing multiple axonemes, although it is unclear to what extent such morphological abnormalities occur *in vivo* (it has been suggested that such observations are artefactual) (Garnham *et al.*, 1967; Sinden, 1975a). Microgametes are highly motile and undergo rapid sinusoidal movements (Sinden & Croll, 1975; Sinden *et al.*, 1978) but are short-lived, only remaining active for approximately 40 minutes after their formation *in vitro* (Sinden, 1978; Sinden, 1983). Microgametes specifically adhere to the surface of uninfected erythrocytes *in vitro*, which possibly facilitates their movement through the bloodmeal *in vivo* (Templeton *et al.*, 1998).

1.4.2 Fertilisation & zygote formation

Micro- and macrogametes rapidly fertilise within the mosquito bloodmeal to form diploid zygotes, typically within 1 hour of the induction of gametogenesis (Sinden, 1978; Sinden, 1983; Aikawa *et al.*, 1984; Sinden, Hartley & Winger, 1985; Sinden & Hartley, 1985; Janse *et al.*, 1986a; Janse *et al.*, 1986b). Whether microgamete migration within the bloodmeal is random or directed is unknown, although no evidence has been found for directed movement (chemotaxis) of microgametes towards macrogametes (Sinden *et al.*, 1978). Several members of a superfamily of molecules, including P230 and P48/45, containing variable numbers of a "6-Cys domain" unique to malaria parasites, are found on the surface of micro- and macrogametes (Kaushal & Carter, 1984; Williamson, Criscio & Kaslow, 1993; Kocken *et al.*, 1993; Carter *et al.*, 1995; Templeton & Kaslow, 1999; van Dijk *et al.*, 2001; Eksi & Williamson, 2002; Williamson, 2003; Gerloff *et al.*, 2005). The exact function of these molecules is currently unknown, but they are putatively gamete-specific receptors mediating fertilisation. Consistent with this hypothesis, microgametes lacking P48/45 have a reduced ability to fertilise macrogametes (van Dijk *et al.*, 2001). Following initial attachment, the plasma membranes of the micro- and macrogametes fuse while the microgamete axoneme and its associated nucleus pass into the macrogamete cytoplasm. During this period the microgamete axoneme continues to writhe but eventually ceases movement and disassembles (Sinden, 1978). Meanwhile, within approximately 30 minutes of fertilisation, the microgamete nucleus passes through the endoplasmic reticulum of the macrogamete and unites with the macrogamete nucleus (Aikawa *et al.*, 1984; Janse *et al.*, 1986b). Endomeiotic replication subsequently ensues during the next 2 to 3 hours to produce a single approximately tetraploid zygote nucleus (Mehlhorn, Peters & Haberkorn, 1980; Aikawa *et al.*, 1984; Sinden *et al.*, 1985; Sinden & Hartley, 1985; Janse *et al.*, 1986a; Janse *et al.*, 1986b). A NIMA-related protein kinase has been identified that is necessary for the induction of this meiotic DNA replication (Reininger *et al.*, 2005). The exact timing of the subsequent meiotic reduction divisions is not well understood: segregation of homologous chromosomes rapidly follows DNA replication but separation and segregation of the sister chromatids has not been clearly identified, although mature ookinetes contain intranuclear microtubules (Davies, 1974; Sinden, 1978; Mehlhorn *et al.*, 1980). Regardless of when the meiotic reduction divisions occur, the single approximately tetraploid nucleus persists throughout the ookinete stage

(Janse *et al.*, 1986b) and is assumed to eventually contain the four haploid meiotic products expected from a conventional two-step meiosis (Walliker *et al.*, 1998).

During the period of macrogamete and zygote formation, expression of the P25 and P28 proteins becomes apparent over the surface of the malaria parasite (Carter & Kaushal, 1984; Vermeulen *et al.*, 1985a; Vervenne *et al.*, 1994; Shaw *et al.*, 1996). These two related molecules, which both contain four tandemly-repeated epidermal growth factor-like domains, persist on the surface of the malaria parasite up to the oocyst stage and have multiple, partially-redundant functions during infection of the mosquito vector (Tomas *et al.*, 2001; Baton & Ranford-Cartwright, 2005a).

1.4.3 Zygote differentiation into the ookinete

The extracellular sessile and spherical zygote gradually transforms over the following 10 to 30 hours, *via* an intermediate "retort-form" (named after the chemist's flask), into a mature motile and invasive banana-shaped ookinete (Gk. "moving egg") (Garnham, Bird & Baker, 1962; Garnham *et al.*, 1969; Canning & Sinden, 1973; Davies, 1974; Sinden, 1978; Gass, 1979; Mehlhorn *et al.*, 1980; Aikawa *et al.*, 1984; Sinden *et al.*, 1985; Sinden *et al.*, 1987; Meis & Ponnudurai, 1989; Chege & Beier, 1994; Robert *et al.*, 1994; Robert *et al.*, 1998; Vernick, Fujioka & Aikawa, 1999; Limviroj *et al.*, 2002). Morphological differentiation of the zygote into the ookinete is regulated by a calcium/calmodulin-dependent protein kinase (Silva-Neto, Atella & Shahabuddin, 2002) and is accompanied by a number of molecular changes in the surface of the malaria parasite (Kaushal *et al.*, 1983a; Carter & Kaushal, 1984; Vermeulen *et al.*, 1985a; Vermeulen *et al.*, 1986; Langer, Li & Vinetz, 2002). The mature ookinete eventually resulting from zygote differentiation is a highly polarised cell, approximately 2 to 4 by 12 to 18 μm, with a specific anterior region (the phylum-defining apical complex) and a distinct cytoskeleton. Initially, a basket of microtubules assembles, from a single MTOC, around the spherical nucleus of the zygote, causing polarisation of the nucleus which becomes cone-shaped (Aikawa *et al.*, 1984). The MTOC, which is at the apex of the nuclear cone, becomes closely apposed to the zygote plasma membrane creating a localised prominence in the zygote surface: an incipient apical complex. Simultaneously, the nucleus retracts to the centre of the zygote leaving the MTOC and its associated basket of microtubules immediately beneath the plasma membrane of the zygote. Within the centre of the apical prominence, several concentric polar rings form from the MTOC and two closely apposed plasma membranes, derived from a single flattened vesicle (Gass, 1979; Raibaud *et al.*, 2001), develop between the

zygote plasma membrane and the microtubules radiating from the MTOC. Together with the outer zygote plasma membrane, these closely apposed plasma membranes, known as the inner membrane complex, form the ookinete pellicle, which is surrounded by a dense surface coat (Gass, 1979; Raibaud *et al.*, 2001). As the apical prominence emerges from the surface of the zygote, the anterior-most region of the inner membrane complex expands to form a posteriorly-tapering cone-shaped collar, which gives rigidity and shape to apical region of the mature ookinete (Canning & Sinden, 1973; Dubremetz *et al.*, 1998). There are approximately 50 to 70 microtubules originating from the MTOC, symmetrically arranged around the circumference of the malaria parasite, which extend longitudinally, from the outer polar ring, beneath the pellicle of the ookinete. The subpellicular network, a skeleton of interwoven filaments associated with the inner membrane complex and the subpellicular microtubules, is presumably concurrently assembled (Khater, Sinden & Dessens, 2004; Vontas *et al.*, 2005a; 2005b). The ookinete pellicle and its associated subpellicular microtubules/network extend from the junction with the zygote body driving the apical prominence from the zygote surface and transforming the malaria parasite into the classical intermediate retort-form of the immature ookinete. The spherical zygote body maintains a single plasma membrane throughout this transformation, and diminishes in size as its entire cytoplasmic contents are drawn into the emerging ookinete. Meanwhile, several large cytoplasmic crystalloids form within the transforming malaria parasite, each consisting of multiply-arrayed small (20-40 nm) lipoprotein particles (Trefiak & Desser, 1973). These organelles persist unchanged through to the early phases of oocyst development, at which time they are no longer apparent within the cytoplasm of the malaria parasite. The function of the crystalloids is unknown; possibly, they are an energy store or precursors of the oocyst capsule. Numerous convoluted membrane-bound tubules also develop within the anterior third of the budding ookinete. Although previously termed rhoptries, these secretory organelles probably comprise a single population of micronemes (Li *et al.*, 2004) that open through the polar rings to the apical surface of the ookinete. A variety of membrane-bound and secreted adhesins, degradative enzymes and lytic proteins are released from the micronemes, during ookinete migration within the mosquito vector (Langer *et al.*, 2000; Limviroj *et al.*, 2002; Yuda & Ishino, 2004; Kadota *et al.*, 2004; Li *et al.*, 2004). The posterior-most region of the ookinete has been reported to be a distinct membrane-delimited compartment within which is a large "round body" containing numerous convoluted membranes (Vernick *et al.*, 1999).

The significance of this posterior compartment is unknown, as it has only been observed in a single ookinete, which was deformed during invasion of the midgut epithelium. Consequently, it is unclear if this structure is a characteristic of morphologically-normal ookinetes within the bloodmeal. Ookinetes also contain membrane-bound haemozoin pigment granules present within the macrogamete and retained from the period of intra-erythrocytic development during the macrogametocyte stage.

The apical complex, pellicle and subpellicular microtubules/network of the mature ookinete are a conserved cellular architecture ("the glideosome") that mediates the *gliding motility* typical of invasive stage apicomplexan parasites (Morrissette & Sibley, 2002; Kappe *et al.*, 2004; Sibley, 2004; Keeley & Soldati, 2004; Carruthers & Blackman, 2005). Gliding motility is a form of substrate-dependent locomotion, which occurs without obvious deformations in cell-shape, and that gives the migratory invasive zoite stages of the malaria parasite their slug-like movement[3]. The glideosome enables malaria parasite penetration and migration through host tissues through extracellular secretion of micronemal adhesins from the apical complex at the anterior of the ookinete. These micronemal adhesins bind to the external substrate and translocate posteriorly over the surface of the malaria parasite through the action of an acto-myosin motor located between the outer membrane and inner membrane complex of the ookinete pellicle (Margos *et al.*, 2000; Kappe *et al.*, 2004; Xu *et al.*, 2005; Vontas *et al.*, 2005a; 2005b). The backward translocation of the micronemal adhesins, which are fixed relative to the external substrate, consequently drives the ookinete forward. Accordingly, the external surface of the mature ookinete can be conceptualised as a single "tubular" caterpillar track running from the anterior to the posterior of the malaria parasite (imagine eversion of a sock as the flow of adhesins over the surface of the ookinete). However, unlike a caterpillar track, the secreted micronemal adhesins are not recycled but cleaved at the posterior end of the ookinete leaving a trail of shed malaria parasite molecules (Carruthers & Blackman, 2005). Apicomplexan gliding motility, therefore, requires continual production of the micronemal adhesins transducing the force of the pellicular acto-myosin motor to the external substrate. The micronemal adhesins mediating gliding motility in ookinetes include the circumsporozoite- and

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[3] For examples of the "slug-like" locomotion of ookinetes (and other apicomplexan parasites) see videos published in the on-line supplementary material to Zieler & Dvorak (2000) and Sibley (2004) available at, respectively, http://dx.doi.org/10.1073/pnas.97.21.11516 and http://dx.doi.org/10.1126/science.1094717.

thrombospondin-related adhesive/anonymous protein-related protein (CTRP), and von Willebrand Factor A-related protein (WARP). CTRP and WARP are both members of a diverse apicomplexan protein family containing variable numbers of thrombospondin- and/or von Willebrand Factor A-related domains (Trottein, Triglia & Cowman, 1995; Naitza *et al.*, 1998; Dessens *et al.*, 1999; Yuda, Sawai & Chinzei, 1999; Templeton, Kaslow & Fidock, 2000; Yuda *et al.*, 2001; Li *et al.*, 2004).

The outer and inner double membranes of the ookinete pellicle contain numerous intramembranous particles (Raibaud *et al.*, 2001). These probably include various components associated with the acto-myosin motor driving gliding motility (Margos *et al.*, 2000; Kappe *et al.*, 2004; Xu *et al.*, 2005; Vontas *et al.*, 2005a; 2005b) and other structural proteins anchoring the subpellicular microtubules to the inner membrane complex (Raibaud *et al.*, 2001). Uniquely among invasive stage malaria parasites, the ookinete inner membrane complex contains large pores regularly intercalated between the subpellicular microtubules (Raibaud *et al.*, 2001). These pores may facilitate molecular trafficking between the inner membrane complex and cytosol during ookinete gliding motility (Raibaud *et al.*, 2001).

1.4.4 Ookinete migration through the bloodmeal

During the approximately 24 hour period of zygote formation and subsequent differentiation into the ookinete, the environment of the mosquito midgut lumen changes markedly. Initially, within the first few hours post-bloodfeeding (pbf), the bloodmeal is compacted into a dense erythrocytic mass through excretion of ingested fluid *via* the processes of pre-diuresis and/or diuresis (Romoser, 1996) while an extracellular peritrophic (Gk. "around food") matrix is secreted from the mosquito midgut epithelium and coalesces around the entire bolus of ingested erythrocytes, compartmentalising the midgut lumen into ecto- (outer) and endo- (inner) peritrophic spaces (Shao, Devenport & Jacobs-Lorena, 2001). Simultaneously, the process of bloodmeal digestion is initiated. Digestion of the bloodmeal within the midgut lumen proceeds by the action of a variety of mosquito digestive proteases (and possibly other degradative enzymes, such as glycosidases and lipases). The mechanisms regulating bloodmeal digestion vary between anopheline and culicine mosquitoes but ultimately result in the production by the midgut epithelium of various trypsins, chymotrypsins, elastases, aminopeptidases and carboxypeptidases (Gooding, 1966a; Gooding, 1966b; Hecker & Rudin, 1979; Borovsky, 1986; Billingsley & Hecker, 1991; Billingsley & Rudin, 1992; Muller *et al.*, 1993a; Muller *et al.*, 1993b; Hörler & Briegel, 1995; Chadee

& Beier, 1995; Muller *et al.*, 1995; Lemos, Cornel & Jacobs-Lorena, 1996; Edwards *et al.*, 1997; Jiang *et al.*, 1997; Moskalyk, 1998; Rosenfeld & Vanderberg, 1998; Noriega & Wells, 1999; Edwards *et al.*, 2000; Shen, Edwards & Jacobs-Lorena, 2000; Vizioli *et al.*, 2001b; Okuda *et al.*, 2002; Noriega *et al.*, 2002; Sanders *et al.*, 2003; Caroci *et al.*, 2003; de Almeida *et al.*, 2003; Ribeiro, 2003; Devenport, Fujioka & Jacobs-Lorena, 2004; Dana *et al.*, 2005; Okuda *et al.*, 2005). The majority of digestive proteases are secreted from the midgut epithelium into the midgut lumen and pass through the semi-permeable peritrophic matrix to enter the endoperitrophic space and digest, from the periphery inwards, the bolus of ingested erythrocytes. In general, the various digestive proteases follow a similar pattern of expression in different mosquito species, initially increasing from low pre-bloodfeeding levels to a peak between approximately 18 to 36 hours after bloodfeeding and then declining to pre-bloodfeeding levels as bloodmeal digestion is completed. However, the levels and the distribution of each of the different types of digestive protease varies within each mosquito species. The serine protease trypsin generally accounts for the majority of endopeptidase activity and is predominantly located within the midgut lumen around the periphery of the bloodmeal (Graf *et al.*, 1986; Billingsley & Hecker, 1991; Hörler & Briegel, 1995; Lemos *et al.*, 1996). In contrast, a significant proportion of aminopeptidase activity is membrane-bound and associated with the midgut epithelium (Billingsley, 1990a; Billingsley & Hecker, 1991; Lemos *et al.*, 1996).

The migration of mature ookinetes from the bloodmeal to the surrounding peritrophic matrix begins from approximately 18 hours pbf, coincident with the peak activity levels of midgut digestive proteases. Essentially nothing is known about how ookinetes attain the peritrophic matrix surrounding the bloodmeal. This is primarily due to the difficulty of studying this phase of the malaria parasite life cycle within the mosquito vector *in situ*. Presumably, ookinetes actively migrate through the bloodmeal using gliding motility. How much time is required by ookinetes to undertake this migration, and whether ookinete movement to the bloodmeal periphery is random or directed, are unknown. Conceivably, chemical and/or viscosity gradients created by the digestion of the bloodmeal might be used by the ookinete to locate the surrounding peritrophic matrix. Whether ookinetes are able to migrate through the undigested central mass of densely-compacted erythrocytes is also unknown. Possibly, ookinetes may be trapped within the bloodmeal until digestion of the immediately surrounding erythrocytes liberates the malaria parasite.

1.4.5 Ookinete penetration of the peritrophic matrix

The type 1 peritrophic matrix (formerly known as the peritrophic membrane) of adult female mosquitoes is an approximately 2 to 10 μm thick extracellular mesh of protein, glycoprotein, proteoglycan, and polysaccharide (Shao *et al.*, 2001). The signal for induction of peritrophic matrix formation is distention of the midgut epithelium following ingestion of the bloodmeal (Freyvogel & Jaquet, 1965; Berner, Rudin & Hecker, 1983), which causes release of vesicles within the apical region of the midgut epithelial cells that contain either pre-formed or *de novo* synthesised precursors (Berner *et al.*, 1983; Devenport *et al.*, 2004; Devenport *et al.*, 2005). Upon secretion into the midgut lumen, the peritrophic matrix precursors coalesce around the bolus of ingested erythrocytes forming a closed but semi-permeable structure that completely surrounds the bloodmeal. A number of peritrophic matrix proteins have been described (Moskalyk, Oo & Jacobs-Lorena, 1996; Shen & Jacobs-Lorena, 1998; Sanders *et al.*, 2003; Devenport *et al.*, 2004; Dana *et al.*, 2005; Shao *et al.*, 2005; Devenport *et al.*, 2005) and a model of peritrophic matrix structure has been proposed in which peritrophins (chitin-binding proteins) act as bridges cross-linking the chitin fibrils believed to be the major component of the peritrophic matrix (Berner *et al.*, 1983; Shahabuddin *et al.*, 1995b; Shen & Jacobs-Lorena, 1998). The thickness and permeability of the peritrophic matrix are thought to be dynamically regulated during bloodmeal digestion through the co-ordination of chitin synthase and chitinase activities derived from the mosquito (Shen & Jacobs-Lorena, 1997; Shen & Jacobs-Lorena, 1998; Ibrahim *et al.*, 2000; Filho *et al.*, 2002). Initially, the peritrophic matrix is weak and only incompletely formed but gradually, with increasing time after bloodfeeding, the peritrophic matrix thickens and hardens. As bloodmeal digestion terminates, the peritrophic matrix is degraded and voided from the midgut along with the remnants of the digested bloodmeal. The time at which the peritrophic matrix is first detectable, the rate of peritrophic matrix formation and degradation, and the rigidity of the peritrophic matrix varies between different mosquito species (Freyvogel & Stäubli, 1965; Freyvogel & Jaquet, 1965; Richardson & Romoser, 1972; Houk, Obie & Hardy, 1979; Perrone & Spielman, 1988). In general, the peritrophic matrix is first detectable between 6 and 18 hours pbf, and fully formed between 12 and 32 hours pbf. The function of the peritrophic matrix is not known, although a variety of roles have been suggested for this mosquito structure including: compartmentalisation of the midgut lumen into distinct physiological spaces, protection of the midgut epithelium from

mechanical damage caused by abrasion with the contents of the midgut lumen, sequestration of toxic heme products resulting from bloodmeal digestion, and a physical barrier to prevent microorganisms within the midgut lumen gaining access to the midgut epithelium (Stohler, 1961; Orihel, 1975; Terra, 2001; Pascoa *et al.*, 2002; Villalon, Ghosh & Jacobs-Lorena, 2003).

The timing of ookinete migration from the bloodmeal to the midgut epithelium means that these malaria parasite stages confront a more or less fully-formed peritrophic matrix. Accordingly, ookinetes have evolved at least one mechanism that enables active and efficient penetration of the peritrophic matrix: micronemal secretion of malaria parasite-derived chitinase(s), locally disrupts the peritrophic matrix enabling ookinete passage to the ectoperitrophic space (Meis & Ponnudurai, 1987b; Sieber *et al.*, 1991; Huber, Cabib & Miller, 1991; Torii *et al.*, 1992; Shahabuddin *et al.*, 1993; Vinetz & Kaslow, 1998; Vinetz *et al.*, 1999; Langer *et al.*, 2000; Vinetz *et al.*, 2000; Dessens *et al.*, 2001; Tsai *et al.*, 2001; Limviroj *et al.*, 2002; Langer *et al.*, 2002; Tsuboi *et al.*, 2003; Li *et al.*, 2004). However, the exact role of ookinete-derived chitinases during mosquito infection is somewhat uncertain (Dessens *et al.*, 2001; Tsai *et al.*, 2001). Additionally, mosquito-derived chitinase and trypsin may influence the success of malaria parasite penetration of the peritrophic matrix (Shahabuddin, Criscio & Kaslow, 1995a; Shahabuddin *et al.*, 1996; Ramasamy *et al.*, 1996a; Langer & Vinetz, 2001; Bhatnagar *et al.*, 2003). Whether other mechanisms (e.g. protease secretion) are also involved in ookinete penetration of the peritrophic matrix is unknown.

1.4.6 Ookinete migration through the microvilli-associated network

After penetration of the peritrophic matrix and entry into the ectoperitrophic space, ookinetes must then pass through the microvilli-associated network, a recently described mesh of fine membranous strands that overlies, and apparently originates from, the microvilli of the apical surface of the midgut epithelium (Roth & Porter, 1964; Zieler *et al.*, 1998; Zieler & Dvorak, 2000; Zieler *et al.*, 2000). The exact composition and function of the microvilli-associated network is uncertain: it has been suggested to facilitate an increase in the surface area of the microvilli (Roth & Porter, 1964) or to act as a protective barrier layer against the contents of the midgut lumen (Zieler *et al.*, 2000). The microvilli-associated network has only been described in detail for aedine mosquitoes (Zieler *et al.*, 2000) and had not previously been reported in anopheline mosquitoes, although this mosquito structure is also apparently found in the latter subfamily of mosquitoes (Zieler & Dvorak, 2000). *P. gallinaceum* ookinetes adhere to

the microvilli-associated network (and/or *vice versa*), the latter appearing to induce annular constrictions in the surface of the malaria parasite (Zieler *et al.*, 1998), and *in vitro* the movement of this avian malaria parasite through the microvilli-associated network is slow in comparison to ookinete movement over the apical surface of the midgut epithelium (Zieler & Dvorak, 2000). There is no evidence that the microvilli-associated network is a barrier to malaria parasite infection of the mosquito vector.

1.4.7 Ookinete invasion of the midgut epithelium

The mosquito midgut epithelium consists of a simple monolayer of polarised epithelial cells possessing an inner (apical) plasma membrane folded into microvilli and a highly convoluted outer (basal) plasma membrane known as the basal labyrinth (Huff, 1934; Pal, 1943; Bertram & Bird, 1961; Freyvogel & Stäubli, 1965; Hecker *et al.*, 1971b; Hecker, 1977; Houk, 1977; Weaver & Scott, 1990a; Billingsley, 1990b; Weaver & Scott, 1990b). Whether the midgut epithelium contains different types of epithelial cells is currently controversial (Shahabuddin, 2002). Studies reporting novel midgut epithelial cell morphologies, including so-called "Ross cells", examined midguts experimentally manipulated *in vitro* (Shahabuddin & Pimenta, 1998; Zieler *et al.*, 1998; Zieler *et al.*, 2000) whereas examination of intact mosquito midguts has failed to identify morphologically distinct midgut epithelial cell types (Bertram & Bird, 1961; Freyvogel & Stäubli, 1965; Hecker, 1977; Zieler & Dvorak, 2000). The lateral plasma membranes of adjacent midgut epithelial cells are connected to one another by intercellular junctions present in the apical region of the midgut epithelium, the exact type and distribution of which vary between different mosquito species (Billingsley, 1990b). Consequently, the midgut epithelium presents a contiguous barrier to the malaria parasite which must be breached if infection of the mosquito vector is to established.

Ookinete recognition, attachment and invasion of the midgut epithelium are generally presumed to be mediated by specific interactions between malaria parasite receptors(s) and mosquito vector ligand(s), although the molecules involved in such putative interactions have not been formally identified. Ookinete attachment to the midgut epithelium is mediated by (as yet uncharacterised) stage-specific receptor(s) (Shahabuddin *et al.*, 1998b; Zieler, Nawrocki & Shahabuddin, 1999). Several candidate malaria parasite molecules have been identified which may act as specific ookinete receptors during invasion of the midgut epithelium, including P25/P28, CTRP, WARP and secreted ookinete adhesive protein (SOAP) (Trottein *et al.*, 1995; Dessens *et al.*,

1999; Yuda, Sakaida & Chinzei, 1999; Yuda *et al.*, 1999; Templeton *et al.*, 2000; Tomas *et al.*, 2001; Yuda *et al.*, 2001; Limviroj *et al.*, 2002; Dessens *et al.*, 2003; Li *et al.*, 2004; Abraham *et al.*, 2004; Danielli *et al.*, 2005; Baton & Ranford-Cartwright, 2005a). Specific antibodies against these malaria parasite molecules and/or gene knockouts reduce or entirely abolish oocyst infection, although the exact functions of P25/P28, CTRP, WARP and SOAP have yet to be determined. The ability of midgut-specific antibodies and various exogenous peptides/proteins to reduce levels of oocyst infection are also interpreted as evidence for the existence of specific mosquito ligand(s) mediating ookinete invasion of the midgut epithelium (Ramasamy & Ramasamy, 1990; Billingsley, 1994; Lal *et al.*, 1994; Srikrishnaraj, Ramasamy & Ramasamy, 1995; Ramasamy *et al.*, 1997; Zieler *et al.*, 1999; Zieler *et al.*, 2000; Zieler *et al.*, 2001; Ghosh, Ribolla & Jacobs-Lorena, 2001; Lal *et al.*, 2001; Almeida & Billingsley, 2002; Dinglasan *et al.*, 2003; Suneja, Gulia & Gakhar, 2003; Kotsyfakis *et al.*, 2005a). In particular, these studies suggest that ookinetes recognise specific carbohydrate moieties present on the luminal surface of the midgut epithelium. Several potential mosquito ligands, located on the luminal surface of the midgut epithelium, have been characterised (AgMUC1, AeIMUC1 and AeG12) (Shen *et al.*, 1999; Morlais & Severson, 2001; Shao *et al.*, 2005; Devenport *et al.*, 2005). Mosquito β integrin and annexins have also been suggested to facilitate the passage of ookinetes across the midgut epithelium (Mahairaki *et al.*, 2005a; Kotsyfakis *et al.*, 2005a; 2005b). Some authors have argued that specific receptors are not involved in ookinete invasion of the midgut epithelium as ookinetes attempt, and sometimes succeed in, invading various cell types *in vitro* (Rosales-Ronquillo & Silverman, 1974; Rosales-Ronquillo, Nienaber & Silverman, 1974; Speer, Rosales-Ronquillo & Silverman, 1975; Maier, Becker-Feldman & Seitz, 1987; Syafruddin *et al.*, 1992; Siden-Kiamos *et al.*, 2000). According to this application of Occam's Razor, *in vivo* ookinetes simply invade whatever cells types are encountered outwith the bloodmeal (Maier *et al.*, 1987).

Several different models have been proposed for the route of ookinete migration across the midgut epithelium. Some of these models date back to the earliest researches on ookinete invasion of the midgut epithelium (discussed briefly in Huff, 1934) whereas others have been proposed only relatively recently (Shahabuddin & Pimenta, 1998; Han *et al.*, 2000). Three basic models have been proposed for the route of ookinete invasion of the midgut epithelium:

(1) solely intercellular migration *between* midgut epithelial cells;

(2) solely intracellular migration *through* midgut epithelial cells; and

(3) initially intracellular through, and then intercellular between, midgut epithelial cells.

Various modifications of the solely intracellular model (2) have also been proposed:

(1) intracellular invasion of *any* midgut epithelial cell;

(2) intracellular invasion of *specific* subpopulations of midgut epithelial cells;

(3) intracellular invasion with or without midgut epithelial cell pathology; and

(4) intracellular invasion through multiple adjacent midgut epithelial cells.

The relationship between these various models is uncertain. Some of these models are necessarily mutually exclusive and it has been suggested that different modes of ookinete invasion across the midgut epithelium may exist for different malaria parasite-mosquito vector combinations (Maier, 1987; Meis & Ponnudurai, 1987a; Meis *et al.*, 1989; Han & Barillas-Mury, 2002; Shahabuddin, 2002). For previous studies on ookinete invasion of the midgut epithelium, the malaria parasite and mosquito species investigated, together with the conclusions of the authors on the route of migration taken by the ookinete and whether damaged epithelial cells were observed, are summarised in Table 1.1.

Recent studies of the rodent malaria *Plasmodium berghei* in *Anopheles stephensi* and the avian malaria *Plasmodium gallinaceum* in *Aedes aegypti* have unequivocally demonstrated that in these laboratory models the route of ookinete invasion is intracellular and results in the induction of apoptotic markers (and other morphologically-abnormal characteristics) in ookinete-invaded midgut epithelial cells (Han *et al.*, 2000; Zieler & Dvorak, 2000; Kumar *et al.*, 2004). In these malaria parasite-mosquito vector combinations, ookinetes invading the midgut epithelium may also migrate intracellularly through multiple adjacent midgut epithelial cells and sometimes be observed with an unusual "stalk-form" morphology (Vernick *et al.*, 1999; Han *et al.*, 2000; Zieler & Dvorak, 2000). *P. berghei* breaches the apical plasma membrane of midgut epithelial cells using membrane attack ookinete protein (MAOP), a protein containing a membrane-attack complex/perforin-related domain that has putative pore-forming capability (Kadota *et al.*, 2004). A tight association forms between the pellicle of the invading ookinete and the apical plasma membrane of the midgut epithelial cell, possibly creating a moving junction that translocates posteriorly over the malaria parasite during internalisation into the midgut epithelial cell (Yuda & Ishino, 2004; Kadota *et al.*, 2004). Although it has been previously stated that ookinetes

Table 1.1 Summary of previous studies on the route of ookinete migration across the mosquito midgut epithelium (continued over page).

Malaria parasite species[a]	Mosquito species	Method[b]	Route[c]	Pathology[d]	References
P. berghei (rodent)	*An. stephensi*	EM	Intra	Yes	Meis *et al.*, (1989); Limviroj *et al.*, (2002).
		CM	Intra/Lateral	Yes	Han *et al.*, (2000); Kumar *et al.*, (2004).
		LM/EM			Kadota *et al.*, (2004).
		VLM			Zieler & Dvorak (2000).
P. cathemerium (avian)	*Culex pipiens*	LM	Inter	No	Huff (1934).
		LM	Intra	Yes	Maier (1973).
P. circumflexum (avian)	*Culiseta annulata*	LM	Intra	No	Reichenow (1932).
P. cynomolgi bastianellii	*An. albimanus*	LM	Intra	No	Omar (1968b).
(monkey)	*An. stephensi*	LM	Intra	No	Omar (1968b).
		EM	Intra	No	Garnham *et al.*, (1962).
P. cynomolgi B (monkey)	*An. gambiae*	EM	Intra	Yes	Paskewitz *et al.*, (1988).
P. falciparum (human)	*An. maculipennis*	LM	Intra	No	Indacochea (1935).
	An. stephensi	EM	Inter	No	Meis & Ponnudurai (1987b); Meis *et al.*, (1989).

[a] The vertebrate host is given in brackets. [b] CM = confocal microscopy; EM = transmission electron microscopy; V/LM = video/light microscopy.
[c] Route of ookinete migration across the midgut epithelium. Intra = intracellular; Inter = intercellular; Both = both intra- and intercellular routes; Lateral = intracellular through multiple adjacent midgut epithelial cells. [d] Pathology of midgut epithelial cells associated with invading ookinetes.

Table 1.1 Continued...

Malaria parasite species	Mosquito species	Method	Route	Pathology	References
P. gallinaceum (avian)	*Ae. aegypti*	LM	Inter	No	Stohler (1957); Howard (1962).
		EM	Intra	No	Garnham *et al.*, (1962); Melhorn *et al.*, (1980).
		LM/EM			Shahabuddin & Pimenta (1998).
		EM	Intra/Lateral	No	Vernick *et al.*, (1999).
		VM		Yes	Zieler & Dvorak (2000).
		EM	Both	No	Torii *et al.*, (1992).
		VLM	Undetermined	No	Freyvogel (1966).
	An. gambiae	EM	Both	Yes	Vernick *et al.*, (1995).
P. yoelii nigeriensis	*An. omorii*	EM	Inter/Both	No	Syafruddin *et al.*, (1991).
(rodent)	*An. stephensi*	EM	Intra	No	Davies (1974).
		EM	Intra	Yes	Becker-Feldman *et al.*, (1985); Maier *et al.*, (1987).
		EM	Equivocal	No	Canning & Sinden (1973).
	unknown	EM	Intra/Both	No	Sinden (1978).
P. yoelii yoelii (rodent)	*An. stephensi*	EM	Intra	No	Garnham *et al.*, (1969).
P. vivax (human)	*An. albimanus*	EM	Intra	No	Gonzalez-Ceron *et al.*, (2001).

initially enter into the midgut epithelial cells *via* a parasitophorous vacuole that does not persist (Sinden, 1984; Sinden, 1998; Dimopoulos *et al.*, 2002b), there is no published evidence that internalisation of the ookinete is accompanied by parasitophorous vacuole formation. As ookinetes have only been reported to be in direct contact with the cytoplasm of the invaded midgut epithelial cells (Mehlhorn *et al.*, 1980; Becker-Feldman, Maier & Seitz, 1985; Maier *et al.*, 1987; Meis *et al.*, 1989; Syafruddin *et al.*, 1991; Torii *et al.*, 1992; Vernick *et al.*, 1995; Vernick *et al.*, 1999; Limviroj *et al.*, 2002), ookinetes probably enter midgut epithelial cells directly without parasitophorous vacuole formation (Yuda & Ishino, 2004). This interpretation is consistent with the apparent absence of rhoptries and dense granules in ookinetes (Li *et al.*, 2004; Hall *et al.*, 2005), secretory organelles thought to contribute to parasitophorous vacuole formation in the invasive zoite stages of apicomplexan parasites that develop intracellularly (Dubremetz *et al.*, 1998; Bannister & Mitchell, 2003; Frevert, 2004). During their intracellular migration, *P. berghei* ookinetes secrete a serine protease, subtilisin 2 (SUB2), and shed the P28 surface molecule, into the cytoplasm of invaded midgut epithelial cells (Han *et al.*, 2000; Han & Barillas-Mury, 2002).

1.4.8 Sporogony: oocyst formation, growth & differentiation

Between approximately 20 and 40 hours pbf, ookinetes emerge from the midgut epithelium and attain the basal surface of the latter, where transformation is initiated into the sedentary and spherical oocyst (Gk. "egg sac") (Garnham *et al.*, 1969; Davies, 1974; Mehlhorn *et al.*, 1980; Rastogi *et al.*, 1988; Meis *et al.*, 1989; Meis & Ponnudurai, 1989; Syafruddin *et al.*, 1991; Torii *et al.*, 1992; Vernick *et al.*, 1995). The extracellular ookinetes cease movement and "round up" beneath the basal lamina, which is relatively thin and electron dense at site of ookinete contact (Limviroj *et al.*, 2002). The basal lamina is an extracellular matrix, partly composed of collagen IV and laminin, which completely surrounds the basal surface of the midgut epithelium. Several ookinete surface proteins, including P25/P28, CTRP and SOAP, have been shown to bind components of the basal lamina and it has been proposed the latter mosquito ligands may trigger the developmental transformation into the oocyst (Warburg & Miller, 1992; Warburg & Schneider, 1993; Adini & Warburg, 1999; Vlachou *et al.*, 2001; Arrighi & Hurd, 2002; Al-Olayan *et al.*, 2002a; Dessens *et al.*, 2003; Arrighi *et al.*, 2005; Mahairaki *et al.*, 2005b). A thick extracellular capsule forms around the young oocyst, the origin and composition of which are controversial (Vanderberg, Rhodin & Yoeli, 1967; Garnham *et al.*, 1969; Mehlhorn *et al.*, 1980).

Probably, the oocyst capsule initially forms from the deposition of malaria parasite-derived material (Meis *et al.*, 1989; Syafruddin *et al.*, 1991), which subsequently incorporates mosquito-derived components of the basal lamina (e.g. laminin) (Adini & Warburg, 1999). The high transglutaminase activity associated with the oocyst capsule has been suggested to cross-link the malaria parasite-derived capsule with mosquito-derived components of the basal lamina (Adini *et al.*, 2001). New basal lamina is also simultaneously deposited beneath the developing oocyst from the underlying midgut epithelium, separating the malaria parasite from the midgut epithelium (Meis *et al.*, 1989; Gare, Piertney & Billingsley, 2003; Xu *et al.*, 2005).

The glideosome, the molecular machinery of motility and host cell invasion, is no longer required in the non-motile vegetative oocyst stage. Accordingly, during the early phases of oocyst formation, the apical complex and attached subpellicular microtubules retract into the malaria parasite cytoplasm and are disassembled, while the inner membrane complex fragments leaving a single outer plasma membrane (Davies, 1974; Sinden, 1978; Meis & Ponnudurai, 1989; Syafruddin *et al.*, 1991; Vernick *et al.*, 1995). Concomitantly, the machinery of DNA replication and protein synthesis are up-regulated (Vanderberg *et al.*, 1967; Hall *et al.*, 2005) for the massive asexual amplification of malaria parasite numbers, known as sporogony (Gk. "seed production"), that occurs within each oocyst (Duncan *et al.*, 1960; Terzakis, Sprinz & Ward, 1966; Terzakis, Sprinz & Ward, 1967; Vanderberg & Rhodin, 1967; Vanderberg *et al.*, 1967; Howells & Davies, 1971; Terzakis, 1971; Bafort, 1971; Terzakis, 1972; Sinden, 1975b; Schrevel, Asfaux-Foucher & Bafort, 1977; Sinden & Strong, 1978; Sinden, 1978; Meszoely *et al.*, 1982; Chen *et al.*, 1984; Nanda, Dass & Sharma, 1985; Rastogi *et al.*, 1988; Posthuma *et al.*, 1988; Klein *et al.*, 1988a; Meszoely *et al.*, 1989; Meis & Ponnudurai, 1989; Meis *et al.*, 1992b; Meszoely *et al.*, 1995). The crystalloids now disappear from the oocyst cytoplasm, the apicoplasts and mitochondria proliferate extensively, and mitotic spindles appear within the nucleus. The presumed four haploid products of meiosis are each assumed to now undergo multiple rounds of endomitosis to produce many hundreds of genomes contained within a single persistent nuclear envelope. During the middle period of sporogony, approximately between 4 to 8 days pbf, the huge polyploid nucleus becomes highly convoluted and lobulized. At this time, small peripheral vacuoles coalesce beneath the oocyst capsule causing inward retraction of the oocyst plasma membrane to create clefts of subcapsular "space". This cleft formation divides the oocyst cytoplasm into one or more islands, known as sporoblasts

(Gk. "seed germs"). The exact nature of sporoblast formation varies between malaria parasite species (Vanderberg *et al.*, 1967; Terzakis, 1971), and there is some controversy about whether cleft formation also occurs by expansion of the endoplasmic reticulum within the oocyst cytoplasm (Sinden & Strong, 1978; Posthuma *et al.*, 1988; Hamilton, Davies & Sinden, 1988; Meis & Ponnudurai, 1989). However, the subsequent process of sporozoite (Gk. "seed animal") budding from the sporoblast plasma membranes is conserved, following a similar pattern to the formation of invasive stages observed in other parts of the malaria parasite life cycle (Bannister & Mitchell, 2003; Frevert, 2004). The single lobulized nucleus has fragmented into numerous small ovoid nuclei, which undergo one final round of mitotic replication. The poles of the two mitotic spindles present within these nuclei align beneath opposing regions of the sporoblast plasma membrane, and direct formation of the sporozoite apical complex and pellicle in a manner similar to the zygote MTOC during transformation into the ookinete (Section 1.4.3). Throughout the oocyst, at numerous discrete foci defined by the MTOC of the dividing nuclei, localised prominences form in the sporoblast plasma membrane: nascent apical complexes created by the assembly of polar rings and two closely-apposed plasma membranes, together with their associated microtubules, immediately beneath the sporoblast plasma membrane. The sporoblast plasma membrane and closely-apposed underlying plasma membrane doublet form, respectively, the outer plasma membrane and inner membrane complex of the pellicle of the mature sporozoite. The subpellicular network, which gives shape and rigidity to the malaria parasite, is also concurrently formed (Khater *et al.*, 2004). Extension of the pellicle and its associated subpellicular microtubules/network from the junction with the single sporoblast plasma membrane drives eversion of the emergent sporozoite into the subcapsular space. As with ookinetes, the closely apposed membranes of the inner membrane complex are derived from a single flattened vesicle (Dubremetz *et al.*, 1979). However, no pores are present within the inner membrane complex of sporozoites. Sporozoites also lack the apical collar present in ookinetes and only possess approximately 14 to 16 subpellicular microtubules, depending on the malaria species, *a*symmetrically arranged around the malaria parasite circumference. The nuclei eventually complete mitosis and divide, with each resultant daughter nucleus migrating into its own sporozoite bud. Secretory organelles (either micronemes and/or rhoptries – the literature is unclear of their exact identity) subsequently develop within the apical region of the emergent sporozoites, anterior to the in-coming nucleus, while dense

granules may also form within the cytoplasm (again, the literature is uncertain as to when these organelles form). Each sporozoite probably also simultaneously acquires a single apicoplast and mitochondrion. Sporozoite budding occurs synchronously throughout the oocyst: a dense pile of narrow, elongated and finely curved daughter malaria parasites, approximately 1 to 2 by 10 to 20 μm, extends from the diminishing islands of sporoblast cytoplasm "resembling sea-anemones" (Meis *et al.*, 1992b).

The rate of oocyst development during sporogony is highly variable and oocysts of markedly differing states of maturity and size can be found within simultaneously infected individual mosquitoes, even under constant environmental conditions (Huff, 1940; Howells & Davies, 1971; Strome & Beaudoin, 1974; Sinden & Strong, 1978; Chen *et al.*, 1984; Collins *et al.*, 1986b; Klein *et al.*, 1988a). This variation in oocyst maturity and size is probably partly due to some ookinetes attaining the basal lamina and transforming into oocysts before other malaria parasites have completed migration across the midgut epithelium (Garnham *et al.*, 1969). However, the rate of oocyst growth for the same malaria parasite strain also varies between mosquito species/strains implying that asynchrony in the timing of ookinete invasion of the midgut epithelium is not the only explanation for the observed variation in oocyst maturity and size (Huff, 1940; Hunninen, 1953; Ward, 1966; Collins *et al.*, 1969b). Eventually, after approximately 7 to 14 days, the time depending on temperature and the malaria parasite species, the final outcome of sporogony is the production of several thousand mature haploid invasive sporozoite stage malaria parasites packed within a single oocyst capsule (Pringle, 1965; Rosenberg & Rungsiwongse, 1991; Bell & Ranford-Cartwright, 2004). A small residuum of vacuolated sporoblast cytoplasm containing remnant nuclear material and the membrane-bound haemozoin retained from the intra-erythrocytic development of the macrogametocyte stage remains within the oocyst.

Two malaria parasite molecules essential for oocyst differentiation and sporozoite formation have been identified. Circumsporozoite protein (CSP) appears on the oocyst plasma membrane prior to the onset of peripheral vacuolisation and is subsequently found on the plasma membrane surrounding emerging and mature sporozoites (Nagasawa *et al.*, 1987; Posthuma *et al.*, 1988; Hamilton *et al.*, 1988; Nagasawa *et al.*, 1988; Meis & Ponnudurai, 1989; Golenda, Starkweather & Wirtz, 1990; Aikawa *et al.*, 1990; Meis *et al.*, 1992a; Simonetti *et al.*, 1993). Sporozoite budding from the sporoblast membrane is prevented and/or disregulated in CSP mutant malaria parasites, suggesting that this molecule co-ordinates assembly of the inner membrane complex

and subpellicular microtubules beneath the plasma membrane of the sporoblast (Menard *et al.*, 1997; Kocken *et al.*, 2002; Thathy *et al.*, 2002; Wang, Fujioka & Nussenzweig, 2005b). Lectin adhesive-like protein (LAP) 1, a member of a superfamily of relatively large proteins containing multiple adhesive domains, is also apparently necessary for sporozoite formation in *P. berghei* malaria parasites but the function of this molecule is less well-understood (Claudianos *et al.*, 2002; Lasonder *et al.*, 2002; Florens *et al.*, 2002; Trueman *et al.*, 2004; Pradel *et al.*, 2004; Dessens, Sinden & Claudianos, 2004). The other members of the LAP family are primarily expressed during various periods of malaria parasite development within the mosquito vector, but the function of these molecules has yet to be determined.

1.4.9 Sporozoite migration to the salivary glands

Developing oocysts are typically located external to the midgut epithelium, protruding into the haemocoelic cavity of the mosquito (Strome & Beaudoin, 1974; Sinden, 1975b; Chen *et al.*, 1984; Rastogi *et al.*, 1988; Meis & Ponnudurai, 1989; Meis *et al.*, 1992b). Although, in some malaria parasite-mosquito vector combinations, oocysts also occur within the midgut epithelium either in intercellular or, apparently, intracellular locations (Vanderberg *et al.*, 1967; Bafort, 1971; Beaudoin, Strome & Tubergen, 1974; Klein *et al.*, 1988a; Meis *et al.*, 1992b). During sporogony, oocysts undergo massive growth from approximately 4 to 8, to 40 to 80 μm or more in diameter: an approximately 1000-fold increase in volume. Consequently, the basal lamina overlying the oocyst stretches, and eventually tears, directly exposing the malaria parasite to the surrounding haemolymph of the mosquito (Strome & Beaudoin, 1974; Sinden, 1975b; Sinden & Strong, 1978; Meis *et al.*, 1989; Meis *et al.*, 1992b; Mahairaki *et al.*, 2005a). Concomitantly, the oocyst capsule becomes progressively thinner and fragmentary (Duncan *et al.*, 1960; Meis *et al.*, 1989) and numerous small holes appear through which individual sporozoites may pass. However, most sporozoites apparently rapidly escape *en masse* through a single large rupture in the oocyst capsule, possibly caused by an accumulation of the smaller perforations, leaving an empty collapsed oocyst capsule on the external surface of the midgut epithelium (Sinden, 1974; Strome & Beaudoin, 1974; Sinden, 1975b; Sinden & Strong, 1978; Chen *et al.*, 1984; Rastogi *et al.*, 1988; Meis *et al.*, 1992b). Sporozoite release from the oocyst is an active process involving both CSP and a cysteine protease derived from the malaria parasite (Aly & Matuschewski, 2005; Wang, Fujioka & Nussenzweig, 2005a). The liberation of sporozoites from different oocysts of the same age within the same mosquito is

asynchronous, and spread over several days, as oocysts of differing maturity release separate broods of malaria parasites into the haemolymph. However, sporozoite migration to the salivary glands is relatively rapid: sporozoites only persist within the haemocoelic cavity for several days following oocyst maturation (Golenda *et al.*, 1990).

Migration of the sporozoites from the oocyst to the salivary glands is poorly understood. These malaria parasite stages primarily accumulate in the thoracic region of the salivary glands (Robert *et al.*, 1988; Golenda *et al.*, 1990). However, sporozoites are found throughout the haemocoelic cavity of the mosquito (Robert *et al.*, 1988; Golenda *et al.*, 1990), inside midgut epithelial cells and even within the midgut lumen (Beaudoin *et al.*, 1974; Sinden & Strong, 1978). Sporozoites may circulate passively in the haemolymph (Golenda *et al.*, 1990) or migrate actively along the internal organs of the mosquito using chemotaxis to locate the salivary glands (Boulanger, Matile & Betschart, 1988; Boulanger *et al.*, 1995; Akaki & Dvorak, 2005). Many oocyst sporozoites fail to successfully migrate to, and invade, the mosquito salivary glands. The reasons for this reduction in malaria parasite numbers is unclear, but may be partly accounted for by mosquito immune responses, including antimicrobial factors present within the haemolymph and phagocytosis by haemocytes (Garnham, 1966; Weathersby, 1967; Hernandez-Martinez *et al.*, 2002; Hillyer, Schmidt & Christensen, 2003).

1.4.10 Sporozoite invasion of the salivary glands

The salivary glands of adult female mosquitoes consist of a pair of three-lobed glands located within the thorax just posterior to the head (Wright, 1969; Janzen & Wright, 1971; Sterling, Aikawa & Vanderberg, 1973; Meis *et al.*, 1992b; Pimenta, Touray & Miller, 1994; Ando *et al.*, 1999; Moreira-Ferro, Marinotti & Bijovsky, 1999). Each gland possesses one median and two lateral lobes, and each lobe comprises a single layer epithelium of polarised secretory cells encircling a central duct. The central ducts of each lobe, of each gland, unite in a common salivary duct, which extends anteriorly into the proboscis of the female mosquito. The lateral lobes of each gland possess morphologically and functionally distinct proximal, intermediate and distal regions, while the median lobes are shorter and consist only of equivalent intermediate and distal regions. The distal regions of the salivary gland lobes are specialised for bloodfeeding while the proximal regions of the salivary gland lobes appear adapted for sugar-feeding (James, 1994; Moreira-Ferro *et al.*, 1999). The secretory cells of the distal regions of the salivary gland lobes possess large apical cavities, which diminish in size and become increasingly microvillated in the more proximal regions of each

salivary gland lobe. The outer haemocoelic surface of the salivary glands is covered by a thin basal lamina. The overall anatomy of the salivary glands of anopheline and culicine mosquitoes is similar, although the length of the central chitinous salivary duct present within each lobe differs between these mosquito families.

Sporozoites accumulate within the distal regions of the median and lateral lobes of the salivary glands (Meis *et al.*, 1992b; Ando *et al.*, 1999; Frischknecht *et al.*, 2004). This implies that relatively specific receptor-ligand interactions mediate malaria parasite targeting and/or invasion of the salivary glands. Several malaria parasite molecules involved in sporozoite invasion of the salivary glands have been identified, including CSP and thrombospondin-related adhesive/anonymous protein (TRAP), which both belong to the same family of apicomplexan adhesins as the ookinete proteins CTRP and WARP. CSP specifically binds to the distal regions of the lateral and median lobes, and mediates malaria parasite species-specific recognition of the salivary glands (Sidjanski, Vanderberg & Sinnis, 1997; Tewari *et al.*, 2002; Myung, Marshall & Sinnis, 2004; Tewari, Rathore & Crisanti, 2005). This suggests that CSP, independent of any function in mediating gliding motility, is a receptor for sporozoite targeting and attachment to the salivary glands. TRAP is a micronemal adhesin that mediates sporozoite gliding motility and is necessary for sporozoite invasion of the salivary glands (Sultan *et al.*, 1997; Kappe *et al.*, 1999; Wengelnik *et al.*, 1999; Mota *et al.*, 2001; Sultan *et al.*, 2001; Matuschewski *et al.*, 2002). However, the exact role of TRAP in sporozoite invasion of the salivary glands is unclear; there is currently little evidence that TRAP is a specific receptor mediating targeting of the sporozoite to the salivary gland. The function of TRAP in salivary gland invasion appears to be related to its role as an adhesin mediating gliding motility. Another molecule called MAEBL, apparently has no role in sporozoite gliding motility, but is required for sporozoite invasion of the salivary glands (Kariu *et al.*, 2002). This implies that MAEBL mediates specific attachment to, and recognition of, the mosquito salivary glands. Mosquito ligands mediating sporozoite invasion of the salivary glands have not been identified but their existence is suggested by the ability of various antibodies, lectins and peptides specific to the latter mosquito organs to block malaria parasite infection (Barreau *et al.*, 1995; Barreau *et al.*, 1999; Brennan *et al.*, 2000; Ghosh *et al.*, 2001). Furthermore, malaria parasites are able to invade the salivary glands of some but not other mosquito species implying that sporozoite invasion of the salivary glands is mediated by recognition of specific mosquito ligands (Rosenberg, 1985). Unlike ookinete recognition and invasion

of the mosquito midgut epithelium (Section 1.4.7), receptor-ligand interactions between sporozoites and the salivary glands are likely to be highly specific in order to enable malaria parasite targeting to these mosquito organs.

Sporozoites initially attach to, and then penetrate, the basal lamina surrounding the haemocoelic surface of the salivary glands (Sterling *et al.*, 1973; Posthuma *et al.*, 1989; Meis *et al.*, 1992b; Pimenta *et al.*, 1994; Ando *et al.*, 1999). The mechanism of sporozoite penetration of the basal lamina is unknown. However, the sporozoite surface coat, including CSP, is shed during penetration of the basal lamina (Golenda *et al.*, 1990; Meis *et al.*, 1992b; Pimenta *et al.*, 1994). Sporozoites subsequently enter into the underlying salivary gland secretory cells through the either the basal and/or lateral plasma membranes of the host cells (Posthuma *et al.*, 1989; Pimenta *et al.*, 1994). Entry into the salivary gland secretory cells proceeds through formation of a moving junction between the sporozoite pellicle and salivary gland cell plasma membrane, which constricts the malaria parasite during internalisation (Klein *et al.*, 1988b; Pimenta *et al.*, 1994). Unlike ookinete invasion of midgut epithelial cells, sporozoites do not directly pass into the cytoplasm of invaded salivary gland secretory cells but enter *via* parasitophorous vacuole formation (Sinden & Strong, 1978; Klein *et al.*, 1988b; Meis & Ponnudurai, 1989; Posthuma *et al.*, 1989; Pimenta *et al.*, 1994; Ando *et al.*, 1999). Sporozoite-invaded salivary gland secretory cells are sometimes swollen and deformed (Vanderberg *et al.*, 1967; Oelerich, 1967; Suwan *et al.*, 2002). However, in other studies sporozoite-infected salivary gland secretory cells appear morphologically normal, especially in the distal-most region of the salivary glands (Vanderberg & Rhodin, 1967; Posthuma *et al.*, 1989; Pimenta *et al.*, 1994). There is no evidence of irreversible destruction similar to that accompanying intracellular ookinete invasion of the midgut epithelium (although detailed studies on this aspect of the malaria parasite life cycle have yet to be published). Possibly, these apparent differences in the pathology of invaded host cells are related to differences in parasitophorous vacuole formation between ookinetes and sporozoites. Given the potentially large numbers of sporozoites invading the salivary glands, the relatively small size of these mosquito organs, and the dependence of malaria parasite transmission on sporozoite residence within the salivary glands, there may be strong selection pressure on sporozoites not to cause irreversible destruction of large numbers of salivary gland secretory cells.

Once sporozoites are internalised within the salivary gland secretory cell, the parasitophorous vacuole disintegrates leaving these malaria parasite stages in direct

contact with the host cell cytoplasm (Sinden & Strong, 1978; Pimenta *et al.*, 1994; Ando *et al.*, 1999). Host cell mitochondria frequently surround sporozoites during intracellular migration through the cytoplasm to the apical plasma membrane of the salivary gland secretory cell (Pimenta *et al.*, 1994; Ando *et al.*, 1999). The apical plasma membrane of the invaded salivary gland secretory cell stretches around the sporozoite during egress into the secretory cavity, in a manner (at least superficially) analogous to budding of enveloped virons from their host cell. The secretory cell-derived plasma membrane surrounding the sporozoite subsequently disintegrates, leaving an extracellular malaria parasite within the secretory cavity of the salivary gland cell (Pimenta *et al.*, 1994). Sporozoites accumulate *en masse* within the secretory cavities of the salivary gland cells, in tightly-associated parallel-rowed bundles of largely non-motile malaria parasites, and subsequently pass into the central secretory ducts into which the surrounding secretory cavities of the salivary gland cells flow (Meis & Ponnudurai, 1989; Pimenta *et al.*, 1994; Ando *et al.*, 1999; Frischknecht *et al.*, 2004).

In anopheline mosquitoes, the chitin-walled secretory ducts terminate, open-ended, within the distal half of the salivary gland lobes (Vanderberg *et al.*, 1967). This provides sporozoites in the distal regions of the salivary glands with direct access to the lumen of the secretory ducts. In contrast, sporozoites located in the more proximal regions of the salivary glands, where the lumen of the salivary gland is surrounded by the chitin wall, are apparently unable to enter the central salivary duct. In aedine mosquitoes, the chitin walls of the secretory ducts extend the entire length of the salivary gland lobes (Pimenta *et al.*, 1994). Consequently, sporozoites in aedine mosquitoes must penetrate the salivary duct walls in order to enter the lumen of the salivary gland. How sporozoites penetrate the chitin-walled salivary duct of aedine mosquitoes is not known, but possibly malaria parasite passage across this mosquito structure is facilitated by the secretion of chitinase (Pimenta *et al.*, 1994). Once within the salivary ducts, sporozoites actively migrate along the secretory duct lumen, towards the proboscis, using gliding motility (Frischknecht *et al.*, 2004). Anteriorly, the salivary gland secretory ducts become increasingly narrow, eventually only allowing the passage of individual sporozoites (Vanderberg *et al.*, 1967; Pimenta *et al.*, 1994). Only the latter sporozoites, within the anterior region of the salivary duct, are ejected into the vertebrate host during salivation; the majority of the malaria parasites remain congregated in secretory cavities of the salivary glands during bloodfeeding

(Frischknecht *et al.*, 2004). Consequently, only a few of the potentially many thousands of sporozoites present within the salivary glands are actually inoculated into vertebrate host each time the mosquito probes for blood (Vanderberg, 1977; Rosenberg *et al.*, 1990; Ponnudurai *et al.*, 1991; Beier *et al.*, 1991a; Beier *et al.*, 1991b; Li, Sina & Rossignol, 1992; Rosenberg, 1992).

1. 5 Factors affecting malaria parasite infection of the mosquito vector

The number of malaria parasites within individual mosquitoes undergoes dramatic changes during the successive stages of malaria parasite development within the mosquito vector (Vaughan, Noden & Beier, 1992; Vaughan, Hensley & Beier, 1994a; Vaughan, Noden & Beier, 1994b; Awono-Ambene & Robert, 1998; Gouagna *et al.*, 1998; Alavi *et al.*, 2003; 2004; Gouagna *et al.*, 2004a). Typically, even within highly susceptible mosquito species, less than 5% of the approximately 10 to 10,000 gametocytes ingested within the bloodmeal will successfully develop into oocysts on the basal surface of the midgut epithelium. The efficiency of malaria parasite development within individual mosquitoes varies markedly, both within and between malaria parasite-mosquito vector combinations. Some mosquitoes may become very heavily infected with oocysts, while other mosquitoes, fed the same gametocytes, exhibit no oocyst infection. Although the reduction in malaria parasite numbers during the transformation from the gametocyte to oocyst stages is well-documented, the reasons for these malaria parasite losses are poorly-understood. Malaria parasite survival is dependent on multiple malaria parasite, mosquito and vertebrate host determinants, which may act at various times during malaria parasite development within the mosquito vector.

1.5.1 Vertebrate host factors

1.5.1.1 Components of the immune system

During gametogenesis, malaria parasites change from an intra- to extracellular existence and consequently become directly exposed to components of the vertebrate host immune system, present within the bloodmeal, ingested by the mosquito vector. Such vertebrate immune components include complement, specific anti-malaria parasite antibodies, leucocytes/phagocytes, cytokines and other inhibitory factors present within the vertebrate host plasma associated with malaria "crisis" (acute asexual erythrocytic stage infection of the vertebrate host).

1.5.1.1.1 Complement

Gametes and zygotes of the malaria parasite are sensitive to both the alternative (antibody-independent) and classical (antibody-dependent) pathways of complement (Rener *et al.*, 1983; Kaushal *et al.*, 1983b; Grotendorst *et al.*, 1986; Grotendorst & Carter, 1987; Quakyi *et al.*, 1987; Read *et al.*, 1994; Tsuboi *et al.*, 1995; Roeffen *et al.*, 1995; Healer *et al.*, 1997; Margos *et al.*, 2001). Gametes and zygotes are initially resistant to the alternative pathway of complement from their *native* (but not non-native) vertebrate hosts but subsequently lose their resistance between 6 to 8 hours pbf (Grotendorst *et al.*, 1986; Grotendorst & Carter, 1987). This increase in zygote sensitivity is apparently preceded by inactivation of the alternative pathway of complement within the bloodmeal, such that developing malaria parasites are not killed by this complement pathway under normal circumstances. However, gametes and zygotes *are* sensitive to the classical pathway of complement (even from their native vertebrate host) in the presence of antibody specific to these malaria parasite stages (Rener *et al.*, 1983; Kaushal *et al.*, 1983b; Quakyi *et al.*, 1987; Read *et al.*, 1994; Roeffen *et al.*, 1995; Healer *et al.*, 1997). In contrast, mature ookinetes regain resistance to the alternative pathway of complement (Grotendorst & Carter, 1987) and there is no published evidence that these malaria parasite stages are sensitive to antibody-mediated complement lysis.

1.5.1.1.2 Antibodies

The presence of specific antibodies within the bloodmeal may also prevent malaria parasite infection of the mosquito by complement-independent mechanisms. Antibodies against the gamete surface antigens, in particular P48/45, cause agglutination of microgametes preventing their release from the residual parasite body during exflagellation, and also inhibit fertilisation possibly through interference of receptor-ligand interactions between male and female gametes (Rener *et al.*, 1980; Kaushal *et al.*, 1983b; Carter *et al.*, 1990). Antibodies against zygote- and/or ookinete-specific antigens also prevent malaria parasite infection of the mosquito (e.g. Vermeulen *et al.*, 1985a; Li *et al.*, 2004; Abraham *et al.*, 2004). The exact mode of action of these particular inhibitory antibodies is largely unknown but presumably depends on inhibiting the function of their respective target antigens. Antibodies against the zygote/ookinete surface proteins P25 and P28 appear to have multiple modes of action interfering with zygote to ookinete differentiation, ookinete migration through

the bloodmeal, penetration of the peritrophic matrix and, possibly, invasion of the midgut epithelium (Sieber *et al.*, 1991; Ranawaka, Alejo-Blanco & Sinden, 1993; Ranawaka, Alejo-Blanco & Sinden, 1994a; Ranawaka *et al.*, 1994b; Baton & Ranford-Cartwright, 2005a). In general, naturally-acquired antibody responses are only found against gamete/zygote antigens expressed during gametocyte development within the vertebrate host (Graves *et al.*, 1988; Carter *et al.*, 1989; Graves *et al.*, 1992; Premawansa *et al.*, 1994). In contrast, specific-antibody responses to zygote and/or ookinete antigens expressed exclusively within the mosquito vector are not observed in natural field settings (Carter *et al.*, 1989). However, in principle, artificially-induced antibody responses can be generated against any malaria parasite antigens expressed within the mosquito vector providing the rationale underlying immunologically-based transmission-blocking interventions (Yoshida *et al.*, 1999; Yoshida *et al.*, 2001).

In addition to their negative effects, the presence of specific antibodies within the bloodmeal, especially at low titres, has also been reported to *enhance* malaria parasite infection of the mosquito vector through unknown mechanisms (Peiris *et al.*, 1988; Naotunne *et al.*, 1990; Lensen *et al.*, 1992; Ranawaka *et al.*, 1993). Antibodies ingested with the bloodmeal are able to pass through the midgut epithelium into the haemolymph, and consequently may also be capable of affecting malaria parasite development outwith the midgut lumen (Hatfield, 1988; Vaughan & Azad, 1988; Vaughan *et al.*, 1988; Beier *et al.*, 1989; Vaughan *et al.*, 1990; Lensen *et al.*, 1992; Davis *et al.*, 1993; Brennan *et al.*, 2000).

1.5.1.1.3 White blood cells

Several studies have reported evidence of phagocytosis of gametocytes, macrogametes, zygotes and/or ookinetes by vertebrate host leucocytes present within the ingested bloodmeal (Sinden & Smalley, 1976; Sinden *et al.*, 1978; Sinden *et al.*, 1985; Ranawaka *et al.*, 1994a; Lensen *et al.*, 1997; Lensen *et al.*, 1998; Healer, Graszynski & Riley, 1999). Phagocytosis by monocytes/macrophages and polymorphonuclear neutrophils is apparently dependent on antibody-mediated opsinisation of the relevant malaria parasites stages (Ranawaka *et al.*, 1994a; Lensen *et al.*, 1997; Healer *et al.*, 1999). However, the importance of phagocytosis in reducing malaria parasite numbers *in vivo* under natural conditions is controversial and possibly of little significance (Lensen *et al.*, 1997; Lensen *et al.*, 1998; Healer *et al.*, 1999).

1.5.1.1.4 Cytokines

Vertebrate host-derived cytokines ingested within the bloodmeal may also alter malaria parasite infectivity by modulating *mosquito* immune responses. For example, recent studies suggest that ingested latent mammalian TGF-β1 is activated within the bloodmeal and reduces the up-regulation of mosquito nitric oxide synthase associated with *P. falciparum* ookinete invasion of the *An. stephensi* midgut epithelium (Luckhart *et al.*, 2003a; Vodovotz *et al.*, 2004; Lieber & Luckhart, 2004).

1.5.1.2 Asexual erythrocytic stage malaria parasites

Malaria parasite infection of the mosquito vector is also influenced by the asexual erythrocytic stages of the malaria parasite, either indirectly through alteration of the blood of the vertebrate host and/or directly *via* modulation of the responses of the mosquito vector (Boete, Paul & Koella, 2004). Additionally, the presence of antimalarial drugs within the vertebrate host may also act against the malaria parasite stages developing within the mosquito vector if ingested in the bloodmeal (Butcher, 1997).

1.5.1.2.1 Inhibition of gametocyte infectivity associated with peak asexual parasitaemia

The infectivity of gametocytes developing *in vivo* is reduced (and sometimes completely eliminated) during the periods of peak asexual parasitaemia and schizogony, known as "crisis" in experimental models and "paroxysm" in natural infections, despite gametocyte densities in the blood simultaneously peaking during these phases of malaria parasite infection of the vertebrate host (Lumsden & Bertram, 1940; Eyles, 1952; Huff & Marchbank, 1955; Wery, 1968; Mendis & Targett, 1979; Rosenberg & Koontz, 1984; Dearsly, Sinden & Self, 1990; Sinden, 1991). This loss of gametocyte infectivity is caused by non-antibody-dependent serum factors (Eyles, 1952; Huff, Marchbank & Shiroishi, 1958; Fleck, Butcher & Sinden, 1994). Butcher, Sinden & Billker (1996) suggested acidosis of the vertebrate host blood, associated with acute asexual parasitaemia, inhibited bicarbonate-dependent exflagellation of microgametocytes. Other studies suggest cytokine-mediated nitric oxide production by leucocytes reduces the ability of gametocytes to transform into gametes and zygotes (Naotunne *et al.*, 1991; Naotunne *et al.*, 1993; Motard *et al.*, 1993; Cao, Tsuboi & Torii, 1998; Cao *et al.*, 1998). Naotunne *et al.* (1993) showed that the latter mechanism may act both in the vertebrate host and within the bloodmeal ingested by the mosquito.

1.5.1.2.2 Haematocrit

Malaria parasite infectivity to the mosquito vector may be indirectly influenced by asexual erythrocytic stage infection through alteration of the haematocrit of the infected-vertebrate host blood (i.e. anaemia) (Rosenberg *et al.*, 1984; Rosenberg & Koontz, 1984; Taylor & Hurd, 2001). Variation in vertebrate host haematocrit may alter the volume of blood ingested by the mosquito and the density of erythrocytes within the bloodmeal, which may conceivably alter fertilisation success and/or the dynamics of bloodmeal digestion. Furthermore, there is evidence that the sex ratio of gametocytes, and the production of gametocytes from asexual erythrocytic stage parasites, is altered by host anaemia (Paul *et al.*, 2000; Robert *et al.*, 2003; Reece *et al.*, 2005).

1.5.1.2.3 Modulation of mosquito immune responses

Alternatively, asexual erythrocytic stage malaria parasites present within the bloodmeal may directly influence malaria parasite infectivity to the mosquito vector through modulation of mosquito immune responses. Several studies have reported that expression of mosquito immune response genes is altered by the presence of asexual erythrocytic stage malaria parasites within the bloodmeal (Crampton & Luckhart, 2001; Bonnet *et al.*, 2001; Tahar *et al.*, 2002; Luckhart *et al.*, 2003a). Malaria parasite-derived glycosylphosphatidylinositols present within the bloodmeal are able to induce nitric oxide expression in *An. stephensi* midgut epithelia *via* mosquito kinases associated with the insulin signalling pathway (Lim *et al.*, 2005). However, the importance of these interactions for determining the level of malaria parasite infection of the mosquito vector have yet to be determined.

1.5.2 Mosquito factors

1.5.2.1 Gametogenesis and fertilization

The ability of mosquitoes to provide a suitable environment for gametogenesis varies: in some mosquito species, the induction or completion of gametogenesis is prevented (Micks *et al.*, 1948; Omar, 1968b; Yoeli, 1973; Alavi *et al.*, 2003). The reasons for this variation are unknown, but it has been suggested that uncharacterised mosquito-derived factors inhibit the formation of malaria parasite gametes in these mosquito species. In some instances, the failure of mosquitoes to provide a suitable environment for gametogenesis is related to the absence of xanthurenic acid (Arai *et al.*, 2001). The mechanisms of mosquito bloodfeeding and bloodmeal processing may influence the development of malaria parasites within the midgut lumen (Chege &

Beier, 1998). Conceivably, these mechanisms may enhance and/or reduce gametogenesis and/or the ability of microgametes to fertilise macrogametes. However, there is currently little evidence that differences in bloodmeal haemolysis, agglutination and compaction effect mosquito susceptibility to malaria parasite infection.

1.5.2.2 Midgut digestive proteases

The coincident timing of malaria parasite development within the bloodmeal and secretion of digestive proteases from the midgut epithelium suggests that the process of bloodmeal digestion might play a significant role (either negative and/or positive) in malaria parasite infection of the mosquito vector. The appreciable variation observed between mosquito species in the absolute level and timing of peak midgut proteolytic activity (Hörler & Briegel, 1995; Chadee & Beier, 1995; Chege, Pumpuni & Beier, 1996; Caroci *et al.*, 2003) further suggests that differences in bloodmeal digestion might account for the differences in susceptibility to malaria parasite infection between different mosquito species. Asexual and sexual intra-erythrocytic stage malaria parasites present within the midgut lumen are destroyed during bloodmeal digestion implying that malaria parasites are susceptible to the activity of midgut digestive proteases (Huff, 1934; Omar, 1968b). However, there is no conclusive evidence that midgut digestive proteases are significant determinants of either the marked attrition in malaria parasite numbers that occurs within individual mosquitoes or the differential susceptibility to malaria parasite infection observed between mosquito species/strains under normal circumstances.

P. gallinaceum ookinetes are susceptible to damage by *Ae. aegypti* midgut proteases both *in vitro* and *in vivo* (Gass, 1977; Gass, 1979; Gass & Yeates, 1979; Yeates & Steiger, 1981). Trypsin was the major cause of malaria parasite damage, although other mosquito digestive proteases may also have been involved in this malaria parasite-mosquito vector combination. Similarly, cultured *P. berghei* ookinetes are also susceptible to proteolytic degradation by exogenous trypsin *in vitro* (Tomas *et al.*, 2001). The sensitivity of the malaria parasites to proteolytic damage is stage-dependent with zygotes and immature retort-form ookinetes being more sensitive to lytic destruction than mature ookinetes. The variation in sensitivity to proteolytic damage between these different developmental stages was apparently attributable to the presence of the thick surface coat of the ookinete pellicle, which is absent from zygotes and the basal body of transforming zygotes/retort-form ookinetes (Gass, 1979). Under normal conditions, most zygotes and immature retort-form ookinetes, located within the

centre of the ingested bolus of ingested erythrocytes, are unlikely to be exposed to high levels of midgut protease until they have completed transformation into the relatively resistant mature ookinete stage as bloodmeal digestion proceeds from the periphery inwards and peak protease levels are typically attained after ookinete formation begins (Huff, 1934; Fisk, 1950; Fisk & Shambaugh, 1952; Stohler, 1957; Gooding, 1966b; Graf *et al.*, 1986). This implies that malaria parasite development within the midgut lumen is adapted to the timing of bloodmeal digestion and that ookinetes have evolved mechanisms to withstand the process of bloodmeal digestion (Gass, 1977). For example, the P25 and P28 surface proteins appear to protect malaria parasites from digestive proteases (Tomas *et al.*, 2001; Danielli *et al.*, 2005). However, zygotes located in the periphery of the bloodmeal may be unable to complete transformation into mature ookinetes before high protease levels are attained within their region of the midgut lumen (Gass & Yeates, 1979). Additionally, the surface coat overlying the pellicle of mature ookinetes is lost after prolonged exposure to digestive proteases implying that invasion of the midgut epithelium must be achieved relatively rapidly in order to avoid proteolytic destruction within the midgut lumen (Gass, 1979; Gass & Yeates, 1979). Several studies have also provided indirect correlative evidence that malaria parasites are sensitive to mosquito midgut digestive proteases. Ponnudurai *et al.* (1989) claimed that levels of *P. falciparum* oocyst infection were higher in *An. stephensi* mosquitoes who took longer to complete bloodmeal digestion while Vaughan, Noden & Beier (1994a) similarly reported that accelerated rates of bloodmeal digestion resulted in fewer *P. falciparum* ookinetes developing into oocysts in *An .gambiae.*

The sensitivity of malaria parasites to midgut proteases *in vitro* is dose-dependent (Gass & Yeates, 1979; Yeates & Steiger, 1981) suggesting that variation in the level and timing of digestive protease activity may explain differences in the level of malaria parasite infection. For example, mosquito strains that have been selected for susceptibility and refractoriness to *Plasmodium* infection sometimes exhibit differences in midgut protease activity. Feldmann, Billingsley & Savelkoul (1990) reported that *An. stephensi* refractory to *P. falciparum* infection had elevated levels of aminopeptidase activity compared to susceptible *An. stephensi.* However, there are a number of other differences in midgut protein expression between these refractory and susceptible strains of *An. stephensi* (Prevot *et al.*, 1998) and aminopeptidase activity may be an incidental character linked to another trait determining susceptibility/refractoriness to malaria parasite infection. A midgut-specific

aminopeptidase has also been shown to be up-regulated in refractory but not susceptible *An. gambiae* in response to *P. berghei* infection, although it is not known if this protease is involved in bloodmeal digestion (Rosenfeld & Vanderberg, 1999). In contrast, a number of studies have failed to find any association between either trypsin and/or aminopeptidase activity levels and susceptibility to malaria parasite infection (Feldmann, Billingsley & Savelkoul, 1990; Rudin, Billingsley & Saladin, 1991; Chege *et al.*, 1996; Rosenfeld & Vanderberg, 1999; Kaplan, Zwiers & Yan, 2001; Somboon & Prapanthadara, 2002).

Mosquito midgut digestive proteases may also *facilitate* malaria parasite infection of the mosquito. For example, digestive proteases may be responsible for the inactivation of complement and degradation of antibodies within the bloodmeal that may otherwise be inhibitory to malaria parasite development (Grotendorst *et al.*, 1986; Grotendorst & Carter, 1987). In many instances, addition of trypsin inhibitors to the infectious bloodmeal reduces and/or prevents oocyst infection of the mosquito vector implying that bloodmeal digestion is necessary for successful malaria parasite development (Rosenberg *et al.*, 1984; Grotendorst *et al.*, 1986; Shahabuddin *et al.*, 1995a; Shahabuddin *et al.*, 1996; Ramasamy *et al.*, 1996a). This has been interpreted as evidence that mosquito-derived trypsins activate the chitinase enzyme secreted by ookinetes to penetrate the peritrophic matrix surrounding the bloodmeal (see Section 1.4.5) (Shahabuddin *et al.*, 1993; Shahabuddin *et al.*, 1995a; Shahabuddin *et al.*, 1996; Ramasamy *et al.*, 1996a). In other malaria parasite-mosquito vector combinations, the presence of trypsin inhibitors has been reported to *increase* the level of oocyst infection consistent with a negative effect of mosquito digestive proteases on malaria parasite development within the bloodmeal (Ramasamy *et al.*, 1996a). However, the effect of adding protease inhibitors to an infectious bloodmeal should be interpreted with caution, as multiple physiological systems are likely to be simultaneously affected. For example, there is now evidence that malaria parasites possess their own proteases necessary for successful infection of the mosquito vector (Han *et al.*, 2000; Eksi *et al.*, 2004; Xu *et al.*, 2005; Vontas *et al.*, 2005a; 2005b) while the mosquito immune system is regulated by a cascade of serine proteases and their associated serpins (Siden-Kiamos *et al.*, 1996; Han *et al.*, 1997; Dimopoulos *et al.*, 1998; Paskewitz, Reese-Stardy & Gorman, 1999; Danielli *et al.*, 2000; Oduol *et al.*, 2000; Gorman, Andreeva & Paskewitz, 2000a; Gorman, Andreeva & Paskewitz, 2000b; Gorman & Paskewitz, 2001; Danielli, Kafatos & Loukeris, 2003; Danielli *et al.*, 2005; Michel *et al.*, 2005).

1.5.2.3 The peritrophic matrix

Potentially, the peritrophic matrix is a significant physical barrier to malaria parasite infection of the mosquito vector (Stohler, 1957; Meis *et al.*, 1989; Billingsley & Rudin, 1992). The variation in the composition, and timing of the formation, of the peritrophic matrix between mosquito species suggests that the peritrophic matrix might also account for the differences in the susceptibility of mosquitoes to malaria parasite infection (Freyvogel & Stäubli, 1965; Berner *et al.*, 1983; Ponnudurai, Billingsley & Rudin, 1988; Rudin & Hecker, 1989; Moskalyk *et al.*, 1996).

Artificially increasing the thickness of the peritrophic matrix reduces the level of oocyst infection, implying that the peritrophic matrix is a barrier to malaria parasite development within the mosquito vector (Billingsley & Rudin, 1992). However, there is little evidence that, under normal circumstances, the peritrophic matrix is an important determinant of the ability of malaria parasites to infect the mosquito vector. In several different malaria parasite-mosquito vector combinations, the absence of the peritrophic matrix does not result in an increase in the level of oocyst infection, implying that the peritrophic matrix is not a barrier to malaria parasite infection (Billingsley & Rudin, 1992; Shahabuddin *et al.*, 1995b; Ramasamy *et al.*, 1996b). Furthermore, disruption of the peritrophic matrix in mosquito species that are refractory to oocyst infection does not alter their susceptibility to this stage of malaria parasite infection, suggesting that other factors account for the refractoriness of these mosquito species (Shahabuddin *et al.*, 1995b).

1.5.2.4 The midgut epithelium

The mosquito midgut epithelium has long been considered the major determinant of malaria parasite infection in the mosquito vector (Huff, 1941). Indeed, a number of investigators observing normal formation of mature ookinetes within the bloodmeal and then reduced levels of oocyst infection have concluded that the midgut epithelium is the cause of mosquito refractoriness to malaria parasite infection (e.g. Nicolaew & Yakowlewa, 1929; Shute, 1940; Rudin *et al.*, 1991; Vaughan *et al.*, 1994a; Vaughan *et al.*, 1994b; Shahabuddin *et al.*, 1995b; Gouagna *et al.*, 1998; Kaplan *et al.*, 2001).

1.5.2.4.1 Receptor-ligand interactions

Specific receptor-ligand interactions have often been cited as a determinant of the ability of ookinetes to invade the midgut epithelium. Ookinetes have been reported to accumulate on the luminal side of the midgut epithelium suggesting that the malaria

parasites were unable to penetrate into the midgut epithelium (Gonzalez-Ceron *et al.*, 2001). Further, polymorphism in the mosquito midgut mucins AgMUC1 and AeIMUC1 is correlated with differences in susceptibility to malaria parasite infection (Shen *et al.*, 1999; Morlais & Severson, 2001; Devenport *et al.*, 2005). However, there is no direct evidence the latter molecules mediate ookinete invasion of the midgut epithelium. Although receptor-ligand interactions are presumably essential for ookinete invasion of the midgut epithelium (i.e. for enabling gliding motility), with respect to mosquito susceptibility to malaria parasite infection, the important issue is how *specific* are the receptor-ligand interactions between ookinetes and the midgut epithelium. Do ookinetes recognise particular ligands unique to a given sub-population of mosquitoes or more general molecular motifs conserved across all mosquito species? The ability of evolutionarily divergent malaria parasite species to successfully infect equally distantly-related mosquito species implies that the receptor-ligand interactions mediating ookinete entry into the midgut epithelium are relatively non-specific (e.g. Gupta *et al.*, 2005) and not a major determinant of the level of malaria parasite infection of the mosquito vector.

1.5.2.4.2 Immune responses

The mosquito midgut epithelium is an immune competent organ capable of mounting considerable antiparasitic responses against invading ookinetes, including a lytic mechanism, melanisation, nitric oxide-mediated killing, and oocyst developmental arrest (Collins *et al.*, 1986a; Paskewitz *et al.*, 1988; Paskewitz *et al.*, 1989; Vernick *et al.*, 1995; Luckhart *et al.*, 1998; Gonzalez-Ceron *et al.*, 2001; Blandin *et al.*, 2004). Whether these various immune responses represent distinct mechanisms of mosquito refractoriness or different manifestations of the same underlying response is currently uncertain. For example, the mosquito recognition protein TEP1 is involved in both lysis and melanisation of ookinetes within the midgut epithelium (Blandin *et al.*, 2004). Recent research has greatly advanced our knowledge of mosquito immune responses and a number immune-related molecules have been identified that influence ookinete survival within the midgut epithelium.

1.5.2.5 Immune responses

Unlike vertebrates, mosquitoes are thought to lack adaptive immunity and only possess innate immune responses mediated by the action of various antimicrobial peptides/proteins (Hoffmann *et al.*, 1999). The *An. gambiae* genome project has

enabled the molecular characterisation of the mosquito immune system (Christophides *et al.*, 2002; Holt *et al.*, 2002; Zdobnov *et al.*, 2002) and the identification of various immune response-related molecules involved in pathogen recognition, signal transduction pathways, and effector mechanisms (for recent detailed reviews see Christophides, Vlachou & Kafatos, 2004; Osta *et al.*, 2004b). Malaria parasite infection of the mosquito has been shown to activate various components of this innate immune response, both within the midgut epithelium and the salivary gland epithelia, as well as systemically (e.g. the fat body and haemolymph) (Dimopoulos *et al.*, 1997; Dimopoulos *et al.*, 1998). High-throughput transcriptional profiling has further identified a large number of molecular changes occurring in mosquito gene expression during malaria parasite infection (Oduol *et al.*, 2000; Bonnet *et al.*, 2001; Tahar *et al.*, 2002; Dimopoulos *et al.*, 2002a; Abraham *et al.*, 2004; Srinivasan *et al.*, 2004; Vlachou *et al.*, 2005; Xu *et al.*, 2005). However, data on the significance of most of these transcriptional changes for the outcome of malaria parasite infection of the mosquito has yet to be published.

1.5.2.5.1 Recognition proteins

A number of mosquito recognition molecules are transcriptionally up-regulated during malaria parasite infection of the mosquito vector, including a peptidoglycan recognition protein, a gram-negative binding protein, three thioester-containing proteins (TEPs), several C-type lectins (CTLs), two galactoside-binding lectins, several fibrinogen-like domain lectins, a scavenger receptor protein and a leucine-rich repeat immune protein (LRIM) (Dimopoulos *et al.*, 1997; Richman *et al.*, 1997; Dimopoulos *et al.*, 1998; Oduol *et al.*, 2000; Tahar *et al.*, 2002; Dimopoulos *et al.*, 2002a; Christophides *et al.*, 2004; Vlachou *et al.*, 2005). Gene silencing experiments have shown that TEP1 and LRIM1 have an inhibitory effect on malaria parasite infection of the midgut epithelium, while two CTLs (CTL4 and CTLMA2) appear to protect malaria parasites from the mosquito immune effector mechanisms (Blandin *et al.*, 2004; Osta, Christophides & Kafatos, 2004a). Whether these recognition proteins are directly toxic to malaria parasites or act as opsonins for down-stream immune effector mechanisms is currently unknown. However, all four recognition proteins are apparently involved in mediating melanisation of malaria parasites within the midgut epithelium. Gene silencing studies on the other recognition proteins that are differentially expressed during malaria parasite infection of the mosquito vector have not been published.

1.5.2.5.2 Signalling pathways

Proteolytic cascades involving feedback mechanisms between serine proteases and their associated inhibitors (serpins) regulate various aspects of the invertebrate immune response, including signalling between different tissues and the pro-phenoloxidase activation required for melanisation (Jiang & Kanost, 2000; Nappi & Christensen, 2005; Christensen *et al.*, 2005). A number of mosquito serine proteases and serpins with putative immune-related functions are differentially expressed following malaria parasite infection (Dimopoulos *et al.*, 1997; Dimopoulos *et al.*, 1998; Oduol *et al.*, 2000; Tahar *et al.*, 2002; Dimopoulos *et al.*, 2002a; Danielli *et al.*, 2003; Christophides *et al.*, 2004). Gene silencing of the serine protease inhibitor SRPN2 results in systemic disregulation of the mosquito melanisation response, but also dramatically reduces the number of ookinetes that successfully invade the midgut epithelium (Michel *et al.*, 2005). SRPN10 is over-expressed and undergoes redistribution from the nucleus to the cytoplasm in ookinete-invaded midgut epithelial cells (Danielli *et al.*, 2005). However, there is no evidence that this serine protease inhibitor regulates the level of malaria parasite infection of the mosquito vector. The roles of the other serpins and serine proteases differentially expressed during malaria parasite infection of the mosquito vector have not been published

Mosquitoes also possess components of the Toll, Imd and JAK/STAT immune signalling pathways (Barillas-Mury *et al.*, 1996; Luo & Zheng, 2000; Christophides *et al.*, 2002; Hoffmann & Reichhart, 2002; Shin *et al.*, 2002; Luna *et al.*, 2002; Shin *et al.*, 2003; Luna *et al.*, 2003; Mizutani *et al.*, 2003; Christophides *et al.*, 2004; Bian *et al.*, 2005; Shin *et al.*, 2005). REL2, a NF-κB-like transcription factor, mediates Imd-dependent regulation of the level of oocyst infection and Imd-independent control of ookinete melanisation (Meister *et al.*, 2005). A member of the STAT family of transcription factors also fails to undergo typical immune-responsive translocation from the cytoplasm to the nucleus in ookinete-invaded midgut epithelial cells (Barillas-Mury *et al.*, 1999; Han *et al.*, 2000).

1.5.2.5.3 Antimicrobial peptides

Various exogenous antimicrobial peptides have been shown to negatively effect the different stages of malaria parasite development that occur within the mosquito vector (Gwadz *et al.*, 1989; Rodriguez *et al.*, 1995; Possani *et al.*, 1998; Boisbouvier *et al.*, 1998; Shahabuddin *et al.*, 1998a; Lowenberger *et al.*, 1999; Conde *et al.*, 2000;

Arrighi *et al.*, 2002; Possani *et al.*, 2002; Shin, Kokoza & Raikhel, 2003; Kim *et al.*, 2004). However, the significance of these observations for understanding malaria infection of the mosquito under normal circumstances are unclear: the antimicrobial peptides studied were either synthetic, naturally-occurring but not derived from mosquitoes, or mosquito-derived but expressed ectopically. For example, several studies showed that malaria parasite stages developing within the bloodmeal were susceptible to antimicrobial peptides (Rodriguez *et al.*, 1995; Arrighi & Hurd, 2002). However, it is not known if endogenous antimicrobial peptides are present within the mosquito midgut lumen and, if so, whether malaria parasites developing within the bloodmeal are exposed to such components of the mosquito immune system. The endogenous antimicrobial peptides defensin, cecropin and (inconsistently) gambicin have all been shown to be transcriptionally up-regulated during malaria parasite infection of the mosquito (Dimopoulos *et al.*, 1997; Richman *et al.*, 1997; Dimopoulos *et al.*, 1998; Vizioli *et al.*, 2000; Vizioli *et al.*, 2001a; Tahar *et al.*, 2002; Christophides *et al.*, 2004). However, the significance of these observations is again uncertain: transcriptional up-regulation of defensin and cecropin occurs in the anterior midgut as well as systemically (rather than the posterior midgut, the site of ookinete invasion), while gene-silencing of defensin has no effect on the level of oocyst infection (Blandin *et al.*, 2002). Surprisingly, despite the availability of recombinant/synthetic mosquito defensin and cecropin, the activity of these antimicrobial peptides against malaria parasites developing *in vitro* has not been published (Vizioli *et al.*, 2000; Vizioli *et al.*, 2001c). Although there is no direct evidence that endogenous antimicrobial peptides limit malaria parasite development within the mosquito vector, levels of *P. falciparum* in naturally-infected *An. gambiae* are associated with particular genetic variants of cecropin (Luckhart *et al.*, 2003b).

1.5.2.5.4 Reactive nitrogen/oxygen intermediates

Ookinete invasion of the mosquito midgut epithelium induces up-regulation of mosquito nitric oxide synthase and the production of nitric oxide within invaded midgut epithelial cells (Luckhart *et al.*, 1998; Dimopoulos *et al.*, 1998; Han *et al.*, 2000; Tahar *et al.*, 2002; Luckhart *et al.*, 2003b; Herrera-Ortiz *et al.*, 2004). Nitric oxide is toxic to ookinetes *in vitro* and correlated with levels of oocyst infection *in vivo* implying that nitric oxide production inhibits malaria parasite infection of the mosquito vector. However, up-regulation of nitric oxide synthase is not always induced following ookinete invasion of midgut epithelial cells (Gupta *et al.*, 2005). Ookinete invasion of

the midgut epithelium also induces up-regulation of mosquito peroxidases, enabling the localised production of superoxide anions within ookinete-invaded midgut epithelial cells (Kumar *et al.*, 2004). Superoxide anions are toxic to ookinetes *in vitro* (Lanz-Mendoza *et al.*, 2002) while a refractory strain of *An. gambiae* is in a chronic state of oxidative stress, constitutively producing high levels of reactive oxygen intermediates, which may account for the melanisation immune response of this mosquito strain to malaria parasite infection (Kumar *et al.*, 2003).

1.5.2.5.5 Haemocytes

Mosquitoes also possess a variety of haemocytes, which are found within the haemocoel (Foley, 1978; Willott *et al.*, 1995; Hernandez *et al.*, 1999; Hillyer & Christensen, 2002). Haemocytes are unlikely to mediate direct immune responses against malaria parasites within the midgut lumen (although haemocytes may be involved in immune signalling between different mosquito tissues). However, haemocytes may be a source of the factors involved in the immune responses against extracellular ookinetes emerging from the basal region of the midgut epithelium (Levashina *et al.*, 2001; Blandin *et al.*, 2004) and may directly mediate immune responses against malaria parasites located within the haemocoel (e.g. phagocytosis of sporozoites) (Hernandez-Martinez *et al.*, 2002; Hillyer *et al.*, 2003).

1.5.3 Malaria parasite factors

Extremely little is known about malaria parasite factors determining the differential infectivity of malaria parasites to the mosquito vector. As described in Section 1. 4, a number of malaria parasite molecules have been identified that are necessary for infection of the mosquito vector, but, with one exception (CSP; Tewari *et al.*, 2005), there is no evidence that the polymorphism observed in these molecules explains the differential infectivity of malaria parasites to mosquitoes. Malaria parasite species also vary in their sensitivity to xanthurenic acid suggesting that the ability to undergo gametogenesis may vary between malaria parasite-mosquito vector combinations (Arai *et al.*, 2001). There is no evidence that malaria parasites differ in their sensitivity to midgut digestive proteases or their ability to penetrate the peritrophic matrix. However, the number and substrate specificity of chitinases apparently differs between malaria parasite species, with some Plasmodia possessing one and others two chitinases (Vinetz *et al.*, 1999; Vinetz *et al.*, 2000; Tsai *et al.*, 2001; Tsuboi *et al.*, 2003). The significance of these observations is unknown. Possibly, the variation in

chitinases indicates differences in the structure of the peritrophic matrix between mosquito vector species (Alavi *et al.*, 2003) or the requirement to invade the chitinised salivary ducts of aedine mosquitoes (Section 1.4.10). Malaria parasite species and strains vary in their sensitivity to mosquito immune responses (Collins *et al.*, 1986a; Gonzalez-Ceron *et al.*, 1999; Gonzalez-Ceron *et al.*, 2000; Gonzalez-Ceron *et al.*, 2001) and there is evidence that malaria parasites can suppress mosquito immune responses (Boete, Paul & Koella, 2002; Boete *et al.*, 2004). The mechanisms underlying these traits are unknown, but CSP is apparently involved in sporozoite evasion of the melanisation immune response (Tewari *et al.*, 2005).

1. 6 Summary

The malaria parasite life cycle within the mosquito vector is being understood in ever greater detail. In particular, great progress is being made in characterising the mosquito immune response and its role in determining the outcome of malaria parasite infection of the mosquito vector, especially during ookinete invasion of the midgut epithelium. A number of differences have been identified between mosquitoes that differ in their susceptibility to malaria parasite infection. In contrast, relatively little is known about malaria parasite factors determining differential infectivity to the mosquito vector.

Chapter 2. Comparative infectivity of different *P. falciparum* clones to *An. albimanus* and *An. stephensi*

2. 1 Introduction

There is a vast body of literature describing the comparative infectivity of various malaria parasite species to diverse mosquito species, far too extensive to be briefly summarised here (for example, reviews by Covell (1927), Huff (1965) and Warren & Collins (1981) provide a taster of the vastness of this literature). Consequently, only those studies investigating the infectivity of malaria parasites to *An. albimanus* and *An. stephensi* will be considered in detail. Most of the latter studies report the infectivity of malaria parasites to either one or other of these two mosquito species (although comparisons were often made with other mosquito species), while relatively few investigators have simultaneously assessed the susceptibility of both mosquitoes to the same malaria parasite species. In the current context, the latter studies are the most informative as they allow direct comparisons to be made about the relative susceptibility of the two mosquito species to malaria parasite infection. Most of the previous studies also only examined mosquitoes for mature oocyst infections. Consequently, where the level of oocyst infection differs markedly between mosquito species, usually no attempt was made to characterise the reasons for the observed variation in the level of malaria parasite infection.

2.1.1 Susceptibility of *An. albimanus* to *Plasmodium* infection

2.1.1.1 Human malaria parasite species

Several investigators have compared the infectivity of various isolates of *P. falciparum* and *P. vivax* to different *An. albimanus* strains using either induced infections of patients receiving malariotherapy or naturally acquired infections of soldiers returning from duty in endemic areas (Boyd, Carr & Rozeboom, 1938; Boyd & Jobbins, 1940; Young *et al.*, 1946; Young & Burgess, 1948; Eyles & Jeffrey, 1949; Eyles & Young, 1950; Jeffrey, Eyles & Young, 1950; Jeffrey, Burgess & Eyles, 1954).

Boyd *et al.* (1938) compared the infectivity of *P. falciparum* and *P. vivax* isolates from Cuba and Florida (Long isolate) to *An. albimanus* from Cuba and Panama (Gorgas strain). Both *An. albimanus* strains were moderately to highly susceptible to *P. falciparum* and *P. vivax* isolates from Cuba (oocyst prevalence 24.6 to 63.6%) but exhibited very low susceptibility to infection with parasites from Florida (oocyst

prevalence 1.5 to 6.1%). In contrast, *An. punctipennis* and *An. quadrimaculatus* from Florida showed high levels of infection regardless of the geographical origin of the malaria parasites (oocyst prevalence 16.7 to 68.2% and 50.8 to 82.0% respectively). Boyd and Jobbins (1940) subsequently compared the infectivity of *P. falciparum* from Florida (Long), Mexico and Panama to *An. albimanus* from Panama (Gorgas) and to *An. quadrimaculatus*. *An. albimanus* showed low levels of susceptibility to all three parasite isolates with the Floridan having the lowest infectivity (oocyst prevalence 7.5, 13.5, and 13.6% respectively) whilst *An. quadrimaculatus* exhibited low to moderate levels of infection (oocyst prevalence 48.9, 57.1 and 17.6% respectively). On the basis of these studies the authors concluded that *An. albimanus* showed a high degree of specificity in its susceptibility to malaria parasite infection, only being infected by "coindigenous" neotropical parasite isolates, while *An. quadrimaculatus* was more generally susceptible, being infected by both nearctic and neotropical malaria parasite strains.

A similar set of investigations by another group of workers were similarly interpreted, although the pattern was somewhat less marked (Young *et al.*, 1946; Young & Burgess, 1948; Eyles & Jeffrey, 1949; Eyles & Young, 1950; Jeffrey *et al.*, 1954). *An. albimanus* from Texas were highly refractory to *P. vivax* from New Guinea (Chesson) (oocyst prevalence 1.4%) while *An. freeborni* (F-1), *An. punctipennis*, *An. pseudopunctipennis* and *An. quadrimaculatus* (Q-1) from the southern United States were moderately to highly susceptible (oocyst prevalence ranged from 24.6 to 93.4%) (Young *et al.*, 1946; Young & Burgess, 1948). Similarly, *An. albimanus* from Panama (A-2; synonymous with Rozeboom's Gorgas?) and Florida (A-3) were highly refractory to infection with the New Guinea (Chesson) isolate of *P. vivax* and other isolates of the same parasite species from the southern United States (St. Elizabeth) and Korea (oocyst prevalence 0.0 to 1.8% and mean oocyst intensity 0.0 to 6.0 per midgut – both mosquito strains combined) (Jeffrey *et al.*, 1954). Again, *An. quadrimaculatus* (Q-1) exposed to the same parasites became heavily infected (oocyst prevalence 60.6 to 85.0% and mean oocyst intensity 15.7 to 246.6 per midgut). The infectivity of *P. falciparum* from Southern Carolina (Santee-Cooper) and Panama (El Limon) to *An. albimanus* (A-2 and A-3), and *An. quadrimaculatus* (Q-1), were also compared by the same workers (Eyles & Jeffrey, 1949; Eyles & Young, 1950; Jeffrey *et al.*, 1950). The level of oocyst infection with the American *P. falciparum* isolate was higher in *An. quadrimaculatus* than in *An. albimanus* (A-2) (oocyst prevalence 58.0 versus 28.0% and mean oocyst

intensity 38.5 versus 15.5 per midgut) (Eyles & Young, 1950). The Panamanian isolate exhibited the reciprocal pattern of infectivity (oocyst prevalence 62.1 versus 79.9% and 76.8 versus 82.3% in *An. quadrimaculatus* versus A-2 and A-3, respectively) (Jeffrey *et al.*, 1950). The Panamanian *An. albimanus* were marginally but significantly more susceptible to the El Limon *P. falciparum* isolate than the same species of mosquito from Florida (oocyst prevalence 86.4 versus 81.2%) (Jeffrey *et al.*, 1950). Analysis of the relationship between the prevalence and intensity of oocyst infection in separate feeding experiments also showed that all Panamanian *An. albimanus* were susceptible to coindigenous parasites (Jeffrey *et al.*, 1950) while greater than 30% of this mosquito strain appeared refractory to the non-indigenous Santee-Cooper isolate (Eyles & Young, 1950). Although these findings supported the conclusions of Boyd and colleagues, the pattern was considerably less marked. The Panamanian *An. albimanus* did exhibit an appreciable, if moderate, susceptibility to the Santee-Cooper isolate of *P. falciparum* and were capable of acting as an efficient vector for this parasite (Eyles & Young, 1950). Similarly, *An. albimanus* from Florida exhibited high susceptibility to the non-indigenous El Limon *P. falciparum* isolate. However, the susceptibility of *An. albimanus* from El Salvador (A-9) to infection with the El Limon *P. falciparum* isolate was less than that of *An. quadrimaculatus* (Q-1) and *An. freeborni* (F-1) (oocyst prevalence 16.0 versus 35.0 and 88.0%, respectively) (Collins *et al.*, 1964). *An. albimanus* (A-9) was also less susceptible than *An. quadrimaculatus* (Q-1) and *An. freeborni* (F-1) to *P. falciparum* isolates from Columbia (oocyst prevalence A-9 versus Q-1 was 0.0 to 36.0 versus 32.0 to 89.0%) (Eyles & Jeffrey, 1949; Collins, 1962), the southern United States (South Carolina; McLendon) (oocyst prevalence 26.8 versus 62.4 and 38.0 to 48.2%, respectively) and Thailand (oocyst prevalence 0.3 to 0.6 versus 86.0 to 87.2 and 55.1 to 68.5%, respectively) (Collins, Jeffrey & Burgess, 1963).

The susceptibility of *An. albimanus* (A-2 and A-3) to *P. ovale* (Donaldson isolate) from the western Pacific (probably the Philippines) has also been investigated (Jeffrey, 1954). The level of infection in *An. albimanus* was less than that in *An. quadrimaculatus* (Q-1) but exhibited a similar moderately high level (oocyst prevalence 33.0 and 46.0 versus 53.0% and mean oocyst intensity 10.8 and 13.4 versus 15.0 per midgut, respectively). The level of infection was marginally higher in the coindigenous *An. albimanus* strain (A-3; Florida) than the non-indigenous (A-2; Panama) strain.

More recently, several studies have investigated the susceptibility of laboratory-reared *An. albimanus* to *P. vivax* using malaria parasites from naturally-infected

individuals (Olano *et al.*, 1985; Gonzalez-Ceron *et al.*, 1999; Gonzalez-Ceron *et al.*, 2000; Gonzalez-Ceron *et al.*, 2001). *An. albimanus* (Cartagena, Columbia) directly fed on *P. falciparum* and *P. vivax*-infected patients developed relatively low levels of oocyst infection (prevalence 8.7 and 19.1%, and mean intensity 12.7 and 17.2 respectively) (Olano *et al.*, 1985). However, no other mosquito species were simultaneously fed on so the low level of infection could result from either low infectiousness of the patients or low susceptibility of *An. albimanus* (Olano *et al.*, 1985). An elegant series of studies has shown that in a region of southern Mexico the distribution of two different variants of *P. vivax* is determined by the differential infectivity of these malaria parasite strains to local *An. albimanus* and *An. pseudopunctipennis* populations (Gonzalez-Ceron *et al.*, 1999; Rodriguez *et al.*, 2000). The *P. vivax* CSP VK210 variant heavily infects *An. albimanus* but frequently fails to infect *An. pseudopunctipennis* while the *P. vivax* CSP VK247 variant shows the opposite pattern of infectivity to these two mosquito species. Each *P. vivax* variant is primarily found only in areas where its respective susceptible mosquito species is present, implying adaptation and differentiation of the malaria parasite population to local mosquito vector species.

The infectivity of several *P. falciparum* clones cultured *in vitro* has also been investigated (Teklehaimanot *et al.*, 1987; Vaughan *et al.*, 1994b; Chege *et al.*, 1996). Vaughan *et al.*, (1994b) reported that *An. albimanus* exhibited very low levels of oocyst infection with the *P. falciparum* NF54 isolate compared to *An. arabiensis, An. dirus, An. freeborni, An. gambiae* (G-3) and *An. stephensi* (Pakistan). Chege *et al.*, (1996) similarly found that *An. albimanus* (Panama) was markedly less susceptible to *P. falciparum* NF54 oocyst infection than *An. freeborni* and *An. gambiae* (prevalence 0.0 to 2.7 versus 14 .0 to 74.7%). *An. albimanus* (Archaie) also exhibited poor susceptibility to the *P. falciparum* W2 clone from Indochina while *An. freeborni* and *An. quadrimaculatus* were heavily infected (prevalence 4.0% versus 62.0 and 78.0%, and mean intensity 1.0 versus 10.8 and 6.2 per midgut, respectively) (Teklehaimanot *et al.*, 1987). In contrast, a number of different *An. albimanus* strains exhibited levels of oocyst infection with the *P. falciparum* HB3 clone equivalent to those observed in *An. freeborni* and *An. quadrimaculatus* (prevalence 6.2 to 19.1% versus 19.7 and 8.8%, respectively) (Teklehaimanot *et al.*, 1987).

A number of studies have also been undertaken using non-human primates experimentally infected with various human isolates of *P. vivax* (Collins *et al.*, 1976;

Collins *et al.*, 1977; Collins *et al.*, 1980a; 1980b; Collins *et al.*, 1985c; Li *et al.*, 2001). *An albimanus* (A-1) did not become infected with the Panama II strain of *P. falciparum* whereas *An. freeborni* was heavily infected (prevalence 0.0 versus 43.2%) (Collins *et al.*, 1973). The susceptibility of five *An. albimanus* strains (Apastepeque/El Salvador, CA-109A/El Salvador, Melara/El Salvador, San Diego-Norte/El Salvador, Escobol/Panama) to *P. falciparum* (Santa Lucia, El Salvador) were compared (Collins *et al.*, 1977). The level of oocyst infection was markedly lower in the *An. albimanus* strains than in *An. freeborni* fed simultaneously (prevalence 21.4 to 29.6 versus 85.9 to 87.4%). *An. balabacensis* and *An. maculatus* were also more heavily infected than the *An. albimanus* strains (prevalence 40.5 and 46.7% respectively). However, another study found that the Santa Lucia isolate of *P. falciparum* gave similar levels of oocyst infection in *An. albimanus* (Escobol/Panama and San Diego-Norte/El Salvador) and *An. freeborni* (no data given) (Collins *et al.*, 1985c).

An. albimanus tends to be markedly more susceptible to *P. vivax* isolates from the New World than the Old World (Warren & Collins, 1981; Li *et al.*, 2001). *P. vivax* isolates from El Salvador (Salvador II) and Colombia (Rio Meta) exhibited moderate infectivity to different *An. albimanus* strains (Collins *et al.*, 1976; Collins *et al.*, 1985c). The Salvador II isolate gave similar levels of oocyst infection in *An. albimanus* (Apastepeque/El Salvador, CA-109A/El Salvador, Melara/El Salvador, Panama I/Gorgas), *An. freeborni* and *An. maculatus* (prevalence 13.6 to 45.5 versus 34.9 to 52.7%, and mean intensity 0.75 to 4.61 versus 2.69 to 7.00 (Collins *et al.*, 1976). The Rio Meta isolate gave slightly lower levels of oocyst infection in *An. albimanus* (Apastepeque/El Salvador, Melara/El Salvador, Panama I/Gorgas) compared to *An. freeborni* and *An. maculatus* (prevalence 10.4 to 20.9 versus 18.9 to 52.0%, and mean intensity 0.27 to 0.97 versus 0.61 to 4.21). In contrast to the findings of earlier studies, *An. albimanus* from Panama were more susceptible to infection with the *P. vivax* Salvador II isolate than any of the El Salvador strains of *An. albimanus* (Collins *et al.*, 1976). Further studies using the Salvador II and Rio Meta *P. vivax* isolates but other *An. albimanus* strains (Arachiae/Haiti, Escobol/Panama, Papayal/Columbia and San Diego-Norte/El Salvador) made similar observations (Collins *et al.*, 1985c). However, *An. albimanus* from Panama was less susceptible than *An. albimanus* from Colombia to the Colombian Rio Meta isolate. Three *An. albimanus* strains (Escobol/Panama, Papayal/Columbia and San Diego-Norte/El Salvador) were also shown to be more susceptible to oocyst infection than *An. albitarsis* (prevalence 12.2 to 65.9 versus 1.7 to

21.0 % respectively) whilst another *An. albimanus* strain (Arachiae/Haiti) was less susceptible (prevalence 3.7 to 21.3 versus 15.9 to 31.6% respectively) (Collins *et al.*, 1985c). In contrast to these studies, a *P. vivax* isolate from West Pakistan exhibited extremely low infectivity to three *An. albimanus* strains (Arachiae/Haiti, Escobol/Panama, and San Diego-Norte/El Salvador) whilst *An. balabacensis*, *An. culicifacies*, *An. freeborni*, and *An. maculatus* moderately infected (prevalence 0.0 to 1.6 versus 25.9 to 27.5% respectively) (Collins *et al.*, 1980b). These studies demonstrated significant variation in the level of oocyst infection *between* geographically different strains of *An. albimanus*. Other studies have further shown the existence of significant variation in susceptibility to *P. falciparum* and *P. vivax* infection *within* different *An. albimanus* strains (Warren *et al.*, 1977; Warren *et al.*, 1979; Chan *et al.*, 1994; Gonzalez-Ceron *et al.*, 2000).

2.1.1.2 Non-human primate malaria parasite species

Several studies have investigated the infectivity of various non-human malaria parasite species to *An. albimanus* (Eyles, 1960; Omar, 1968a; Collins *et al.*, 1969a; Collins *et al.*, 1985a; Collins, Warren & Galland, 1999).

P. brasilianum, a malaria parasite of New World monkeys believed to be synonymous with the human parasite *P. malariae* (Ayala *et al.*, 1998; Leclerc *et al.*, 2004), failed to infect *An. albimanus* (A-9) but produced moderate to heavy infections in *An. freeborni* (F-1), *An. maculatus*, *An. quadrimaculatus* (Q-1), *An. stephensi* (India) and *An. sundaicus* (oocyst prevalence 9 to 83% and mean intensity 4.1 to 84.2 per midgut) (Collins *et al.*, 1969a). Another isolate of *P. brasilianum* (Peruvian I/CDC) was also unable to infect another strain of *An. albimanus* (San-Diego-Norte, El Salvador) but two further *An. albimanus* strains (Arachiae, Haiti and Escobol, Panama) were infected with oocysts (prevalence 2.4 and 11.1% and mean intensity 0.01 and 0.23 respectively) (Collins *et al.*, 1985a). In contrast, *An. culicifacies*, *An. dirus*, *An. freeborni*, *An. gambiae* (G-3), *An. maculatus*, *An. quadrimaculatus* (Q-2), and *An. stephensi* (India) exhibited higher levels of oocyst infection (prevalence 9.7 to 54.8% and mean intensity 0.70 to 10.30) (Collins *et al.*, 1985a).

P. cynomolgi, a South Asian malaria of macaques related to *P. vivax* (Escalante *et al.*, 1998; Perkins & Schall, 2002; Leclerc *et al.*, 2004; Escalante *et al.*, 2005), has been investigated on several occasions for its infectivity to *An. albimanus* (Eyles, 1960; Omar, 1968a; Collins *et al.*, 1999). Two strains of *An. albimanus*, from Mexico and Panama, had very low susceptibility to *P. cynomolgi bastianellii* compared to *An.*

gambiae, *An. atroparvus*, *An. sacharovi* and *An. stephensi* (Iraq) (prevalence 0.7 to 2.3 versus 40.0 to 82.1% and mean intensity 1 to 10 and 13.8 to 120.8 per midgut respectively) (Omar, 1968a). *An. albimanus* (A-9 from El Salvador?) also had low susceptibility to *P. cynomolgi bastianellii* compared to *An. quadrimaculatus* (Q-1) (oocyst prevalence 2.1 versus 65.0% and mean oocyst intensity 0 to 4.0 versus 3.0 to 469.0 per midgut respectively) (Eyles, 1960). In contrast, the Berok isolate of *P. cynomolgi* showed a slightly increased ability to infect *An. albimanus* (A-9?) (prevalence 11.8% and oocyst range 0 to 8 per midgut) although the level of infection was still markedly lower than that in other anophelines tested such as *An. freeborni* (F-1) and *An. stephensi* (India) (prevalence 40.9 and 45.2% and oocyst range 0 to 500 and 0 to 200 per midgut respectively) (Collins *et al.*, 1999). Studies with the R O strain of *P. cynomolgi* have also shown that *An. albimanus* is refractory to this malaria parasite while *An. freeborni* and *An. stephensi* are both heavily infected (Collins, Jones & Dobrovolony, 1965).

P. gonderi, a malaria parasite of African monkeys distantly related to the *vivax*-like malarias (Leclerc *et al.*, 2004; Escalante *et al.*, 2005), exhibited poor infectivity to *An. albimanus* but relatively high infectivity to *An. atroparvus*, *An. dirus, An. freeborni, An. maculatus*, *An. quadrimaculatus* and *An. stephensi* (prevalence 2.4 versus 21.5 to 37.0% and mean intensity 0.1 versus 2.2 to 10.8 per midgut respectively) (Collins & Contacos, 1980). *An. albimanus* and *An. quadrimaculatus* have been shown to have relatively poor susceptibility to *P. inui*, a malaria parasite of Asian monkeys distantly related to *vivax*-like malarias (Leclerc *et al.*, 2004; Escalante *et al.*, 2005), while *An. freeborni* and *An. stephensi* exhibit heavy oocyst infections with this malaria parasite species (Eyles, 1960; Collins *et al.*, 1966). *An. albimanus* (El Salvador) also failed to become infected with the related Asian monkey malaria *P. coatneyi*, whereas *An. dirus, An. freeborni, An. gambiae*, *An. maculatus*, and *An. stephensi* (India) all exhibited low to moderate levels of oocyst infection (prevalence 6.6 to 24.8%) (Collins *et al.*, 2001).

2.1.1.3 Avian and rodent malaria parasite species

The susceptibility of *An. albimanus* to several avian and rodent malaria parasite species has also been investigated (Hunninen, 1951; Hunninen, 1953; Eyles, 1960; Bafort, 1971; Nayar & Young, 1984; Vaughan, Narum & Azad, 1991; Vaughan *et al.*, 1994a; Noden *et al.*, 1995).

An. albimanus was slightly less susceptible than *Cu. pipiens* to infection with the avian malaria parasite *P. relictum,* but far more susceptible than *An. crucians*, *An.*

freeborni and *An. quadrimaculatus* (oocyst prevalence 80.0 versus 33.0 to 54.0% and median oocyst intensity 119 versus 5 to 30 per midgut respectively for the anopheline species only) (Hunninen, 1951). *An. albimanus* (A-9 from El Salvador?) also had low susceptibility to the avian malaria *P. gallinaceum* compared to *Ae. aegypti* (oocyst prevalence 6.0 versus 87.0% and mean oocyst intensity 0 to 6.2 versus 18 to 100 per midgut respectively) and *An. freeborni* (F-1) (oocyst prevalence 60.0 % and mean oocyst intensity 95.0 per midgut) (Eyles, 1960).

The rodent malaria parasite *P. berghei* (NK-65) was found to be unable to produce mature oocyst infections in *An. albimanus* (strain not given) (Nayar & Young, 1984). Similarly, *An. albimanus* (A-2) also failed to become infected with oocysts of *P. berghei* (ANKA) while *An. freeborni* (F-1) and *An. stephensi* (Dutch) were heavily infected (oocyst prevalence 40.0 to 94.0% and geometric mean oocyst intensity 18.0 to 78.0 per midgut) (Vaughan *et al.*, 1991). *P. yoelii yoelii* (17XL and NL), another rodent malaria closely related *P. berghei* (Perkins & Schall, 2002; Leclerc *et al.*, 2004), exhibited extremely low levels of infection in *An. albimanus* compared to that in *An. dirus*, *An. freeborni*, *An. gambiae* and *An. stephensi* (Pakistan) (oocyst prevalence 0.0 to 18.0 versus 35.0 to 65.3%) (Vaughan *et al.*, 1994a; Noden *et al.*, 1995).

2.1.2 Susceptibility of *An. stephensi* to *Plasmodium* infection

As well as the studies described above in which *An .albimanus* was studied, a number of other investigations have shown that *An. stephensi* exhibits moderate to high susceptibility to oocyst infection with a wide variety of mammalian malaria parasites, including *P. falciparum*, *P. vivax*, *P. malariae*, *P. brasilianum*, *P. cynomolgi*, *P. fieldi*, *P. inui*, *P. knowlesi*, *P. simium*, *P. berghei*, and *P. yoelii nigeriensis* (Collins *et al.*, 1965; Collins, Contacos & Guinn, 1967; Collins *et al.*, 1968a; Collins *et al.*, 1968b; Collins, Contacos & Guinn, 1969; Rutledge, Hayes & Ward, 1970; Collins *et al.*, 1972; Collins *et al.*, 1973; Rutledge, Ward & Hayes, 1973; Collins, Contacos & Richardson, 1975; Warren & Collins, 1981; Collins *et al.*, 1981; Ponnudurai *et al.*, 1982; Collins *et al.*, 1983; Collins *et al.*, 1984; Collins *et al.*, 1985b; Ichimori, 1989; Ichimori *et al.*, 1989; Puri, Kamboj & Dutta, 1989; Collins *et al.*, 1993; Takken *et al.*, 1999; Collins *et al.*, 2002; Toma *et al.*, 2002; Alavi *et al.*, 2003). In contrast, like *An. albimanus*, *An. stephensi* exhibits poor susceptibility to oocyst infection with the avian malaria parasite *P. gallinaceum* (Russell & Nath Mohan, 1942; Rudin *et al.*, 1991; Alavi *et al.*, 2003).

2.1.3 Comparative susceptibility of *An. albimanus* and *An. stephensi* to *Plasmodium* infection

The few studies directly comparing the susceptibility of *An. albimanus* and *An. stephensi* to malaria parasite infection confirm the impression given by indirect comparisons that *An. albimanus* is markedly more refractory to mature oocyst infection than *An. stephensi*. Omar (1968b) reported much higher levels of *P. cynomolgi bastianellii* infection in *An. stephensi* (Iraq) compared to *An. albimanus* (Mexico) (oocyst prevalence 61.1 versus 1.7%, and median oocyst intensity 59.6 versus 0.0 per midgut respectively). Two strains of *An. albimanus* failed to become infected with the rodent malaria parasite *P. vinckei vinckei* while *An. stephensi* was heavily infected (oocyst prevalence 0.0 versus 70.0 to 100.0%) (Bafort, 1971).

2.1.4 Summary

An. albimanus is generally markedly more refractory to malaria parasite infection than other mosquito species examined. Many malaria parasite species/strains fail to establish mature oocyst infections in *An. albimanus* and malaria parasites to which this mosquito is susceptible often exhibit only comparatively low levels of mature oocyst infection. Furthermore, *An. albimanus* tends to be susceptible only to infection with naturally-encountered malaria parasites (i.e. those from the same or nearby geographical location and/or preferred hosts) suggesting a relatively high degree of specificity in the relationship between this mosquito species and the malaria parasites investigated. In comparison, other mosquito species investigated tended to be markedly more receptive to malaria parasite infection regardless of the latter's geographical and/or vertebrate host origin.

In contrast to *An. albimanus*, *An. stephensi* is markedly more susceptible to malaria parasite infection than many other mosquito species examined, with many malaria parasite species/strains establishing high levels of mature oocyst infection in this mosquito species. *An. stephensi* is highly susceptible to infection with many malaria parasites to which it is not naturally exposed suggesting a relatively low degree of specificity in the relationship between this mosquito species and the malaria parasites investigated.

The few previous studies directly comparing the infectivity of malaria parasites to *An. albimanus* and *An. stephensi* support the interpretation that *An. albimanus* is relatively refractory, and *An. stephensi* relatively susceptible, to mature oocyst infection.

2. 2 Materials & Methods

All materials and methods followed established standard protocols for malaria parasite culture and mosquito infection (Carter, Ranford-Cartwright & Alano, 1993). Full details of the various solutions and media used for malaria parasite culture are given in Appendix 1.

2.2.1 *In vitro* culture of *P. falciparum*

P. falciparum clones 3D7A, HB3B-B2 and 7G8 were cultured *in vitro* under conditions permissive for the development of asexual erythrocytic parasite stages and mature gametocytes infective to mosquitoes as previously described (Haynes *et al.*, 1976; Trager & Jensen, 1976; Ifediba & Vanderberg, 1981; Ponnudurai *et al.*, 1982; Carter *et al.*, 1993). All procedures related to culture of erythrocytic stage malaria parasites, including handling of human blood and serum, were performed within a class II microbiological safety cabinet according to standard laboratory protocols as delineated within local COSHH forms. As the infectivity of gametocytes of a given malaria parasite clone varies from culture to culture (Ponnudurai *et al.*, 1989; Vaughan *et al.*, 1992), in all experimental feeds *An. albimanus* and *An. stephensi* were simultaneously fed gametocytes from the same cultures in order to ensure comparability between the two mosquito species.

2.2.1.1 *P. falciparum* clones

The 3D7A clone was previously derived from the isolate NF54 by limiting dilution (Rosario, 1981; Walliker *et al.*, 1987). NF54 was isolated in 1978 from a woman who had never left the Netherlands (Delemarre & van der Kaay, 1979; Ponnudurai, Leeuwenberg & Meuwissen, 1981). As malaria was not endemic in the Netherlands at this time, such that autochthonous transmission was not believed to occur, and the woman lived close to Schiphol airport near Amsterdam, the infection was assumed to be a case of airport malaria (Delemarre & van der Kaay, 1979): an infected tropical anopheline mosquito was probably imported *via* an airplane into the Netherlands from a malaria-endemic area. The exact geographical origin of the NF54 isolate, and hence the 3D7A clone, is, therefore, unknown.

The HB3B-B2 clone was derived from the isolate Honduras 1/CDC (H1) by microscopic selection (Trager *et al.*, 1981; Bhasin & Trager, 1984) and then re-cloned by limiting dilution after mosquito passage through a chimpanzee (Walliker *et al.*,

1987). H1 was isolated in 1980 from an individual in Honduras (Bhasin & Trager, 1984). No other published information is available about the origin of this isolate.

The 7G8 clone was derived from isolate IMTM 22 by limiting dilution (Burkot, Williams & Schneider, 1984). IMTM 22 was isolated from a 12 year old boy in Manaus, Brazil, on 12th March 1980 (Burkot *et al.*, 1984). No other information is available about the isolation of IMTM 22.

2.2.1.2 Long-term storage of parasite stocks

Asexual erythrocytic stage malaria parasite stocks were kept in liquid nitrogen and thawed/frozen as required according to previously published protocols (Aley *et al.*, 1984).

2.2.1.2.1 Thawing malaria parasites

Malaria parasite stabilates frozen in liquid nitrogen were thawed quickly at 37°C, mixed at a 4:1 ratio with thawing solution I, left to stand for 2 minutes at room temperature, then mixed at a 1:9 ratio with thawing solution II, pelleted at 1500 g for 5 minutes, supernatant removed, resuspended at a 1:9 ratio with thawing solution III, pelleted, supernatant removed, and resuspended in 3.0 ml of "complete" RPMI 1640 medium supplemented with 0.1 ml of fresh washed human erythrocytes. The cells resuspended in complete RPMI medium were transferred to a culture flask, gassed and maintained in an incubator (see Section 2.2.1.3). The culture medium was changed daily and the parasitaemia was checked from the third day after thawing. Once the parasitaemia reached greater than 1%, the culture was bulked up to 5 ml and henceforth maintained as described below (Section 2.2.1.3).

2.2.1.2.2 Freezing malaria parasites

Asexual erythrocytic parasite stages were frozen as follows: high parasitaemia cultures rich in ring stage parasites were pelleted at 1500 g for 5 minutes, the supernatant was removed, and the pellet was resuspended at a 1:1 ratio with freezing solution. 0.3 to 0.5 ml of the mixture was aliquoted into 1.2 ml CRIS cyrovials (Bibby Sterilin Ltd., UK, Cat. No. 1 072 3 8030) and immediately placed into liquid nitrogen.

2.2.1.3 *In vitro* culture of asexual erythrocytic stage parasites

Asexual erythrocytic parasite stages were cultured according to previously published protocols (Haynes *et al.*, 1976; Trager & Jensen, 1976) with minor modifications. Five millilitre volume stock cultures, at 5.0% haematocrit in complete RPMI 1640 medium, were maintained in 25 cm^2 plug-seal tissue culture flasks (Corning

Inc., NY, USA, Cat. No. 430168) and kept in an incubator at 37°C. Culture medium was changed daily with complete RPMI 1640 medium, and the flasks individually gassed with a mixture of 1% O_2, 3% CO_2 and 96% N_2 (BOC Ltd., UK) after each change of medium. Parasitaemia was monitored using Giemsa-stained thin smears and maintained between 0.2 and 8.0% by diluting the cultures approximately every other day with washed human erythrocytes suspended in complete RPMI 1640 medium.

Fresh whole human blood was obtained weekly from Dr. Peterkin and staff at the Glasgow and West of Scotland Blood Transfusion service, Scottish National Blood Transfusion Service. Erythrocytes from the whole blood were washed three times, to remove plasma and white blood cells, by centrifuging at 1500 g for 10 to 15 minutes, withdrawing the supernatant and buffy coat from the erythrocyte pellet, and resuspending the latter in an equal volume of complete/incomplete RPMI 1640 medium. Washed erythrocytes up to four weeks of age were used for maintaining asexual erythrocytic malaria parasite stage cultures. Erythrocytes of all blood group types were used.

2.2.1.4 *In vitro* culture of sexual erythrocytic stage parasites

Sexual erythrocytic parasite stages (gametocytes) were cultured according to previously published protocols (Ifediba & Vanderberg, 1981; Ponnudurai *et al.*, 1982; Carter *et al.*, 1993) with minor modifications. Fifteen millilitre volume gametocyte cultures, at 6.0% haematocrit in complete RPMI 1640 medium, were initiated at 0.5 to 0.7% parasitaemia (depending on which parasite stages were present in the inoculum – lower if predominantly schizonts and higher if predominantly ring-stage parasites). Fresh washed human erythrocytes, less than one week old, were used to initiate gametocyte cultures. The cultures were maintained in 75 cm^2 plug-seal tissue culture flasks (Corning Inc., Cat. No. 430720) and kept in an incubator at 37°C. Culture medium was changed daily, and the flasks individually gassed, as for asexual parasite stages. Parasitaemia and gametocyte development were monitored regularly using Giemsa-stained thin smears. Once the parasitaemia was greater than approximately 4.0% and the parasites appeared stressed as determined by morphology (usually the third day after initiation of the culture), the culture was bulked up to 25 ml through the addition of extra complete RPMI 1640 medium alone. The culture was subsequently maintained at this volume, without the addition of further erythrocytes, until harvesting of the mature gametocytes for mosquito infection.

2.2.2 Maintenance of mosquito colonies

2.2.2.1 Mosquito species

The *An. albimanus* (Weidemann) strain originated from Panama, Central America. This mosquito strain derives from a colony established at the London School of Hygiene and Tropical Medicine in 1986 which itself was founded from a colony held at the Centre for Disease Control, Atlanta, Georgia USA for twenty years previously (*personal communication*, Barbara Sawyer, LSHTM).

The *An. stephensi* (Liston) Dutch SDA500 strain was obtained from Imperial College, London. The Imperial College stock came from a colony in The Netherlands, where the Sind strain of *An. stephensi* had been selected for susceptibility to *P. falciparum* (Feldmann & Ponnudurai, 1989), with some outcrossing to the Kasur strain as a precaution against inbreeding depression. The Sind strain was derived from various laboratory strains collected in Sind province., Pakistan, and the Kasur strain originated from a collection from Kasur, Punjab, Pakistan.

2.2.2.2 Insectary conditions and rearing of mosquitoes

An. albimanus and *An. stephensi* mosquito colonies were maintained under standard laboratory conditions. Both mosquito species were maintained in a temperature and humidity controlled insectary at 26 ± 1°C and 70-80 % relative humidity in a 12:12 hour light/dark cycle. Larvae were maintained in plastic boxes, 5 x 16 x 16 cm, filled with approximately 400 ml of distilled H_2O. $NaHCO_3$ was added to the water to reduce acidity. Larvae were initially reared at relatively high densities, which were subsequently reduced as the larvae grew such that approximately several hundred fourth instar larvae were present within each plastic box. *An. albimanus* were maintained on ground Tetramin®, and fed twice daily, throughout the period of larval development. For *An. stephensi*, young larvae were fed once daily with Liquifry while older larvae were given ground Tetramin® once per day. Pupae were collected daily and placed into mesh cages, 30 x 30 x 30 cm, for adult emergence. Several thousand adults were kept within each stock cage. Adult mosquitoes were provided *ad libitum* with a solution of 5.0% glucose/0.05% para-amino-benzoic acid (PABA), and were membrane-fed fresh washed human erythrocytes resuspended to 40% haematocrit in heat-inactivated pooled human AB serum once or twice per week. Small plastic dessert bowls lined with filter paper and partly filled with water were placed overnight into the adult stock cages two days after bloodfeeding for oviposition. Any pupae bowls present

were covered with an open-ended paper hat or plastic funnel to prevent gravid females laying their eggs within these otherwise alternative oviposition sites. Once laid, eggs were subsequently removed from the stock cages. Hatched larvae were transferred to plastic boxes and reared as described above. Initially, all plastics used for rearing larvae were washed with the detergent Bactosol (Johnson Wax Professional Ltd, Surrey, England). However, this detergent apparently had a detrimental effect on *An. albimanus* larvae (but not on *An. stephensi* larvae) and was no longer used to clean plastics for this mosquito species. Plastics for *An. albimanus* were subsequently washed with hot water only and given plenty of scrubbing!

2.2.3 Infection of mosquitoes with malaria parasites

2.2.3.1 Collection of adult female mosquitoes

Five to seven days prior to infection with the malaria parasites, mosquito pupae were collected from the stock colony, placed into separate small experimental mesh cages, 20 x 20 x 20 cm, and allowed to emerge. Two or three days prior to malaria parasite infection, the emerged adult female mosquitoes were collected from the small experimental cages, using an aspirator (pooter) and a bowl of luke-warm water placed atop the cage to attract the females and hence enable their separation from the males present within the cage. The adult female mosquitoes so collected were transferred to sealed waxed cartons covered with mesh (Carter *et al.*, 1993). These mosquitoes were subsequently kept in a separate secure insectary room but maintained under the same conditions as the stock colonies (Section 2.2.2.2). The mosquitoes were provided *ad libitum* with a solution of 5.0% glucose/0.05% PABA throughout the duration of the experiment, except for the 24 hours prior to infection when distilled water only was given to encourage engorgement from the membrane-feeders.

2.2.3.2 Membrane-feeding mosquitoes cultured gametocytes

Five to seven days after emergence from pupae, adult female mosquitoes were offered infectious cultured gametocytes through membrane-feeders according to previously published protocols (Ponnudurai *et al.*, 1982; Carter *et al.*, 1993). All subsequent stages involving manipulation of the parasites were performed at 37°C to prevent premature gametocyte activation and the induction of gametogenesis prior to mosquito ingestion. Fourteen and seventeen day old gametocyte cultures were pelleted at 1500 g for 5 minutes, the supernatant removed and pellets mixed by resuspension at a 1:1 ratio with pre-warmed pooled heat-inactivated human AB serum. This mixture was

then diluted with pre-heated fresh washed human erythrocytes resuspended to 40% haematocrit in pooled heat-inactivated human AB serum. The diluted gametocytes were immediately transferred to glass membrane-feeders covered with baudruche membrane (Long & Long, Belleville, NJ, USA) and attached to a water bath pre-heated to 37°C. The adult female mosquitoes previously transferred to wax cartons (Section 2.2.3.1) were allowed to engorge from the membrane-feeders. When performing paired feeds in which *An. albimanus* and *An. stephensi* were simultaneously fed the same diluted gametocyte cultures, the latter was offered to each mosquito species through separate membrane-feeders. During this time, a sample of the bloodmeal that had been allowed to stand at room temperature was examined for exflagellation of the cultured gametocytes using a light microscope at 400X magnification. After 15 to 30 minutes, the mosquitoes were removed from the membrane-feeders. Unfed and partially fed mosquitoes were removed from the cartons within two hours of bloodfeeding and killed by freezing. The fully gorged mosquitoes were kept, and maintained under the conditions described above (Section 2.2.3.1).

2.2.3.3 Mosquito dissection & microscopic determination of oocyst infection

Mosquito dissections and microscopic examination for mature oocysts were performed as previously described (Carter *et al.*, 1993). Ten pbf, mosquitoes fed gametocytes were anaesthetised with chloroform (Fisher Scientific, Cat. No. C/4960/17), dipped into 70% (v/v) ethanol (Fisher Scientific, Cat. No. E/0650DF/17) in ddH_2O, submerged in phosphate buffered saline (PBS) and kept on ice until dissection. Mosquito midguts were dissected on a glass slide, in a drop of PBS, with syringe needles, using a dissecting light microscope (Leica, MZ 9_5) at 12.5X magnification. After dissection, midguts were transferred to a fresh drop of PBS using stretched glass capillaries (Camlab Ltd., Cat. No. DMP.002) and mounted on a glass slide with a cover slip. The midguts were examined without staining at 400X magnification using a compound light microscope (Lietz, Laborlux 11) with the condenser lowered to improve contrast.

2.2.4 Statistical analysis

The distribution of oocysts within mosquito host populations varies according to the level of malaria parasite infection (for examples, see Figure 2.1) (Eyles, 1951; Rutledge, Ward & Buckwalter, 1973; Medley *et al.*, 1993; Billingsley *et al.*, 1994). When the level of malaria parasite infection within the mosquito host population is low,

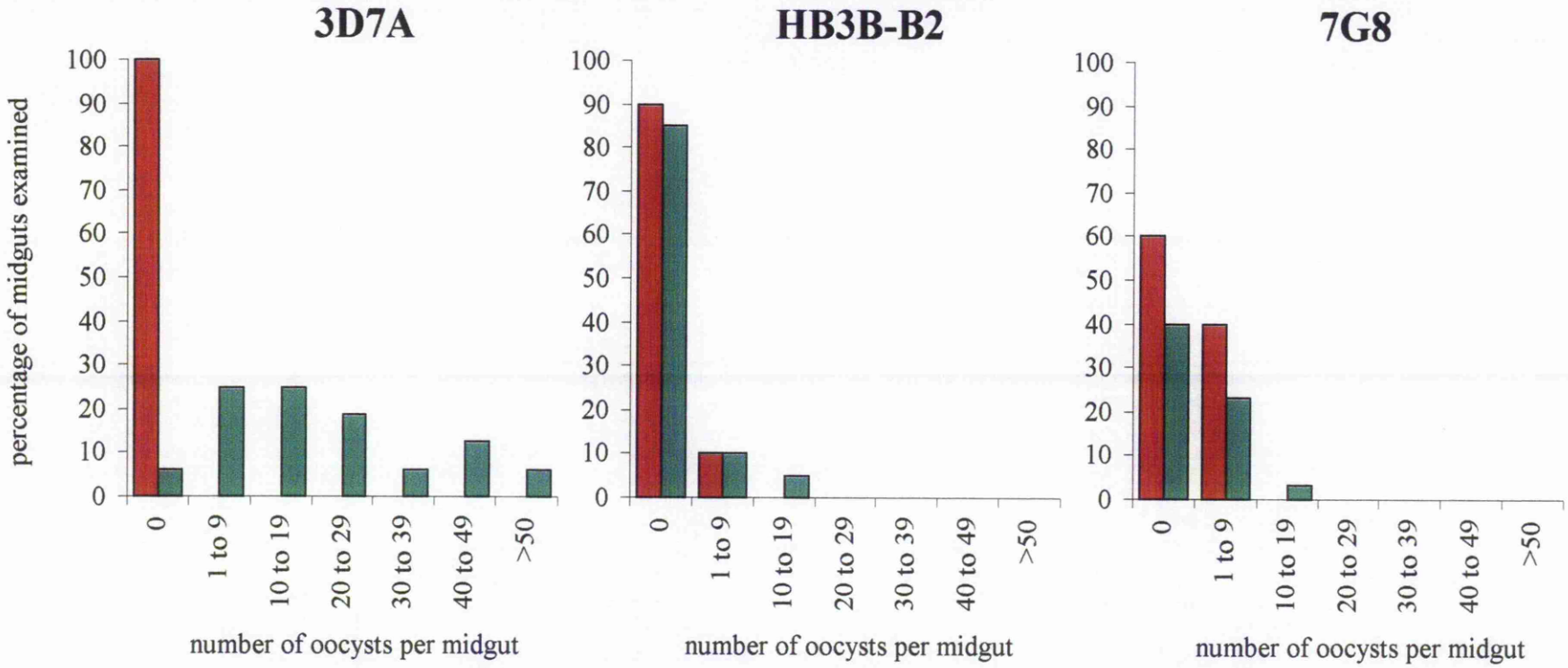


Figure 2.1 Comparison of the distributions of mature oocyst infection of three different *P. falciparum* clones at day 10 post-bloodfeeding in *An. albimanus* and *An. stephensi*.

Graphs showing the distribution of mature oocyst infection for the *P. falciparum* clones 3D7A, HB3B-B2 and 7G8 at day 10 pbf in (●) *An. albimanus* and (●) *An. stephensi*. The results of a single representative experimental feed are shown for each of the malaria parasite clones: the 3D7A graph shows experimental feed 2 from Table 2.1; the HB3B-B2 graph shows experimental feed 2 from Table 2.2; and the 7G8 graph shows experimental feed 2 from Table 2.3.

oocysts are overdispersed approximating a negative binomial distribution. In contrast, as the level of malaria parasite infection within mosquito population increases, the distribution of oocysts between individual mosquitoes tends towards a normal distribution. As most of the experimental feeds undertaken gave low or intermediate levels of oocyst infection, and sometimes different distributions in the two mosquito species tested, the levels of oocyst infection in *An. albimanus* and *An. stephensi* were compared using "distribution free" non-parametric tests to avoid violation of the assumptions of normality required for most common parametric tests.

The prevalence of *P. falciparum* oocyst infection observed in *An. albimanus* and *An. stephensi* at day 10 pbf was compared using a two-tailed Fisher's Exact test because of low counts for some observational categories. The intensity of *P. falciparum* oocyst infection in *An. albimanus* and *An. stephensi* at day 10 pbf was compared using a two-tailed Mann-Whitney *U* test. Both statistical tests were performed using XLStat version 7.5.2 (Addinsoft, 1995-2004).

2. 3 Results

2.3.1 Comparison of the infectivty of the *P. falciparum* 3D7A clone to *An. albimanus* and *An. stephensi*

The levels of oocyst infection for the *P. falciparum* 3D7A clone in *An. albimanus* and *An. stephensi* at day 10 pbf from three experimental feeds are shown in Table 2.1. The prevalence and intensity of oocyst infection within each experimental feed were highly significantly different between the two mosquito species (Table 2.1). Overall, none of the 52 *An. albimanus* fed *P. falciparum* 3D7A gametocytes were infected with oocysts at day 10 pbf. In contrast, 54 of the 68 *An. stephensi* fed *P. falciparum* 3D7A gametocytes were infected with oocysts. The average prevalence of oocyst infection for the three experimental feeds was 69.3% in *An. stephensi*. Furthermore, the intensity of oocyst infection in *An. stephensi* was generally relatively high with an average median of 11.8 oocysts per midgut for the three experimental feeds.

2.3.2 Comparison of the infectivty of the *P. falciparum* HB3B-B2 clone to *An. albimanus* and *An. stephensi*

The levels of oocyst infection for the *P. falciparum* HB3B-B2 clone in *An. albimanus* and *An. stephensi* at day 10 pbf from three experimental feeds are shown in Table 2.2. The prevalence and intensity of oocyst infection in two of the experimental

Table 2.1 Comparison of the levels of mature oocyst infection of the *P. falciparum* 3D7A clone at day 10 post-bloodfeeding in *An. albimanus* and *An. stephensi*.

Experimental feed	Mosquito species	n[a]	Prevalence[b]	P value[c]	Intensity[d]	z statistic[e]	P value[f]
1	***An. albimanus***	20	0.0	<0.0001*	0.0	-4.227	<0.0001*
	An. stephensi	20	65.0		1.5 (0-36)		
2	***An. albimanus***	16	0.0	<0.0001*	0.0	-4.905	<0.0001*
	An. stephensi	16	93.8		16.5 (0-62)		
3	***An. albimanus***	16	0.0	<0.0001*	0.0	-5.228	<0.0001*
	An. stephensi	32	81.3		20.0 (0-78)		

[a] The number of midguts examined.
[b] The percentage of midguts examined harbouring at least one oocyst.
[c] Comparison of the prevalence of oocyst infection in *An. albimanus* and *An. stephensi* using a two-tailed Fisher's Exact test, with $\alpha = 0.05$.
[d] Median number of oocysts observed per midgut including uninfected midguts. Numbers in brackets give the range of oocysts observed on each midgut.
[e, f] Comparison of the intensity of oocyst infection in *An. albimanus* and *An. stephensi* using a two-tailed Mann-Whitney U test, with $\alpha = 0.05$, where the critical value of $z = \pm 1.960$.
* Indicates statistically significant differences between *An. albimanus* and *An. stephensi*.

Table 2.2 Comparison of the levels of mature oocyst infection of the *P. falciparum* HB3B-B2 clone at day 10 post-bloodfeeding in *An. albimanus* and *An. stephensi*.

Experimental feed	Mosquito species	n[a]	Prevalence[b]	P value[c]	Intensity[d]	z statistic[e]	P value[f]
1	*An. albimanus*	20	0.0	0.004*	0.0	-2.863	0.004*
	An. stephensi	20	35.0		0.0 (0-2)		
2	*An. albimanus*	20	10.0	1.000	0.0 (0-1)	-0.565	0.572
	An. stephensi	20	15.0		0.0 (0-12)		
3	*An. albimanus*	32	3.1	<0.0001*	0.0 (0-1)	-5.665	<0.0001*
	An. stephensi	20	80.0		4.0 (0-20)		

[a] The number of midguts examined.
[b] The percentage of midguts examined harbouring at least one oocyst.
[c] Comparison of the prevalence of oocyst infection in *An. albimanus* and *An. stephensi* using a two-tailed Fisher's Exact test, with $\alpha = 0.05$.
[d] Median number of oocysts observed per midgut including uninfected midguts. Numbers in brackets give the range of oocysts observed on each midgut.
[e, f] Comparison of the intensity of oocyst infection in *An. albimanus* and *An. stephensi* using a two-tailed Mann-Whitney U test, with $\alpha = 0.05$, where the critical value of $z = \pm 1.960$.
* Indicates statistically significant differences between *An. albimanus* and *An. stephensi*.

feeds differed significantly between the two mosquito species, while neither of these parameters were significantly different between *An. albimanus* and *An. stephensi* in the remaining experimental feed (Table 2.2). Overall, 3 of 72 *An. albimanus* and 26 of 60 of *An. stephensi* were infected with oocysts. The average prevalence of oocyst infection for the three experimental feeds was 4.2% in *An. albimanus* and 69.3% in *An. stephensi*. In one of the experimental feeds *An. albimanus* was not infected with oocysts, while oocysts were found in *An. stephensi* in all three experimental feeds. The intensity of oocyst infection was generally low in both *An. albimanus* and *An. stephensi* and similarly somewhat variable between experimental feeds. The average median number of oocysts per midgut for the three experimental feeds was 0.0 for *An. albimanus* and 1.3 for *An. stephensi*.

2.3.3 Comparison of the infectivity of the *P. falciparum* 7G8 clone to *An. albimanus* and *An. stephensi*

The levels of oocyst infection for the *P. falciparum* 7G8 clone in *An. albimanus* and *An. stephensi* at day 10 pbf from two experimental feeds are shown in Table 2.3. The prevalence and intensity of oocyst infection within each experimental feed differed significantly between *An. albimanus* and *An. stephensi* in one but not the other of the experimental feeds (Table 2.3). Overall, oocysts were found in 13 of 54 *An. albimanus* and 23 of 44 *An. stephensi* and both mosquito species were infected with oocysts in both experimental feeds. The average prevalence of oocyst infection for the two experimental feeds was 24.1% in *An. albimanus* and 52.3% in *An. stephensi*. The intensity of oocyst infection was low in both *An. albimanus* and *An. stephensi*, the median number of oocysts per midgut averaging 0.0 for *An. albimanus* and 2.7 for *An. stephensi* for the two experimental feeds.

2. 4 Discussion

Comparison of the levels of mature oocyst infection of three different *P. falciparum* clones in *An. albimanus* and *An. stephensi* revealed differences between the malaria parasite clones in the ability to infect the mosquitoes and differences between the two mosquito species in their susceptibility to malaria parasite infection.

The absence of *P. falciparum* 3D7A oocyst infection in *An. albimanus* is consistent with the findings of the previous studies by Vaughan *et al.* (1994b) and Chege & Beier (1994) who also found that the NF54 isolate (and clones derived

Table 2.3 Comparison of the levels of mature oocyst infection of the *P. falciparum* 7G8 clone at day 10 post-bloodfeeding in *An. albimanus* and *An. stephensi*.

Experimental feed	Mosquito species	n[a]	Prevalence[b]	P value[c]	Intensity[d]	z statistic[e]	P value[f]
1	***An. albimanus***	24	4.2	<0.0001*	0.0 (0-1)	-4.314	<0.0001*
	An. stephensi	24	62.5		5.0 (0-38)		
2	***An. albimanus***	30	40.0	1.000	0.0 (0-6)	-0.470	0.638
	An. stephensi	20	40.0		0.0 (0-10)		

[a] The number of midguts examined.
[b] The percentage of midguts examined harbouring at least one oocyst.
[c] Comparison of the prevalence of oocyst infection in *An. albimanus* and *An. stephensi* using a two-tailed Fisher's Exact test, with $\alpha = 0.05$.
[d] Median number of oocysts observed per midgut including uninfected midguts. Numbers in brackets give the range of oocysts observed on each midgut.
[e, f] Comparison of the intensity of oocyst infection in *An. albimanus* and *An. stephensi* using a two-tailed Mann-Whitney U test, with $\alpha = 0.05$, where the critical value of $z = \pm 1.960$.
* Indicates statistically significant differences between *An. albimanus* and *An. stephensi*.

therefrom) exhibited extremely poor infectivity to this mosquito species. Unlike the present study, Vaughan *et al.* (1994b) and Chege, Pumpuni & Beier (1996) observed very low levels of oocyst infection in *An. albimanus*. This could be due to differences either between the malaria parasite clones and/or the strains of *An. albimanus* used in the respective studies. However, the *P. falciparum* 3D7A clone used in the current study is derived from the NF54 isolate (Section 2.2.1.1), although different clones taken from a single isolate can vary in their biological characteristics (Graves, Carter & McNeill, 1984; Davis *et al.*, 1992). The strain of *An. albimanus* used by Vaughan *et al.* (1994b) was not explicitly identified, although it was held at the CDC in Atlanta, the original laboratory home of the Panamanian *An. albimanus* used in the current study while a Panamanian strain of *An. albimanus* of unknown provenance was used by Chege, Pumpuni & Beier (1996). An alternative explanation for the differences between the observations of previous studies and the results presented here is that *P. falciparum* 3D7A oocyst infection in *An. albimanus* could be extremely rare and insufficient mosquitoes were dissected in the current work to detect such a low level of oocyst infection.

Although the previous study by Vaughan *et al.* (Vaughan *et al.*, 1994b) also showed that the *P. falciparum* NF54 isolate/derived clones could infect *An. stephensi*, the susceptibility of *An. albimanus* and *An. stephensi* to these malaria parasites were not directly compared. The results of the paired feedings presented here enables a direct comparison to be made and unequivocally demonstrates that *An. stephensi* is markedly more susceptible to oocyst infection with *P. falciparum* 3D7A than *An. albimanus*.[1]

In contrast to the results with the *P. falciparum* 3D7A clone, the *P. falciparum* HB3B-B2 clone was able to establish mature oocyst infections in both *An. albimanus* and *An. stephensi* at similar if somewhat variable levels. This observation confirms the results of previous studies showing that various strains of *An. albimanus*, including

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[1] The infectivty of cultured gametocytes is known to vary (Ponnudurai *et al.*, 1982; Graves *et al.*, 1984; Ponnudurai *et al.*, 1989), and, potentially, this could account for the failure of *An. albimanus* to become infected with the *P. falciparum* 3D7A clone. However, the heavy oocyst infections observed in *An. stephensi* demonstrates that the cultured gametocytes simultaneously fed to *An. albimanus* were fully functional and capable of establishing mature oocyst infections. Consequently, the failure of *An. albimanus* to become infected with the *P. falciparum* 3D7A clone was not due to the poor infectivity of this malaria parasite clone and must be due to a difference in susceptibility to *P. falciparum* 3D7A between this mosquito species and *An. stephensi*.

Panama Escobol, and *An. stephensi* are both susceptible to this malaria parasite clone (Teklehaimanot *et al.*, 1987). This observation, together with the novel finding that *An. albimanus* and *An. stephensi* are similarly susceptible to the *P. falciparum* 7G8 clone, demonstrates that *An. albimanus* is susceptible to malaria parasite infection. Furthermore, the levels of oocyst infection of *P. falciparum* HB3B-B2 and 7G8 in *An. stephensi* were appreciably lower in comparison to *P. falciparum* 3D7A.

Therefore, taken together, the results presented in this Chapter imply that:

(1) there are differences between the three *P. falciparum* clones investigated in the absolute ability to establish oocyst infections in *An. albimanus*;
(2) there are differences between the three *P. falciparum* clones investigated in level of oocyst infection in *An. stephensi*; and
(3) there are differences between *An. albimanus* and *An. stephensi* in susceptibility to some of the *P. falciparum* clones investigated.

2. 5 Summary

The infectivity of three different laboratory clones of *P. falciparum* to two different mosquito species, *An. albimanus* and *An. stephensi*, were compared by simultaneously feeding *in vitro* cultured gametocytes of each malaria parasite clone to both mosquito species.

The *P. falciparum* 3D7A clone was unable to establish mature oocyst infections at day 10 pbf in *An. albimanus* while a high levels of oocyst infection were observed for this malaria parasite clone in *An. stephensi*. In contrast to the *P. falciparum* 3D7A clone, the *P. falciparum* HB3B-B2 and 7G8 clones exhibited markedly lower mature oocyst infections at day 10 pbf and infected both mosquito species, although the relative susceptibility of *An. albimanus* and *An. stephensi* for these malaria parasite clones was somewhat more variable.

These observations demonstrate that variation exists between different malaria parasite clones, and different mosquito species, that determine the outcome of malaria parasite infection in the mosquito vector.

Chapter 3. Direct immunofluorescence microscopy of the development of the *P. falciparum* 3D7A clone in *An. albimanus* and *An. stephensi*

3. 1 Introduction

The results presented in the previous Chapter demonstrated that the *P. falciparum* 3D7A clone is unable (or, at least, very unlikely) to establish mature oocyst infections in *An. albimanus* but successfully develops to this stage in *An. stephensi*. The reasons for the refractoriness of *An. albimanus*, and the susceptibility of *An. stephensi*, to infection with the *P. falciparum* 3D7A clone are not known. However, the development of other malaria parasite species/isolates/clones in *An. albimanus* have been investigated.

3.1.1 Previous studies of *Plasmodium* development in *An. albimanus*

As reviewed in Chapter 2, *An. albimanus* tends to be markedly more resistant to mature oocyst infection in general than other mosquito species that have been previously investigated. However, despite the large number of studies, the reasons for the general resistance of *An. albimanus* to malaria parasite infection are largely unknown and have seldom been investigated in detail.

Only two studies have directly investigated whether *An. albimanus* provides a suitable environment for the induction of gametogenesis (Omar, 1968b; Mendis, Noden & Beier, 1994). Exflagellation and fertilisation of *P. cynomolgi bastianellii* occurs with equivalent efficiency within the bloodmeals of both *An. albimanus* and *An. stephensi* (Omar, 1968b). Homogenised midguts from these two mosquito species are also equally capable of inducing exflagellation *in vitro* of cultured *P. falciparum* (NF54 isolate) gametocytes (Mendis *et al.*, 1994). These studies indicate that gametogenesis is not inhibited within the midgut lumen of *An. albimanus*, at least for the malaria parasite species that have been investigated. Therefore, other factors must explain the resistance of *An. albimanus* to mature oocyst infection with these malaria parasite strains.

Several investigations have shown that the formation of mature ookinetes within the bloodmeal of *An. albimanus* is broadly similar to that in other susceptible mosquito species (Eyles & Young, 1950; Omar, 1968b; Vaughan *et al.*, 1991; Vaughan *et al.*, 1994a; Vaughan *et al.*, 1994b; Gonzalez-Ceron *et al.*, 2001). These studies, therefore, provide indirect evidence that the processes of gametogenesis and fertilisation occur

successfully in *An. albimanus* and that malaria parasite infection does not fail at either of these stages in this mosquito species.

Eyles and Young (1950) reported that *P. falciparum* (Santee-Cooper isolate) produced similar densities of ookinetes in both *An. albimanus* and *An. quadrimaculatus* despite lower levels of oocyst infection in the former mosquito species. Likewise, Vaughan *et al.* (1994b) and Chege, Pumpuni & Beier (1996) observed similar densities of ookinetes within the bloodmeals of *An. albimanus*, *An .gambiae* and *An. freeborni* fed the same *P. falciparum* gametocyte cultures (NF54 isolate and unspecified clones derived therefrom). Omar (1968b) also found similar densities of *P. cynomolgi bastianellii* ookinetes developing within the midguts of *An. albimanus* and *An. stephensi* simultaneously fed gametocytes of this malaria parasite species while Gonzalez-Ceron *et al.* (2001) reported equivalent densities of *P. vivax* (VK247 CSP variant) ookinetes within the bloodmeals of *An. albimanus* and *An. pseudopunctipennis*. In all of these studies, the levels of oocyst infection were markedly lower in *An. albimanus* than in the other mosquito species tested (Chapter 2, Section 2.1.1) (Eyles & Young, 1950; Omar, 1968b; Vaughan *et al.*, 1994b; Gonzalez-Ceron *et al.*, 2001).

In contrast, Vaughan *et al.* (1991) found significantly higher densities of *P. berghei* (ANKA) ookinetes within the bloodmeals of *An. albimanus* and *An. freeborni* compared to *An. stephensi*, although the kinetics of ookinete development were similar in all three mosquito species examined. Conversely, the density of *P. yoelii yoelii* (17XNL) ookinetes was substantially lower in *An. albimanus* and *An. dirus* compared to *An. freeborni*, *An. gambiae* and *An. stephensi* (Vaughan *et al.*, 1994a). In the latter study, the kinetics of ookinete development for this malaria parasite species differed in *An. albimanus* and *An dirus* compared to the other three anopheline mosquito species investigated, with densities of mature ookinetes peaking approximately 4 hours later and exhibiting a broader flatter plateau in the former mosquito species (Vaughan *et al.*, 1994a). Furthermore, *P. yoelii yoelii* (17XNL) ookinete densities appeared to decline more rapidly in *An. albimanus* than in the other anopheline mosquitoes, such that few ookinetes were apparent within the bloodmeal from 20 hours pbf onwards (Vaughan *et al.*, 1994a). However, despite forming numerous ookinetes, neither *P. berghei* nor *P. yoelii yoelii* established oocyst infections in *An. albimanus* in these particular studies (Vaughan *et al.*, 1991; Vaughan *et al.*, 1994a).

Although these studies demonstrated that the formation of mature ookinetes occurs more or less normally in *An. albimanus*, these investigations, with the two

exceptions described below, did not identify the exact stage during infection at which the malaria parasites subsequently failed to develop into mature oocysts. The published data are consistent with malaria parasite infection failing before, during or after ookinete invasion of the mosquito midgut epithelium. Vaughan *et al.* (1994a) claimed that factors unrelated to the peritrophic matrix and midgut digestive enzymes explained the failure of *P. yoelii yoelii* to establish mature oocyst infections in *An. albimanus*. The relatively rapid rate of ookinete formation for this malaria parasite species (8 to 16 hours pbf at 24°C) was argued to enable midgut invasion to occur before either of these factors could have any appreciable detrimental effect on malaria parasite infection (Vaughan *et al.*, 1994a). Instead, the failure of *P. yoelii yoelii* to infect *An. albimanus* was proposed to result directly from (unspecified) "molecular [in]compatibilities" between the ookinete and the midgut epithelium (Vaughan *et al.*, 1994a). However, ookinete invasion of the midgut epithelium was not directly investigated and no other evidence was provided to support the interpretation of a "midgut barrier" to malaria parasite infection in this mosquito species.

Only two other studies have attempted to characterise in greater detail the reasons for the failure of mature oocyst infections to develop in *An. albimanus* (Omar, 1968b; Gonzalez-Ceron *et al.*, 2001). Omar (1968b) used histological examination of Giemsa-stained sections of midguts challenged with *P. cynomolgi bastianellii* gametocytes to investigate ookinete migration from the bloodmeal and subsequent invasion of the midgut epithelium. In *An. stephensi*, all stages of ookinete migration to the basal surface of the midgut epithelium were observed (Omar, 1968b). In contrast, in *An. albimanus*, ookinetes were only very rarely observed beyond the peritrophic matrix and migrating across the midgut epithelium (Omar, 1968b). The majority of ookinetes in *An. albimanus* apparently degenerated within the endoperitrophic space of the midgut lumen. Omar (1968b) concluded that processes related to bloodmeal digestion primarily accounted for the refractoriness of *An. albimanus* to oocyst infection with *P. cynomolgi bastianellii*, although no direct evidence was provided to support this assertion. Gonzalez-Ceron *et al.* (2001) presented evidence that in *An. albimanus*, *P. vivax* (VK247 CSP variant) ookinetes were destroyed during migration across the midgut epithelium and the establishment of early oocysts on the basal surface of the midgut epithelium. Although the prevalence of oocyst infection was lower in *An. albimanus* compared to *An. pseudopunctipennis*, the intensity of oocyst infection in infected mosquitoes was higher in *An. albimanus* (but most of the oocysts in *An.*

albimanus were degenerate) (Gonzalez-Ceron *et al.*, 2001). Significantly higher numbers of ookinetes were also observed on the apical surface of the midgut epithelium of *An. albimanus* suggesting that *P. vivax* (VK247 CSP variant) ookinete invasion of the midgut epithelium was impeded in this mosquito species (Gonzalez-Ceron *et al.*, 2001). These two studies, therefore, demonstrate that there are at least three different stages during which ookinetes fail to establish mature oocyst infection in *An. albimanus*.

Several studies also suggest that once established, the growth and development of oocysts may be inhibited in *An. albimanus* (Eyles & Young, 1950; Hunninen, 1953; Eyles, 1960; Omar, 1968a). Eyles and Young (1950) noted for *P. falciparum* (Santee-Cooper isolate) that oocyst size and maturity tended to be retarded in *An. albimanus* compared to *An. quadrimaculatus*. Similar observations of retarded oocyst growth and development in *An. albimanus* have also been made for *P. cynomolgi bastianellii* (compared to *An. gambiae*, *An. l. atroparvus*, *An. sacharovi* and *An. stephensi*) (Omar, 1968a), *P. gallinaceum* (compared to *Ae. aegypti*) (Eyles, 1960) and *P. relictum* (compared to *Cu. pipiens*) (Hunninen, 1953).

There is also some evidence that sporozoites are adversely affected by development within *An. albimanus* (Noden *et al.*, 1995). *P. yoelii yoelii* (17XL and NL strains) sporozoites isolated from the salivary glands of *An. albimanus* selected for susceptibility to oocyst infection were unable to induce infections when subsequently inoculated into mice, even when high densities of parasites were used (Noden *et al.*, 1995). However, low densities of sporozoites of the same malaria parasite species taken from simultaneously infected *An. stephensi* successfully established patent infections in mice suggesting that the infectivity of sporozoites was adversely affected by development within *An. albimanus* (Noden *et al.*, 1995). Incubation of sporozoites isolated from one mosquito species in salivary gland homogenates from the other mosquito species was unable to resolve whether the non-infectiousness of sporozoites from *An. albimanus* resulted from the absence of a factor required for parasite development or the presence of toxic substances (Noden *et al.*, 1995).

Other studies have also found that sporozoites of malaria parasite species to which *An. albimanus* is refractory can develop within the salivary glands of this mosquito species (Omar, 1968a; Omar, 1968b). *An. albimanus* infected with sporozoites of *P. cynomolgi bastianellii* were seldom capable of transmitting parasite infection (Omar, 1968a). However, this was possibly due to the low densities of salivary gland

sporozoites resulting from minimal oocyst infections rather than non-infectivty of the sporozoites *per se* as with *P. yoelii yoelii* above.

In summary, previous research on the development of *Plasmodium* spp. in *An. albimanus* suggests that the ookinete to oocyst transition is the key stage during which many malaria parasite species fail to infect this mosquito species. In all instances that have been investigated, mature ookinetes successfully form within the midgut of *An. albimanus*: gametogenesis, fertilization and ookinete development proceed normally within this mosquito species and at equivalent levels to susceptible control mosquito species. However, the mature ookinetes that form within *An. albimanus* typically fail to establish viable oocyst infections. Several apparently independent mechanisms of refractoriness may operate on the malaria parasite during the developmental transition between the ookinete and oocyst stages within *An. albimanus*. These mechanisms of refractoriness vary between, and within, malaria parasite species and may operate before, during and/or after ookinete invasion of the midgut epithelium. However, the precise nature of the mechanisms of refractoriness that act during the ookinete to oocyst transition in *An. albimanus* have not yet been elucidated for any of the malaria parasite species investigated.

3.1.2 Techniques for investigating *Plasmodium* development in the mosquito

The studies described above, and others, have used a variety of techniques to investigate malaria parasite development within the mosquito vector depending on the stage of malaria parasite infection being examined and the particular contingencies of the malaria parasite-mosquito vector system being investigated.

Examination of Giemsa-stained smears of the bloodmeal contents is the simplest method for enumerating and monitoring ookinete development within the midgut lumen (Omar, 1968b; Vaughan *et al.*, 1991; Beier *et al.*, 1992; Gonzalez-Ceron *et al.*, 2001; Alavi *et al.*, 2003; Zollner *et al.*, 2005). However, the relevant malaria parasite stages are typically extremely rare in comparison to the number or erythrocytes present and difficult to discern amongst the substantial cellular debris of the partially digested bloodmeal. Consequently, Giemsa-stained bloodmeal smears are "agonizingly slow to read", rapidly give rise to "microscopist fatigue" and lack precision compared to other methods used to enumerate ookinete densities (Zollner *et al.*, 2005). However, Giemsa-stained bloodmeal smears have the advantage of being permanent preparations ready for examination whenever a dampening of the spirits is required. An alternative method is

to treat the bloodmeal with a solution of Tris-Acetic acid, that lyses uninfected erythrocytes while leaving the malaria parasites intact, thus removing the most of the cellular components of the bloodmeal and making identification of the ookinetes much easier (Vaughan *et al.*, 1991; Beier *et al.*, 1992; Vaughan *et al.*, 1992; Chege & Beier, 1994; Vaughan *et al.*, 1994a; Vaughan *et al.*, 1994b). The resulting suspension is examined immediately in a haemocytometer using phase-contrast microscopy. With both the Giemsa-stained blood smear and Tris-Acetic acid/haemocytometer methods, ookinetes are the only malaria parasite stages that can be reliably identified and unactivated late-stage *P. falciparum* gametocytes can be difficult to distinguish from ookinetes by the latter technique.

An alternative and preferred, if more sophisticated, method for monitoring malaria parasite development within the mosquito is to use fluorescence microscopy, either labelling the malaria parasites with specific fluorescein-conjugated antibodies or genetically transforming them with a construct expressing green fluorescent protein (GFP) (Chege & Beier, 1994; Robert *et al.*, 1994; Gouagna *et al.*, 1999; Han *et al.*, 2000; Natarajan *et al.*, 2001; Alavi *et al.*, 2003; Vlachou *et al.*, 2004; Frischknecht *et al.*, 2004; Franke-Fayard *et al.*, 2004; Gouagna *et al.*, 2004a). Fluorescent microscopy techniques also have the advantage of enabling relatively quick and easy methods for investigating malaria parasite infection of the midgut epithelium, in contrast to the traditional (and extremely laborious and labour intensive) light and/or electron microscopic examination of histological sections from infected midguts (Indacochea, 1935; Stohler, 1957; Omar, 1968b; Maier, 1973; Meis & Ponnudurai, 1989). However, fluorescent microscopy techniques require the existence of appropriate antibodies or genetically-transformed malaria parasite lines. GFP-expressing lines of the rodent malaria *P. berghei* have recently been developed for routine investigation of malaria parasites within their vertebrate and invertebrate hosts (Amino, Menard & Frischknecht, 2005). Although not in routine use, a transgenic GFP-expressing line is also available for the less tractable *P. falciparum* system (VanWye & Haldar, 1997; Kadekoppala *et al.*, 2000).

In order to characterise the reasons for the failure of the *P. falciparum* 3D7A clone to establish mature oocyst infections in *An. albimanus*, the development of this malaria parasite clone within this mosquito species, and in *An. stephensi*, were simultaneously studied in greater detail using direct immunofluorescence microscopy.

This method was chosen because of the unavailability of an appropriate genetically-modified GFP-expressing line of the *P. falciparum* 3D7A clone.

3. 2 Materials & Methods

Initially, it was planned to investigate the development of the *P. falciparum* 3D7A clone in *An. albimanus* and *An. stephensi* using a line of this malaria parasite that had been previously transformed with a chimeric human dihydrofolate reductase/*Aequorea victoria* green fluorescent protein construct (*Pf*HDGFP) (Kadekoppala *et al.*, 2000). A frozen aliquot of this *P. falciparum* 3D7 GFP-expressing line was obtained from the Malaria Research and Reference Reagent Resource Center (MR4; www.malaria.mr4.org) (Cat. No. MRA-317). However, despite several attempts, gametocytes could not be cultured from this transgenic malaria parasite line, probably because of the prolonged period of *in vitro* asexual erythrocytic stage culture that these malaria parasites would have previously undergone during genetic transformation.[1] Consequently, as an appropriate FITC-labelled mAb was already available, development of the *P. falciparum* 3D7A clone in *An. albimanus* and *An. stephensi* was subsequently investigated using direct immunofluorescence microscopy.

3.2.1 Parasite culture & mosquito infection

Malaria parasite culture and mosquito infections were performed as described in Chapter 2, Section 2. 2. As before, for each infection experiment, *An. albimanus* and *An. stephensi* were simultaneously fed gametocytes derived from the same cultures to enable direct comparisons to be made between the two mosquito species.

3.2.2 Direct immunofluorescence microscopy

The initial stages of *P. falciparum* development within *An. albimanus* and *An. stephensi* were compared using the fluorescein isothiocyanate (FITC)-conjugated α-Pfs25 monoclonal antibody (mAb) 32F72 (Vermeulen *et al.*, 1985a). The FITC-labelled α-Pfs25 mAb was a kind gift from Drs. Anton Lensen and Will Roeffen,

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[1] Prolonged *in vitro* asexual erythrocytic stage culture of *P. falciparum* frequently results in the loss of either gametocytogenesis and/or infectivity of mature gametocytes probably through adaptation of the malaria parasites to the conditions of *in vitro* culture which is frequently accompanied by deletions/rearrangements of genomic DNA (Jensen, 1979; Ponnudurai *et al.*, 1982; Graves *et al.*, 1984; Wellems *et al.*, 1988; Day *et al.*, 1993; Alano *et al.*, 1995b).

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Pfs25 is the *P. falciparum* orthologue of the *Plasmodium* genus-specific protein known as P25 (for a review of P25, and its paralogue P28, see Baton & Ranford-Cartwright, 2005a). Pfs25 is expressed at very low levels within the cytoplasm of immature and mature gametocytes but undergoes significant up-regulation and redistribution following macrogametocyte activation and transformation into macrogametes (Vermeulen *et al.*, 1985a). From about 2 hours after macrogamete formation, Pfs25 is found on the malaria parasite surface (as well as within the cytoplasm) persisting throughout the subsequent period of zygote differentiation into the ookinete (Vermeulen *et al.*, 1985a). Pfs25 continues to be expressed on the ookinete surface during migration from the bloodmeal and across the midgut epithelium (Meis & Ponnudurai, 1987b) and during the subsequent oocyst stages of the malaria parasite (Lensen *et al.*, 1992; Gouagna *et al.*, 1999). As Pfs25 is not expressed by asexual erythrocytic stage malaria parasites (Vermeulen *et al.*, 1985a), α-Pfs25 antibodies have been used to specifically label and, hence, monitor the different stages of malaria parasite development within the mosquito (Chege & Beier, 1994; Robert *et al.*, 1994; Robert *et al.*, 1995; Awono-Ambene & Robert, 1998; Gouagna *et al.*, 1998; Robert *et al.*, 1998; Gouagna *et al.*, 1999; Okech *et al.*, 2004a; Gouagna *et al.*, 2004a; Gouagna *et al.*, 2004b; Okech *et al.*, 2004b).

Preparation of samples for immunofluorescence microscopy of different developmental stages of the malaria parasite was carried out as described below. All samples were examined under epifluorescence using a Zeiss Axioskop light microscope fitted with a 100 watt mercury-arc lamp and Zeiss Filter Set 09. Images were captured using a Photometrics CoolSnap™ digital camera and Improvision® Openlab™ version 2 software. Further processing of the images was performed using Adobe® Photoshop® version 5.5.

3.2.2.1 Malaria parasite development within the bloodmeal

Previous field studies using FITC-labelled α-Pfs25 mAb to monitor malaria parasite development within the mosquito bloodmeal have used a technique in which the blood-fed midguts are homogenised in PBS, directly (without fixation) incubated with the labelled mAb, washed, resuspended and the resulting sample examined as a "wet preparation" (Robert *et al.*, 1995). In the current context, this technique was found

to have a number of drawbacks. First, a large number of malaria parasite stages (greater than 90%) were apparently lost during the washing step employed in this method when performed by the writer. Second, the number of malaria parasite stages present within each bloodmeal was considerably higher under laboratory conditions than that reported in the previous field studies and was far too great to be sensibly enumerated by counting all the parasites present within the entire specimen. Third, the prepared specimens were temporary and had to be examined immediately limiting the number of individuals that could be sampled at any given time. Consequently, a modified protocol was developed based upon a previously published protocol using indirect immunofluorescent detection of fixed specimens (Chege & Beier, 1994). The advantages of this technique were that (i) few parasite stages appeared to be lost during sample processing, (ii) a known fraction of each midgut sample could be examined within a reasonable time and used to estimate the total number of parasites present within the whole sample, and (iii) specimens could be stored indefinitely enabling sampling of many individuals over extended periods of time.

For each mosquito species, six adult females were dissected every six hours after ingestion of the infectious gametocyte stage malaria parasites until the process of bloodmeal digestion was complete (approximately 42 to 60 hours pbf depending on the mosquito species). Individual mosquitoes were knocked down with chloroform, dipped into 70% ethanol, submerged in PBS, and kept on ice until the midguts were dissected in PBS at pH 7.2. Dissected intact midguts, including the entire bloodmeal contents, were individually homogenised in 40 µl of sterile PBS by gentle repeated pipetting. Sterile 0.5 ml "non-stick" low retention hydrophobic tubes (Alpha Laboratory Supplies, Cat No. LW2400AS) and sterile "non-stick" low retention hydrophobic pipette tips (Alpha Laboratory Supplies, Cat No. LW1720S) were used to minimise malaria parasite adhesion to plastic surfaces and their consequent loss during sample preparation. A further 280 µl of sterile PBS was subsequently added to each midgut homogenate. One thirty-secondth of this diluted midgut homogenate (10 µl) was then spotted, in duplicate, onto Teflon®-printed microwell glass slides (VWR International, Cat. No. 631-0470) previously treated with 3-aminopropyltriethoxysilane (APES) according to the supplier's instructions (Sigma, Cat. No. A3648). This dilution and volume of midgut homogenate was empirically determined to: (i) provide a density of malaria parasite stages countable within approximately 15 minutes, and (ii) prevent overlaying of the cells within the sample, which inhibited adhesion of the cells to the glass slide and

resulted in sample loss during subsequent incubation with the labelled mAb. The sample slides were then air-dried, fixed in ice cold acetone for 2 minutes, allowed to air-dry again and then placed into a desiccator for at least 24 hours. Clean untreated sample-free glass slides were subsequently placed over the upper surface of the sample slides (to prevent anything sticking to the APES-treated surface and the sample sticking to another treated slide). Both slides were then wrapped in tissue and aluminium foil, placed in an airtight sealable plastic bag containing silica gel and either kept at room temperature or frozen at -25°C until examination for the relevant malaria parasite stages. The slides were read at leisure by direct fluorescence microscopy using the FITC-labelled α-Pfs25 mAb. If previously frozen, slides were allowed to equilibrate to room temperature before addition of the mAb. Ten microlitres of an appropriate dilution of the mAb, determined by titration against control slides, was then added to each microwell. Slides were incubated in a dark moist chamber at room temperature for 40 minutes and then each well was washed once by adding and removing 80 µl of sterile PBS. Several microlitres of 2.5 mg/ml of 1,4-diazabicyclo[2.2.2]octane (DABCO) (Sigma, Cat. No. D2522) in glycerol were then added to each well as an anti-fade reagent and the slides mounted with a cover slip before examination under epifluorescence at 400X magnification. The total number of round forms, retort-forms and mature ookinetes in each spotted sample was counted. Average values for the densities of each malaria parasite stage present within each midgut examined were calculated from the two replicates. These average values were then multiplied by the dilution factor of the sample (1 in 32) to give an estimate of the total number of each malaria parasite stage present within the entire bloodmeal.

3.2.2.2 Ookinete invasion of the midgut epithelium

The mosquito midgut epithelia were prepared for immunofluorescence as previously described (Han *et al.*, 2000; Han *et al.*, 2001). Between 24 and 32 hours pbf, midguts were dissected in sterile PBS and immediately fixed for 30 seconds in fresh 4.0% paraformaldehyde (Sigma, Cat. No. P6148) in 100mM 1,4-piperazinediethanesulfonic acid (PIPES; Sigma, Cat. No. P6757) buffer at pH 7.4, 2 mM $MgSO_4$ and 1mM ethylene glycol-bis(b-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA; Sigma Cat. No. E4378). The midguts were then cut open longitudinally using a scalpel blade and the luminal contents (the bloodmeal and peritrophic matrix) were removed. The resulting midgut epithelial sheets were placed into the wells of 96-

well microtitre plates (Sigma, Cat. No. M0156) containing 100 µl of 4.0% paraformaldehyde/PIPES/$MgSO_4$/EGTA solution and fixed for a further 30 minutes at room temperature. All subsequent stages of sample preparation were performed within the same wells of the microtitre plates. After fixation, the midguts were washed twice for 5 minutes each in sterile PBS. The midguts were then blocked at room temperature in 1.0% bovine serum albumin (BSA) (Sigma, Cat. No. A9418) and 0.1% Triton X-100 (Sigma Cat. No. X100) in PBS at pH 7.5 (PBS-T) for 2 hours. The midguts were then washed twice for 5 minutes each in PBT, and incubated with FITC-labelled α-Pfs25 mAb for 1 hour at room temperature in a dark moist chamber. The midguts were finally washed again, twice for 5 minutes each in PBS-T, and then mounted on a glass slide with 2.5 mg/ml of DABCO in glycerol before examination under epifluorescence at 400X, 600X and 1000X magnification.

3.2.2.3 Early oocyst formation

Whole midguts were examined for early oocysts between 56 and 76 hours pbf as previously described (Awono-Ambene & Robert, 1998; Gouagna *et al.*, 1998; Gouagna *et al.*, 1999). Freshly dissected and unfixed midguts were incubated with 20 µl of FITC-labelled α-Pfs25 mAb in 0.05% Evan's blue (as counter-stain) at room temperature for 30 minutes in a dark moist chamber. Incubations were performed in the wells of 96-well flat-bottomed microtitre assay plates (Sigma, Cat. No. M0156). The midguts were subsequently washed twice with 80 µl of sterile PBS, transferred to a glass slide using a pipette and then mounted individually with 25 µl of fresh sterile PBS and a cover slip before examination. Samples were examined at 400X and 1000X using oil-immersion under epifluorescence and then bright-field white light to confirm the presence of haemozoin pigment granules (Gouagna *et al.*, 1999).

3.2.3 Estimation of total protein content per midgut

The total protein content of the midgut samples used to monitor malaria parasite development within the bloodmeal was estimated using a bicinchoninic acid protein assay kit (Sigma, Cat. No. BCA-1) according to the manufacturer's instructions. The midgut homogenate remaining after preparing the slides for direct immunofluorescence microscopy was diluted to an equivalent of 500 µl PBS per midgut. The protein content of the midgut samples was estimated using known concentrations of bovine serum albumin (BSA) as standards (stock solution of BSA was diluted in PBS to give concentrations of 0, 100, 200, 300, 400, 500, 600 and 700 µg/ml). Fifty microlitres of

each BSA standard and diluted midgut homogenate were added in duplicate to 96-well flat-bottomed assay plates (Sigma, Cat. No. M0156). Two hundred microlitres of BCA working reagent were then added to each well, the assay plate sealed and incubated at 37°C for 30 mins. The assay plates were subsequently read at 570 nm using an MRX Revelation Plate Reader (Dynex Technologies, Inc., USA).

3.2.4 Estimation of malaria parasite survival between different life-cycle stages

In order to estimate the survival of malaria parasites during development within the mosquito vector, an estimate of the number of macrogametocytes ingested during bloodfeeding was required. Accordingly, the mean total number of macrogametocytes ingested per mosquito was calculated as follows: macrogametocytaemia of the bloodmeal x mean erythrocyte density of the bloodmeal x mean bloodmeal volume.

The macrogametocytaemia of the bloodmeal was determined by light microscopical examination of Giemsa-stained thin smears made from the diluted malaria parasite culture immediately prior to membrane-feeding the mosquitoes.

The average erythrocyte density of the bloodmeal was calculated by taking the mean of duplicate red blood cell haemocytometer counts of the diluted parasite culture fed to the mosquitoes. Briefly, 5 µl of the bloodmeal were mixed with 495 µl of PBS and 10 µl of the diluted sample loaded into a haemocytometer. The number of erythrocytes in two separate 0.1 µl samples was recorded and used to calculate the total number of erythrocytes per µl of the bloodmeal.

The mean bloodmeal volume per midgut was calculated using Drabkin's haemoglobinometric method (Briegel, Lea & Klowden, 1979). Briefly, 480 µl of Drabkin's solution, prepared according to the manufacturer's instructions (Sigma, Cat. No. 525-2), was added to blood-fed midguts dissected immediately after engorgement and homogenised in 20 µl of PBS at pH 7.2. Known volumes of the diluted culture fed to the mosquitoes were used as standards. After incubation at room temperature for 30 minutes, the 200 µl of each sample were transferred in duplicate to 96-well flat-bottomed plates and read at 540 nm using the MRX Revelation Plate Reader.

3.2.5 Statistical analysis

All statistical tests were performed using XLStat© version 7.5.2 (Addinsoft, 1995-2004).

3.2.5.1 Comparison of the levels of *P. falciparum* 3D7A infection

3.2.5.1.1 Malaria parasite development within the bloodmeal

Counts of the numbers of round forms, and immature retort-form and mature ookinetes observed within the bloodmeals of *An. albimanus* and *An. stephensi* exhibited no evidence of non-normality, and had equivalent variances between the mosquito species. However, between time points, there was significant heteroscedasticity, both within and between the two mosquito species (data not shown) such that the means and variances of the sample groups were positively correlated. Various transformation procedures were applied to the data, including rank transformation, but none of these completely eliminated the problem of heteroscedasticity. Consequently, it was not possible to analyse the data using parametric two-way ANOVA and no appropriate non-parametric two-way ANOVA procedure (with an associated multiple comparison testing procedure) was known to, or could be found by, the author.

Instead, planned orthogonal (i.e. independent) comparisons were performed, within each time point, between the two mosquito species, for each of the three developmental stages of the malaria parasite investigated. The prevalence of the malaria parasites stages was compared using a two-tailed Fisher's exact test because of the small sample sizes (6 observations for each mosquito species). The intensity of the malaria parasites stages was compared using an unpaired two-tailed Student's *t* test (assuming equal variances between the sample groups). In order to control the family-wise Type I error rate when performing multiple tests on the same set of data, the sequential Bonferroni procedure was used to appropriately adjust the significance values of the Fisher's Exact and *t* tests (Holm, 1979; Quinn & Keough, 2002; Morrison, 2002). In all instances, a nominal value of $\alpha = 0.05$ was used for the overall family-wise level of significance. However, the appropriateness of adjusting significance values when performing multiple *planned* comparisons (rather than "data snooping" i.e. performing all possible comparisons without specific *a priori* hypotheses) is questioned by some authors for being unnecessarily conservative and reducing power, especially when the comparisons made are orthogonal and small in relation to the total set of all possible pairwise comparisons (Quinn & Keough, 2002). Consequently, the results of both the unadjusted and adjusted significance tests are presented. In most instances, "significant" unadjusted *P* values remained significant after adjustment for multiple comparison testing. However, in some instances, *P* values were no longer significant

after adjustment for multiple comparison testing.

3.2.5.1.2 Early and late oocyst infections

The prevalence of *P. falciparum* 3D7A oocyst infection observed in *An. albimanus* and *An. stephensi* at different times pbf was compared using either one- or two-tailed Fisher's Exact tests, depending on the hypothesis being tested. Comparison of the prevalence of *P. falciparum* 3D7A infection between more than two groups of mosquitoes was performed using chi-square test.

The intensity of *P. falciparum* 3D7A oocyst infection in *An. albimanus* and *An. stephensi* at different times pbf was compared using non-parametric tests of location. When the intensity of malaria parasite infection was being compared between two groups of mosquitoes, either a one- or two-tailed Mann-Whitney *U* test was employed. When the intensity of malaria parasite infection was being compared between three or more groups of mosquitoes, a Kruskal-Wallis test was used.

3.2.5.2 Comparison of total protein content per midgut between mosquito species

For reasons similar to those outlined in Section 3.2.5.1.1 above for statistical analysis of the development of different malaria parasite stages within the bloodmeal, the estimated total protein content per midgut was compared, within time points, between *An. albimanus* and *An. stephensi*, using *t* tests with significance levels adjusted for multiple comparisons as previously described.

3. 3 Results

The development of the *P. falciparum* 3D7A clone was compared in *An. albimanus* and *An. stephensi* using direct fluorescence microscopy. Due to logistical constraints, malaria parasite development within the bloodmeal, ookinete invasion of the midgut epithelium, and levels of early oocyst infection were analysed separately in multiple independent experimental feeds rather than simultaneously within the same cohort of experimentally infected mosquitoes.

3.3.1 Comparison of *P. falciparum* 3D7A development in *An. albimanus* and *An. stephensi*

3.3.1.1 *P. falciparum* 3D7A development within the bloodmeal

The development of *P. falciparum* 3D7A within the bloodmeal of *An. albimanus* and *An. stephensi* was compared in two separate experimental feeds. However, the

results from only one these experimental feeds are presented here, as the bloodmeal slides from the second experimental feed have not been fully analysed at the time of writing.

As previously reported, three distinct morphological types of FITC-positive malaria parasites could be detected within the bloodmeals of mosquitoes membrane-fed infectious gametocytes: spherical "round forms", intermediate immature retort-form ookinetes, and mature banana-shaped ookinetes (Figure 3.1) (Vermeulen *et al.*, 1985a). The round forms represent an heterogeneous population of unfertilised and fertilised macrogametes, which are indistinguishable from one another on the basis of α-Pfs25 mAb labelling (Vermeulen *et al.*, 1985a; Fries *et al.*, 1989). The morphology of the round forms changed with increasing time pbf, these malaria parasite stages being relatively large at 6 hours pbf and then gradually diminishing in size and becoming increasingly irregular in outline as bloodmeal digestion proceeded.

The results for the one experimental feed for which all samples have been analysed are shown in Figure 3.2 to Figure 3.7. Overall, the pattern of malaria parasite development within the bloodmeal was broadly similar in *An. albimanus* and *An. stephensi*, in particular, during the early stages of bloodmeal digestion (up to 24 hrs pbf inclusive). However, comparison of the levels of the three developmental stages of the malaria parasite revealed that the kinetics of *P. falciparum* 3D7A disappearance from the midgut lumen differed significantly between these two mosquito species, especially during the latter stages of bloodmeal digestion (from 30 hours pbf inclusive). A detailed analysis of the data upon which these conclusions are based is given in the following two Sections (3.3.1.1.1 and 3.3.1.1.2).

3.3.1.1.1 Prevalence of P. falciparum *3D7A developmental stages within the bloodmeal*

The prevalence of the three different developmental stages of *P. falciparum* 3D7A observed within the bloodmeals of *An. albimanus* and *An. stephensi* is shown in Figure 3.2 and compared in Figure 3.3.

Initially, the prevalence of round forms, and immature retort-form and mature ookinetes was indistinguishable between *An. albimanus* and *An. stephensi*. Between 6 and 24 hours pbf, the prevalence of both round forms and retort-form ookinetes was 100% in both mosquito species. No mature ookinetes were seen at 6 and 12 hours pbf in either mosquito species. Mature ookinetes first became apparent within the

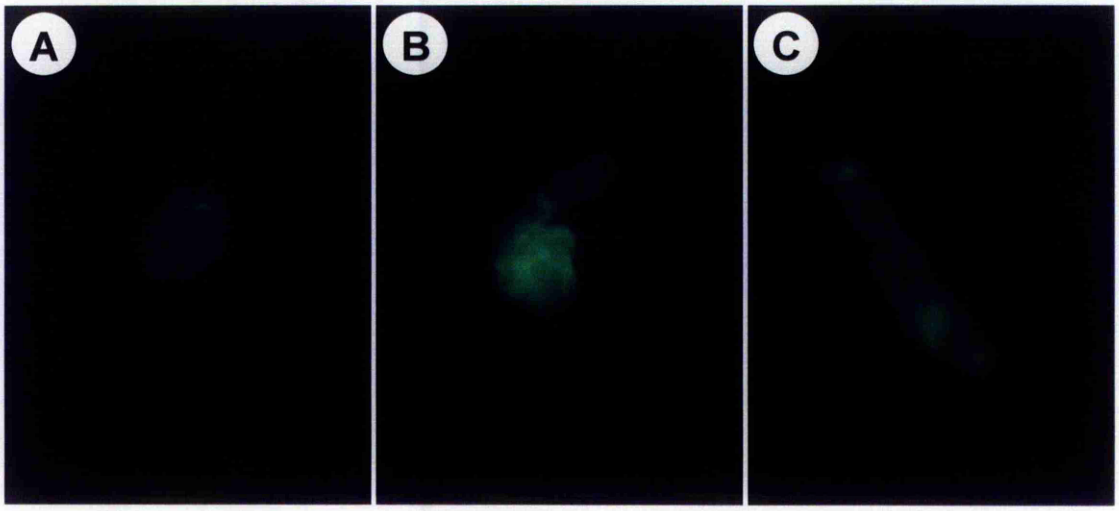


Figure 3.1 Direct immunofluorescence microscopy of the different developmental stages of *P. falciparum* 3D7A within the bloodmeal of *An.albimanus*.

Three developmental stages of *P. falciparum* 3D7A could be detected in smears of bloodmeals taken from *An. albimanus* and prepared for direct immunofluorescence microscopy using the FITC-labelled α-Pfs25 mAb: **(A)** round forms (unfertilised macrogametes and zygotes), **(B)** immature "retort-form" ookinetes, and **(C)** mature ookinetes. Similar malaria parasite stages were also detected within bloodmeals from *An. stephensi* simultaneously fed the same *P. falciparum* 3D7A gametocyte cultures (not shown). 1000X. 24 hours pbf.

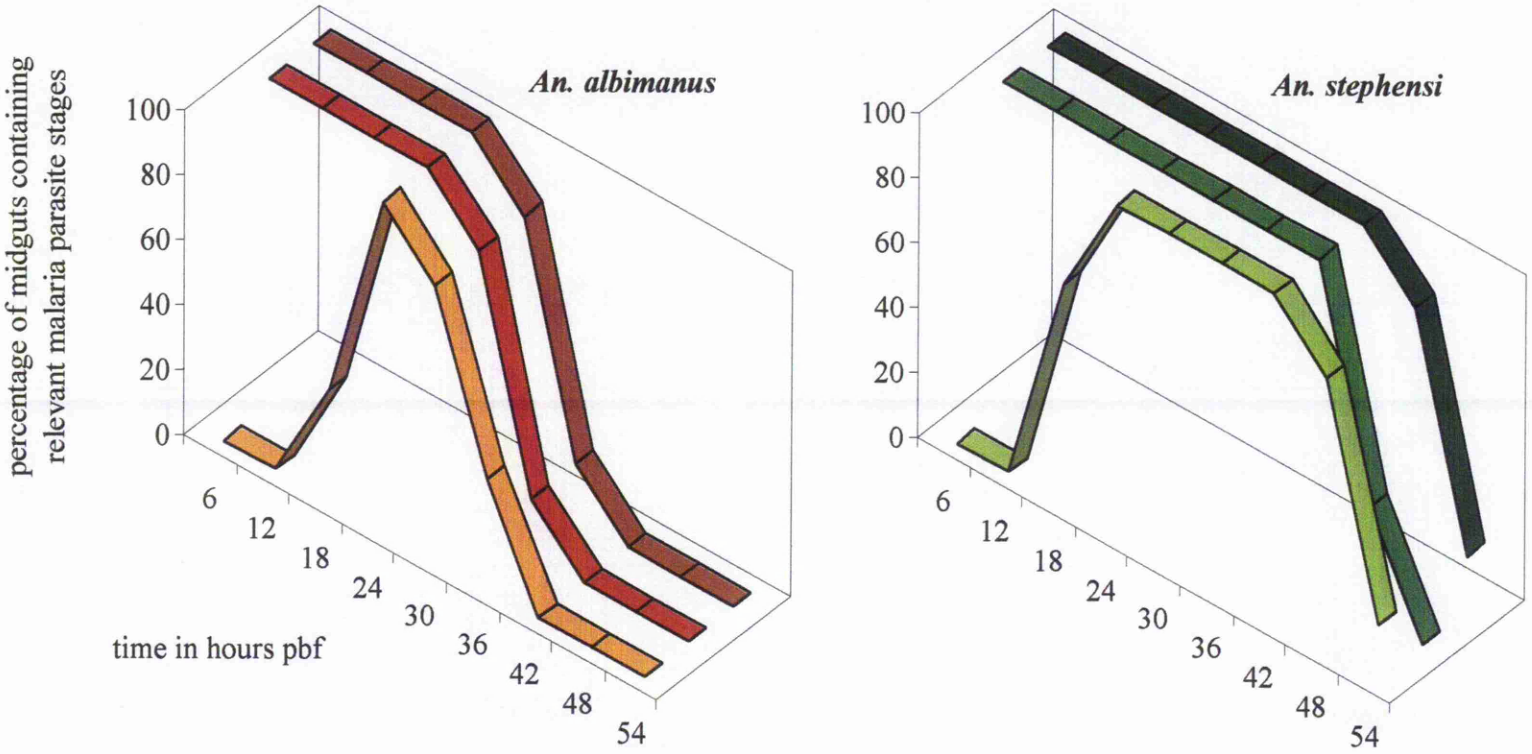


Figure 3.2 The prevalence of different *P. falciparum* 3D7A developmental stages within the bloodmeals of *An. albimanus* and *An. stephensi*.

Graphs showing the prevalence of different *P. falciparum* 3D7A developmental stages found within the bloodmeals of *An. albimanus* and *An. stephensi* with increasing time after blood-feeding. Key: Red = *An. albimanus* (●) round forms, (●) retort-form ookinetes, and (●) mature ookinetes; Green = *An. stephensi* (●) round forms, (●) retort-forms, and (●) mature ookinetes. For all time points, $n = 6$ for both mosquito species.

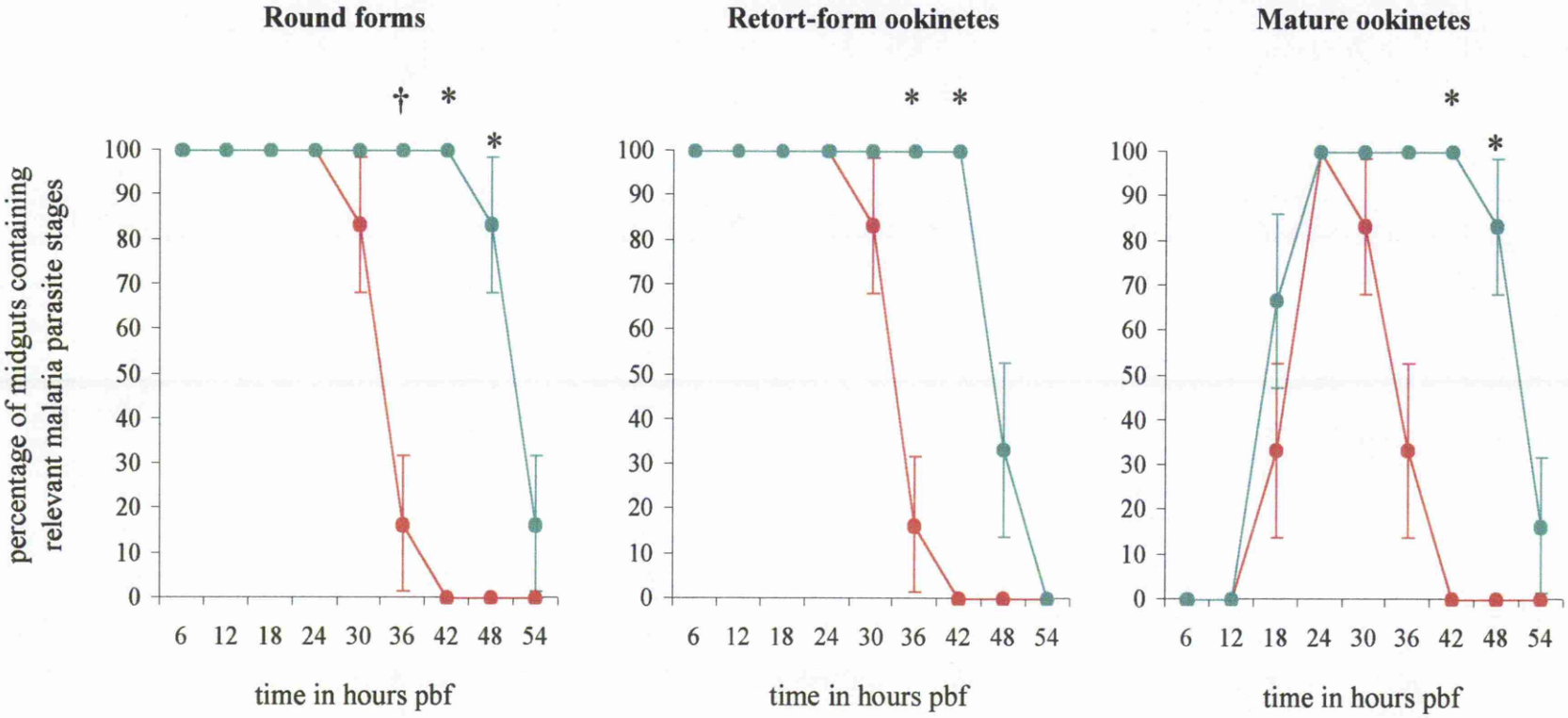


Figure 3.3 Comparison of the prevalence of *P. falciparum* 3D7A malaria parasites within bloodmeals from *An. albimanus* and *An. stephensi*.

Graphs comparing the prevalences of different *P. falciparum* 3D7A developmental stages within the bloodmeals of *An. albimanus* and *An. stephensi*. Key: (●) *An. albimanus*; and (●) *An. stephensi*. Crosses and asterisks indicate "significant" differences between *An. albimanus* and *An. stephensi* using *P* values unadjusted or adjusted, respectively, for multiple comparisons (see Section 3.2.5 for details). For all time points, $n = 6$ for both mosquito species. Data shown are the same as those presented in Figure 3.2 arranged according to malaria parasite stage. Error bars indicate standard errors of the proportion.

bloodmeal at 18 hours pbf, attaining approximately 50% prevalence in both mosquito species, increasing to a peak 100% prevalence at 24 hours pbf. Neither the prevalence of round forms, immature retort-forms or mature ookinetes differed significantly between *An. albimanus* and *An. stephensi* during the first 24 hours pbf (Figure 3.3).

During the second 24 hours pbf, significant differences became apparent in the prevalences of round forms, immature retort-forms and mature ookinetes observed in *An. albimanus* and *An. stephensi* (Figure 3.3). In *An. albimanus*, from 24 hours pbf exclusive, the prevalences of all three of these developmental stages simultaneously began to decline while 100% of the *An. stephensi* examined continued to harbour all of the relevant malaria parasite stages until 42 hours pbf. After 36 hours pbf, round forms, immature retort-forms and mature ookinetes were no longer found within the midguts of *An. albimanus*. These developmental stages of the malaria parasite were still present within *An. stephensi* until at least 48 hours pbf. The prevalences of round forms, retort-forms and mature ookinetes did not differ significantly between *An. albimanus* and *An. stephensi* at 30 hours pbf (Fisher's Exact test, $P > 0.567$ for each developmental stage). Between 36 and 48 hours pbf the prevalences of these three malaria parasite stages differed significantly between the two mosquito species (Figure 3.3).[1] At 54 hours pbf, when the prevalences of all three of these developmental stages of the malaria parasite in *An. stephensi* was less than 20%, there were no significant differences in the prevalences of round forms, retort-forms and mature ookinetes between this mosquito species and *An. albimanus*.

3.3.1.1.2 Intensity of P. falciparum *3D7A developmental stages within the bloodmeal*

The intensity of the three different developmental stages of *P. falciparum* 3D7A observed within the bloodmeals of *An. albimanus* and *An. stephensi* are shown in Figure 3.4 and compared in Figure 3.5. In general, the intensity of round forms, and immature retort-form and mature ookinetes followed a pattern of change over time commensurate with the alterations in the prevalences of these three malaria parasite stages observed

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[1] The prevalence of mature ookinetes observed at 36 hours pbf was not significantly different between *An. albimanus* and *An. stephensi* as shown in Figure 3.3. However, the observed P value was 0.061 with $\alpha = 0.05$ unadjusted for multiple comparison testing. This comparison remained non-significant following adjustment using the sequential Bonferroni procedure to maintain the family-wise Type 1error rate at the same overall level of significance (for this particular comparison, with the nominal family-wise significance level set at $\alpha = 0.05$, $P_{crit} = 0.0100$).

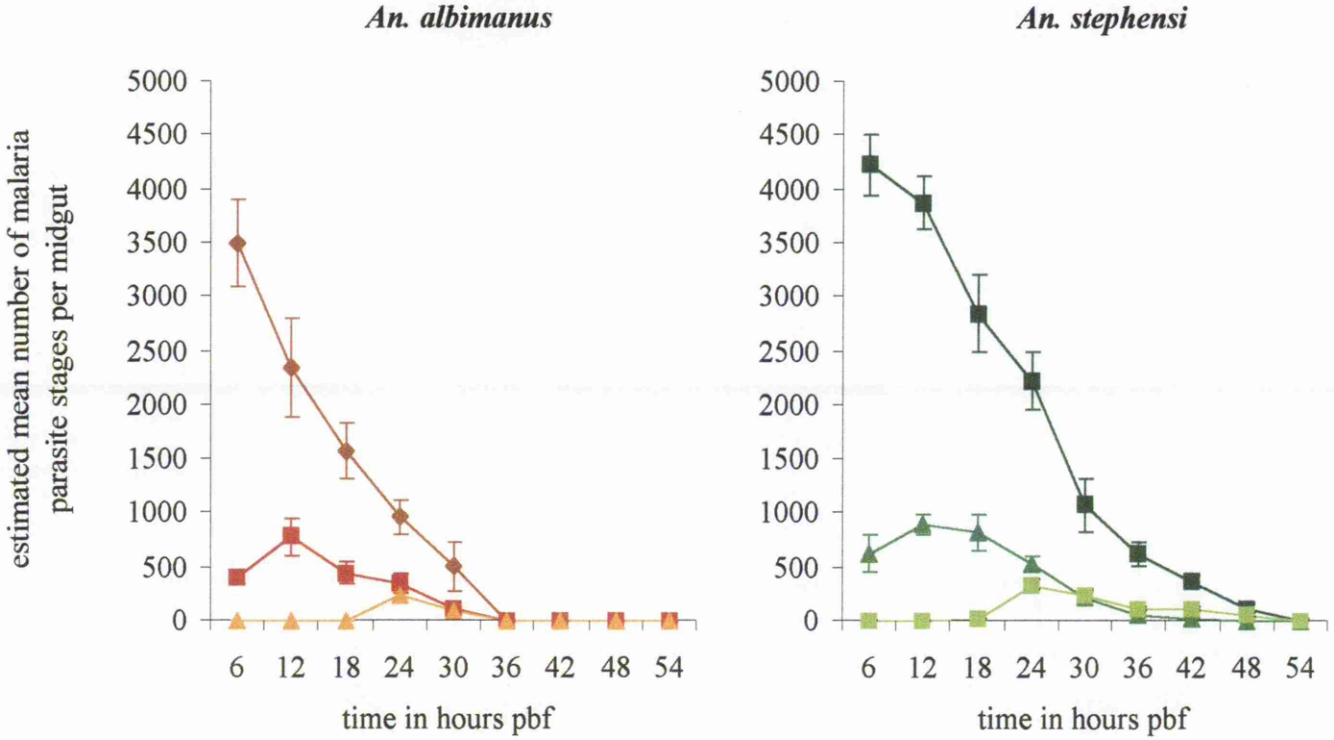


Figure 3.4 The intensity of different *P. falciparum* 3D7A developmental stages within the bloodmeals of *An. albimanus* and *An. stephensi*.

Graphs showing the estimated average (mean) numbers of different *P. falciparum* 3D7A developmental stages found within the bloodmeals of *An. albimanus* and *An. stephensi* with increasing time after blood-feeding. Key: Red = *An. albimanus* (●) round forms, (●) retort-form ookinetes, and (●) mature ookinetes; Green = *An. stephensi* (●) round forms, (●) retort-forms, and (●) mature ookinetes. For all time points, $n = 6$ for both mosquito species. Error bars indicate standard errors of the mean.

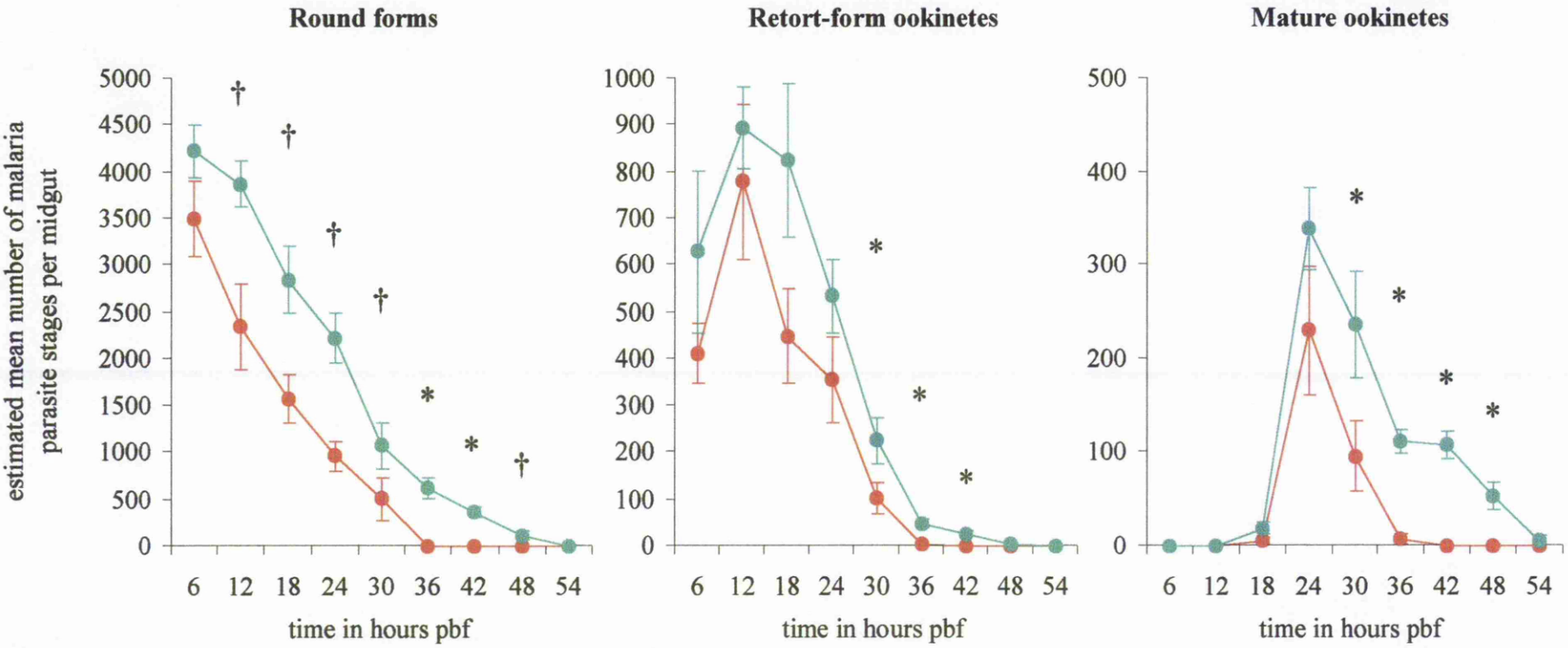


Figure 3.5 Comparison of the intensity of *P. falciparum* 3D7A malaria parasites within bloodmeals from *An. albimanus* and *An. stephensi*.

Graphs comparing the intensities of different *P. falciparum* 3D7A developmental stages within the bloodmeals of *An. albimanus* and *An. stephensi*. Note different scales of the y axis in the three graphs. Key: (●) *An. albimanus*; and (●) *An. stephensi*. Crosses and asterisks indicate "significant" differences between *An. albimanus* and *An. stephensi* using *P* values unadjusted or adjusted, respectively, for multiple comparisons (see Section 3.2.5 for details). For all time points, $n = 6$ for both mosquito species. Error bars indicate standard errors of the mean. Data shown are the same as those presented in Figure 3.4 arranged according to malaria parasite stage rather than mosquito species.

within the two mosquito species (Figure 3.4).

In both *An. albimanus* and *An. stephensi*, the intensity of round forms was highest at 6 hours pbf and declined steadily and consistently thereafter with increasing time pbf. The intensity of immature retort-form ookinetes, early in the process of differentiation into mature ookinetes, was already high at 6 hours pbf, but peaked at 12 hours pbf in both mosquito species, before also declining with increasing time pbf as differentiation into mature ookinetes was completed. As the numbers of retort-forms began to decline at 18 hours pbf, mature ookinetes simultaneously appeared within the bloodmeals of *An. albimanus* and *An. stephensi*, reaching a peak density at 24 hours pbf and then declining over the remaining period of bloodmeal digestion.

Initially, the mean numbers of round forms, retort-forms and mature ookinetes per midgut did not differ significantly between *An. albimanus* and *An. stephensi* with similar peak densities of all three of these malaria parasite stages within both mosquito species (Figure 3.5). However, with increasing time pbf, in particular, from 30 hours pbf onwards, the mean numbers of round forms, retort-forms and mature ookinetes per midgut exhibited significant differences between *An. albimanus* and *An. stephensi*, being markedly lower in the former mosquito species (Figure 3.5).[1]

3.3.1.1.3 Relative and absolute densities of P. falciparum *3D7A developmental stages within the bloodmeal*

The proportion of FITC-positive malaria parasites that were observed as round forms, and immature retort-form or mature ookinetes changed over time (Figure 3.6). For the first 30 hours pbf, the change in the proportion of FITC-positive malaria parasites observed in different developmental stages was similar in both *An. albimanus* and *An. stephensi*: the proportion of FITC-positive malaria parasites that were round forms declined constantly, while the proportion of retort-form ookinetes rose and then declined, and the proportion of mature ookinetes rose throughout this time period. However, the total number of FITC-positive malaria parasites differed significantly

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[1] The difference in the intensity of immature retort-form ookinetes between *An. albimanus* and *An. stephensi* at 18 and 24 hours pbf was not significant when α was unadjusted for multiple comparison testing ($P = 0.0815$ and $P = 0.0856$, respectively) nor after adjustment of the P values using the sequential Bonferroni procedure. For the two particular comparisons in question, for a nominal family-wise significance level of $\alpha = 0.05$, $P_{crit} = 0.0100$ and 0.0125 respectively.

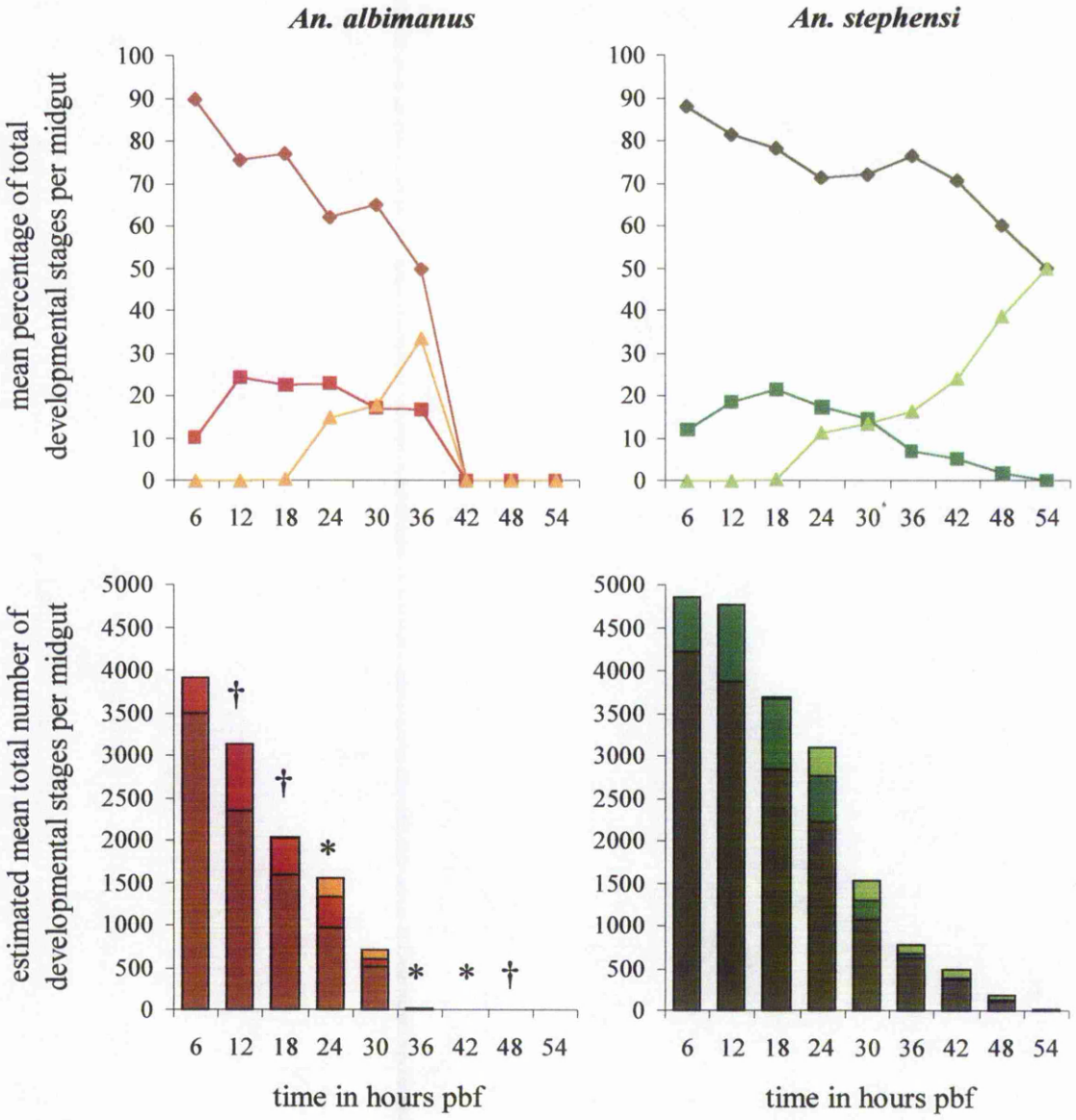


Figure 3.6 Comparison of the proportion and total density of different *P. falciparum* 3D7A developmental stages within the bloodmeals of *An. albimanus* and *An. stephensi*. Upper graphs: show the average relative contribution (mean percentage) of the three developmental stages investigated to the total (combined) number of these stages observed per midgut. Lower graphs: show the absolute contribution of each of the three developmental stages investigated to the estimated average total (combined) number of these stages observed per midgut. Key: Red = *An. albimanus* (●) round forms, (●) retort-form ookinetes, and (●) mature ookinetes; Green = *An. stephensi* (●) round forms, (●) retort-forms, and (●) mature ookinetes. For all time points, $n = 6$ for both mosquito species. Crosses and asterisks indicate "significant" differences between *An. albimanus* and *An. stephensi* in the total (combined) number of developmental stages using P values unadjusted or adjusted, respectively, for multiple comparisons.

between *An. albimanus* and *An. stephensi* (Figure 3.6).

3.3.1.1.4 Estimation of the total protein content of midguts during bloodmeal digestion

The rate of bloodmeal digestion was quantitatively compared between *An. albimanus* and *An. stephensi* by estimating the total protein content of the same midgut samples used to monitor the development of round forms, retort-forms and mature ookinetes within the bloodmeals of these two mosquito species (Figure 3.7).

Overall, the pattern of change over time in the average estimated total protein content per midgut examined was broadly similar in both mosquito species, decreasing within increasing time pbf and mirrored observations made from direct visual inspection of dissected midguts. Although the total amount of protein per midgut was not significantly different between *An. albimanus* and *An. stephensi* at 6 hours pbf, the observed difference between the two mosquito species was borderline (two-tailed *t* test, $t_{0.05,\pm1.812} = -2.135$, $P = 0.0585$ unadjusted for multiple comparison testing; with adjustment for multiple testing, and a nominal family-wise significance level set at $\alpha = 0.05$, $P_{crit} = 0.0250$ for this particular comparison). However, the total amount of protein per midgut differed significantly between *An. albimanus* and *An. stephensi* throughout most of the remaining period of time investigated (12 to 48 hours pbf) (Figure 3.7). A similar pattern of bloodmeal digestion, and differences between the two mosquito species, as estimated by total protein content, was observed in the other experimental feed for which the analysis of *P. falciparum* 3D7A development within the bloodmeal has not yet been completed (data not shown).

The total protein content per midgut was compared with the densities of the different developmental stages of the malaria parasite observed within the same midgut samples. The decline in protein content per midgut over time closely mirrored the decline in the number of round forms per midgut during the same period, within both mosquito species (Figure 3.7). In contrast, changes in the numbers of retort-forms and mature ookinetes, at least during the first 24 hours pbf, exhibited no obvious association with the protein content per midgut.

The quantitative measurement of bloodmeal digestion reported here also correlated with qualitative changes observed in the colour, size and shape of the bloodmeal in dissected midguts. With increasing the time pbf, the bloodmeal became increasingly darker, changing from a bright crimson red to a dark, off black, ruddy brown. Concomitantly, the bloodmeal shrank in size and the midgut became

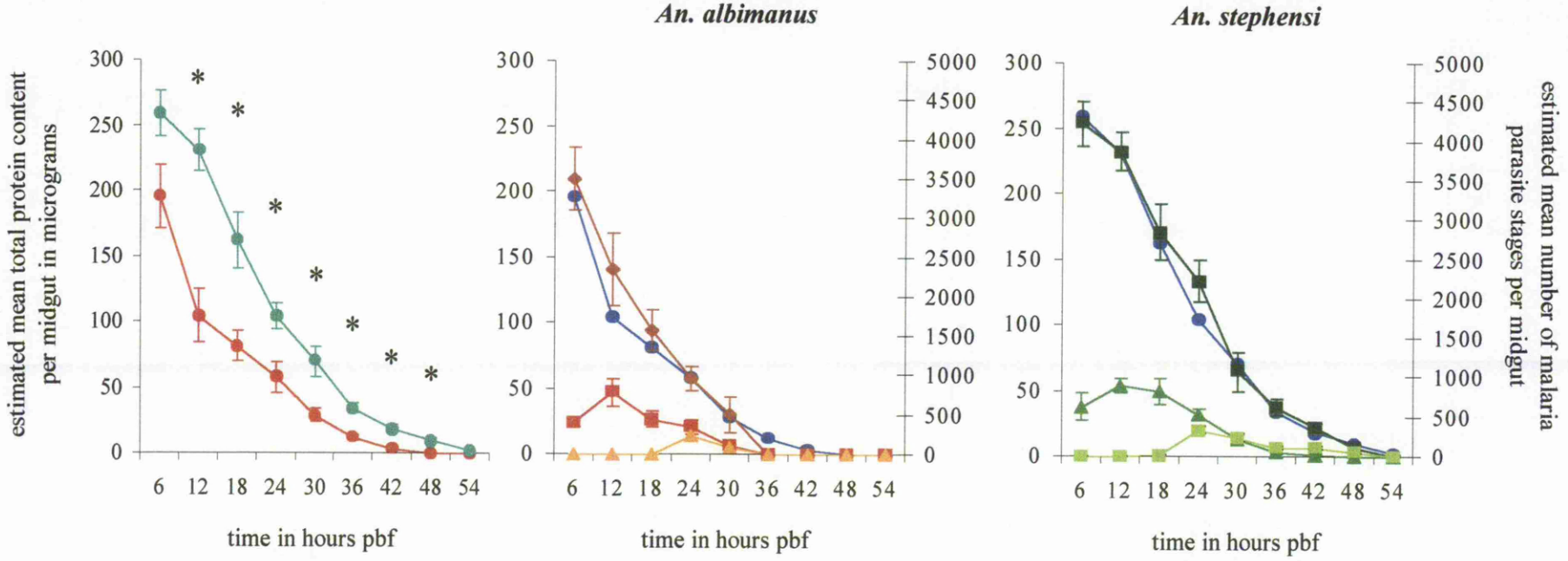


Figure 3.7 Comparison of the rate of bloodmeal digestion and the intensities of different *P. falciparum* 3D7A developmental stages within the bloodmeals of *An. albimanus* and *An. stephensi*.

Left-hand graph: shows change over time in the estimated average (mean) total protein content per midgut for each mosquito species. Key: (●) *An. albimanus*; and (●) *An. stephensi*. Asterisks indicate significant differences between *An. albimanus* and *An. stephensi* using *P* values adjusted for multiple comparisons. Right-hand graphs: show the curves of average total protein content for each mosquito species (●) from the left-hand graph superimposed on the graphs from Figure 3.5 showing the average (mean) numbers of different *P. falciparum* 3D7A developmental stages within the *same* bloodmeals from *An. albimanus* and *An. stephensi* analysed for protein content (for key see Figure 3.5). Note the size of the scales is constant between graphs. For all time points, $n = 6$ for both mosquito species. Error bars indicate standard errors of the mean.

increasingly less distended until at later time points only small dark remnant fragments of the bloodmeal could be observed within the midgut lumen. Additionally, midguts became increasingly "rigid" between approximately 24 to 36 hours pbf, presumably due to formation and hardening of the peritrophic matrix. These visual characteristics of progressive bloodmeal digestion were apparent approximately 6 to 12 hours earlier in *An. albimanus* compared to *An. stephensi*. Accordingly, in *An. albimanus* the majority of the bloodmeal had disappeared from midguts as early as 36 hrs pbf and was no longer visually present within the midguts by 42 hours pbf. In contrast, remnants of the bloodmeal could still be found in *An. stephensi* as late as 54 hours pbf and was only visually absent from all midguts examined at 60 hours pbf.

3.3.1.1.5 Bloodmeal volume of An. albimanus *and* An. stephensi

The volume of blood ingested during bloodfeeding was determined using haemoglobinometry in the same cohort of mosquitoes used to investigate malaria parasite development within the bloodmeal. *An. albimanus* ingested significantly smaller bloodmeals than *An. stephensi* (mean 1.82 and 3.66 µl respectively; two-tailed *t* test assuming equal variances, $t_{0.05, 2.045} = -4.407$, d.f. = 29, $P = 0.00013$). Again, this quantitative measurement correlated with direct observation of the dissected blood-fed midguts which were appreciably larger in *An. stephensi* compared to *An. albimanus*.

3.3.1.1.6 Levels of oocyst infection on day 10 post-bloodfeeding

The levels of mature oocyst infection observed at day 10 pbf in the two experimental feeds used to investigate the development of *P. falciparum* 3D7A within the bloodmeals of *An. albimanus* and *An. stephensi* are shown in Table 3.1. Similar to the observations reported in Chapter 2, no oocysts were observed in *An. albimanus* while *An. stephensi* was heavily infected at day 10 pbf. In both experimental feeds, the prevalence and intensity of oocyst infection at day 10 pbf differed significantly between *An. albimanus* and *An. stephensi* (experimental feeds 1 and 2 in Table 3.1).

3.3.1.2 *P. falciparum* 3D7A ookinete invasion of the midgut epithelium

Midgut epithelia from several separate experimental feeds were processed for immunofluorescent detection of malaria parasites using the FITC-labelled α-Pfs25 mAb. In total, 20 mosquitoes (10 *An. albimanus* and 10 *An. stephensi*) from two experimental feeds were examined. However, no FITC-positive malaria parasites, or midgut epithelial cells, were detected in midgut epithelia from either *An. albimanus* or

Table 3.1 Comparison of the levels of mature oocyst infection observed at day 10 post-bloodfeeding in the cohorts of *An. albimanus* and *An. stephensi* used to investigate *P. falciparum* 3D7A development within the bloodmeal and invasion of the midgut epithelium.

Experimental feed	Mosquito species	n[a]	Prevalence[b]	P value[c]	Intensity[d]	z statistic[e]	P value[f]
1	*An. albimanus*	17	0.0	<0.0001*	0.0	-5.553	<0.0001*
	An. stephensi	28	96.4		38.0		
2	*An. albimanus*	32	0.0	<0.0001*	0.0	-6.283	<0.0001*
	An. stephensi	32	81.3		8.5		
3	*An. albimanus*	16	0.0	<0.0001*	0.0	-4.655	<0.0001*
	An. stephensi	16	87.5		31.0		
4	*An. albimanus*	16	0.0	<0.0001*	0.0	-5.156	<0.0001*
	An. stephensi	16	100.0		67.5		

[a] The number of midguts examined.

[b] The percentage of midguts examined harbouring at least one oocyst.

[c] Comparison of the prevalence of oocyst infection in *An. albimanus* and *An. stephensi* using a two-tailed Fisher's Exact test, with $\alpha = 0.05$.

[d] Median number of oocysts observed per midgut (including uninfected midguts).

[e, f] Comparison of the intensity of oocyst infection in *An. albimanus* and *An. stephensi* using a two-tailed Mann-Whitney U test, with $\alpha = 0.05$, where the critical value of $z = \pm 1.960$.

* Indicates statistically significant differences between *An. albimanus* and *An. stephensi*.

An. stephensi sampled between 24 and 32 hours pbf in either of the experimental feeds investigated. Despite the absence of FITC-positive malaria parasites within the mosquito midguts, at day 10 pbf high levels of mature oocyst infection were observed in the same cohorts of *An. stephensi* prepared for immunofluorescence microscopy (experimental feeds 3 and 4 in Table 3.1).

In order to test whether the mode of fixation affected detection of malaria parasites using the FITC-labelled α-Pfs25 mAb, paired spots of midgut homogenates from both *An. albimanus* and *An. stephensi* fed *P. falciparum* 3D7A gametocytes were prepared on slides as described in Section 3.2.2.1 above. However, for each individual mosquito, one spot was fixed with acetone while the other spot was fixed with 4.0% paraformaldehyde. Both spots were then simultaneously incubated with the FITC-labelled α-Pfs25 mAb and examined under epifluorescence for malaria parasites as described in Section 3.2.2.1. The three expected FITC-positive malaria parasite forms were detected in acetone-fixed samples while no fluorescence was observed in the paired paraformaldehyde-fixed samples derived from the same mosquito bloodmeals (data not shown).

3.3.1.3 *P. falciparum* 3D7A early oocyst formation

3.3.1.3.1 Levels of oocyst infection on days 2 and 3 post-bloodfeeding

The levels of early oocyst infection for the *P. falciparum* 3D7A clone in *An. albimanus* and *An. stephensi* were determined using the FITC-labelled α-Pfs25 mAb in four separate experimental feeds (Table 3.2 and Table 3.3). Most of the FITC-positive malaria parasites detected on the basal surface of the midgut epithelium had the spherical form of typical oocyst stage malaria parasites (Figure 3.8). However, some FITC-positive malaria parasites on the basal surface of the midgut epithelium had an elliptical or elongated shape. Most of the malaria parasites observed exhibited precise circumferential immunofluorescence, although occasionally lines of FITC-positive material were associated with some oocysts. A small number of the FITC-positive malaria parasites found on the basal surface of the midgut epithelium appeared morphologically abnormal, either irregular in shape and/or "fractured".

In none of the four experimental feeds undertaken were FITC-positive malaria parasites detected on the basal surface of the midgut epithelium of *An. albimanus* sampled between 56 and 76 hours pbf (Table 3.2). In contrast, numerous FITC-positive malaria parasites were found on the basal surface of the midgut epithelium of

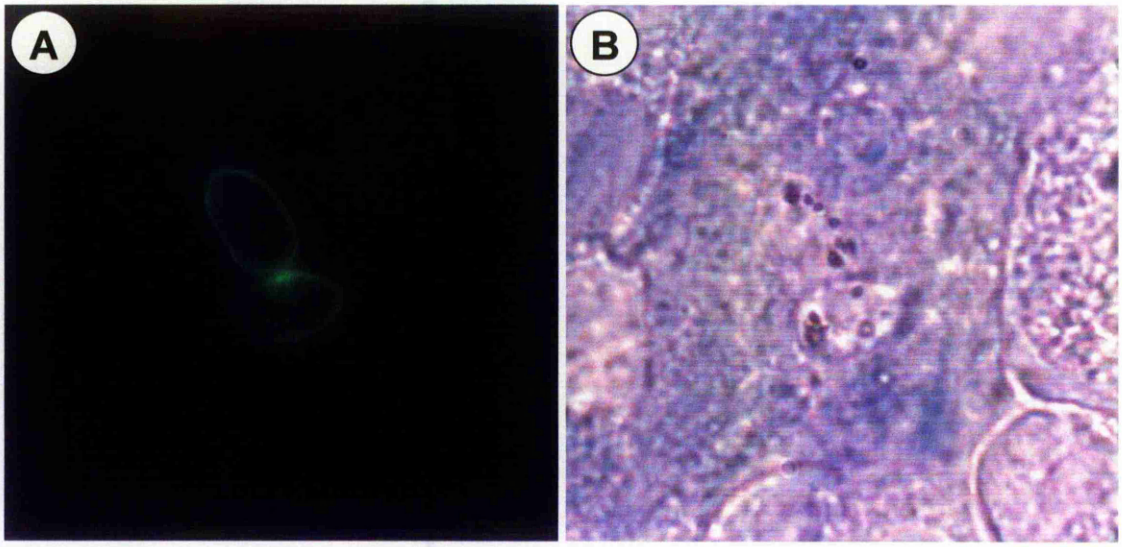


Figure 3.8 Direct immunofluorescence microscopy of early *P. falciparum* 3D7A oocysts on the midgut of *An. stephensi*.

P. falciparum 3D7A early oocysts could be detected on the midguts of *An. stephensi* prepared for direct immunofluorescence microscopy using the FITC-labelled α-Pfs25 mAb. **(A)** Two FITC-labelled α-Pfs25 mAb positive oocysts fluorescing under uv-illumination. **(B)** The same field shown in **A** under white light bright-field illumination. Arcs of haemozoin pigment granules are visible within the oocysts. Early oocysts were not observed on the midguts of *An. albimanus* simultaneously infected with the same *P. falciparum* 3D7A gametocyte cultures. 1000X. 62 hours pbf.

Table 3.2 Comparison of the prevalence of early and late *P. falciparum* 3D7A oocyst infection between *An. albimanus* and *An. stephensi.*

	Time (pbf)		*An. albimanus*		*An. stephensi*		
Experimental feed	**Hours**	**Days**	***n*[a]**	**Prevalence[b]**	***n***	**Prevalence**	***P* value[c]**
5	76	3	7	0.0	9	77.8	0.0032*
	-	10	62	0.0	64	73.4	<0.0001*
6	56 & 62	2	20	0.0	20	95.0	<0.0001*
	-	10	24	0.0	24	95.8	<0.0001*
7	56 & 62	2	27	0.0	20	95.0	<0.0001*
	-	10	32	0.0	34	94.1	<0.0001*
8	56 & 62	2	24	0.0	22	81.8	<0.0001*
	-	10	40	0.0	40	90.0	<0.0001*

[a] The number of midguts examined.

[b] The percentage of midguts examined containing at least one oocyst stage malaria parasite.

[c] Comparison of the prevalence of oocyst infection at each time point, within each experimental feed, between *An. albimanus* and *An. stephensi*, using a two-tailed Fisher's Exact test, with $\alpha = 0.05$.

* Indicates statistically significant differences between *An. albimanus* and *An. stephensi.*

Table 3.3 Comparison of the intensity of early and late *P. falciparum* 3D7A oocyst infection between *An. albimanus* and *An. stephensi*.

	Time (pbf)		*An. albimanus*		*An. stephensi*			
Experimental feed	**Hours**	**Days**	***n*[a]**	**Intensity[b]**	***n***	**Intensity**	***z* statistic[c]**	***P* value**
5	76	3	7	0.0	9	3.0	-2.860	0.0042
	-	10	62	0.0	64	5.5	-8.191	<0.0001*
6	56 & 62	2	20	0.0	20	21.5	-5.558	<0.0001*
	-	10	24	0.0	24	23.5	-6.142	<0.0001*
7	56 & 62	2	27	0.0	20	38.5	-6.215	<0.0001*
	-	10	32	0.0	34	45.5	-7.070	<0.0001*
8	56 & 62	2	24	0.0	22	11.5	-5.397	<0.0001*
	-	10	40	0.0	40	16.5	-7.589	<0.0001*

[a] The number of midguts examined.

[b] Median number of oocyst stage malaria parasites observed per midgut (including uninfected midguts).

[c] Comparison of the intensity of oocyst infection at each time point, within each experimental feed, between *An. albimanus* and *An. stephensi*, using a two-tailed Mann-Whitney *U* test, with $\alpha = 0.05$, where the critical value of $z = \pm 1.960$.

* Indicates statistically significant differences between *An. albimanus* and *An. stephensi*.

An. stephensi sampled over same period of time (Table 3.2 and Table 3.3). In all four experimental feeds, both the prevalence and intensity of early oocyst infection were significantly different between *An. albimanus* and *An. stephensi* (Table 3.2 and Table 3.3, respectively).

3.3.1.3.2 Levels of oocyst infection on day 10 post-bloodfeeding

The levels of mature (late) oocyst infection observed at day 10 pbf in the four experimental feeds used to investigate the formation of early oocysts of *P. falciparum* 3D7A in *An. albimanus* and *An. stephensi* are also shown in Table 3.2 and Table 3.3. Similar to the observations reported in Chapter 2, and to those in Section 3.3.1.3.1 above, at days 2 and 3 pbf no oocysts were observed in *An. albimanus* at day 10 pbf and *An. stephensi* was heavily infected at this time. In all four experimental feeds, the prevalence and intensity of oocyst infection at day 10 pbf differed significantly between *An. albimanus* and *An. stephensi* (Table 3.2 and Table 3.3, respectively). For one of the experimental feeds, the oocyst distributions observed in *An. albimanus* and *An. stephensi* at days 2 and 10 pbf are shown in Figure 3.9.

3.3.2 Population dynamics of *P. falciparum* 3D7A development

The levels of *P. falciparum* 3D7A infection observed in the experimental feeds described in Section 3.3.1 were also compared *within* each mosquito species to provide estimates of the stage-specific survival rates for the different developmental stages of the malaria parasite that occur between ingestion of gametocytes and the formation of mature oocysts on the basal surface of the midgut epithelium.

3.3.2.1 *P. falciparum* 3D7A stage-specific survival in *An. albimanus*

The population dynamics of *P. falciparum* 3D7A development within *An. albimanus* could only be described up to the mature ookinete stage, as no oocysts were observed within this mosquito species.

The average intensities of malaria parasite infection obtained from experimental feed 1 presented in Section 3.3.1.1 were used to estimate the stage-specific survival rates ("yields") for *P. falciparum* 3D7A in *An. albimanus*. Based upon the average bloodmeal volume for this mosquito species (Section 3.3.1.1.5), *An. albimanus* was estimated to have ingested, on average, approximately 4000 macrogametocytes per individual, in experimental feed 1 (Table 3.4). Nearly 100% of these macrogametocytes apparently underwent gametogenesis and successfully developed into macrogametes.

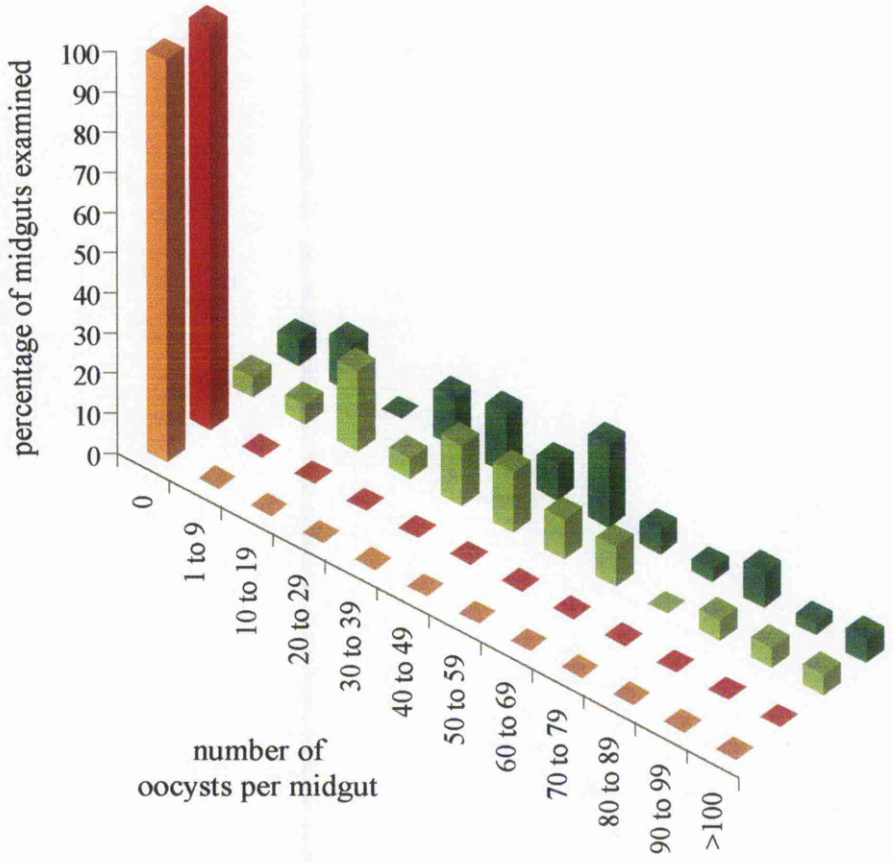


Figure 3.9 Comparison of the distributions of early and late *P. falciparum* 3D7A oocyst infections observed in *An. albimanus* and *An. stephensi*.

Histogram showing the distributions of early and late *P. falciparum* 3D7A oocyst infections observed in *An. albimanus* and *An. stephensi*. Key: ( ) *An. albimanus* at day 2 pbf; ( ) *An. albimanus* at day 10 pbf; ( ) *An. stephensi* at day 2 pbf; and ( ) *An. stephensi* at day 10 pbf. The results of experimental feed 3 are shown (see Table 3.1 to Table 3.4). Although the shape of the distribution of oocysts in *An. stephensi* varied between experimental feeds, the results from the other three experimental feeds not shown were otherwise similar to those presented here (i.e. no oocysts in *An. albimanus* at either time point and similar oocyst distributions in *An. stephensi* at both time points within each experimental feed).

Table 3.4 Estimated survival rates of different *P. falciparum* 3D7A developmental stages within *An. albimanus* and *An. stephensi*.

	Time (pbf)		*An. albimanus*		*An. stephensi*	
Malaria parasite stage	**Hours**	**Days**	**Intensity[a]**	**Yield[b]**	**Intensity**	**Yield**
Macrogametocytes[c]	0	-	3934.8	-	7890.8	-
Macrogametes[d]	6	-	3904.0	99.2	4853.3	61.5
Ookinetes[e]	24	-	230.0	5.9	338.7	7.0
Oocysts	-	10	0.0	0.0	38.0	11.2
Overall[f]				0.0		0.5

[a] The average number of the relevant malaria parasite stage observed per midgut (including uninfected midguts). Means are given for macrogametocytes, macrogametes and ookinetes while the median is given for oocysts. The data shown are drawn from experimental feed 1. [b] Percentage of malaria parasite stages surviving from the preceding to the current stage of development e.g. the yield for ookinetes = 100 x (the mean number of ookinetes observed ÷ the mean number macrogametes). [c] Estimated as the product of mosquito-specific average bloodmeal volume, and the erythrocyte, and macrogametocytaemia, of the blood fed to the mosquitoes. [d] Estimated as the sum of all malaria parasite stages within the bloodmeal positive for the FITC-labelled α-Pfs25 mAb at 6 hrs pbf (i.e. round forms + immature retort-form ookinetes). [e] The number of ookinetes observed within the bloodmeal. [f] The percentage of ingested macrogametocytes surviving to become mature oocysts at day 10 pbf, equal to the product of all the yields between the sequential stages of malaria parasite development given in the rows immediately above.

However, transformation into mature ookinetes within the bloodmeal was less successful with only approximately 6% of macrogametes completing the transition into this malaria parasite stage. Subsequent survival of the ookinetes formed within this mosquito species could not be further characterised, other than to say that none of these malaria parasite stages developed into (early) oocysts (Section 3.3.1.3).

3.3.2.2 *P. falciparum* 3D7A stage-specific survival in *An. stephensi*

The population dynamics of *P. falciparum* 3D7A development within *An. stephensi* could be described up to and including oocyst development within this mosquito species.

The average intensities of malaria parasite infection obtained from experimental feed 1 presented in Section 3.3.1.1 were used to estimate the stage-specific survival rates for *P. falciparum* 3D7A in *An. stephensi*. Based upon the average bloodmeal volume for this mosquito species (Section 3.3.1.1.5), *An. stephensi* was estimated to have ingested, on average, approximately 8000 macrogametocytes per individual, in experimental feed 1 (Table 3.4). In contrast to *An. albimanus*, only approximately 60% of ingested macrogametocytes transformed into macrogametes while a roughly similar proportion of the latter malaria parasite stages (7%) subsequently developed into ookinetes within the bloodmeal of *An. stephensi*. Approximately 10% of ookinetes within the bloodmeal successfully migrated to the basal surface of the midgut epithelium and transformed into oocyst stage malaria parasites present at day 10 pbf.

Comparison of the levels of early and late *P. falciparum* 3D7A oocyst infection within *An. stephensi* derived from experimental feeds 5 to 8 revealed that neither the prevalence nor the intensity of oocyst infection differed significantly, between the two time points examined, within any of the four experimental feeds undertaken (Table 3.5 and Table 3.6).[1] This suggests that approximately 100% of early oocysts established on the basal surface of the midgut epithelium at day 2 pbf survive to maturity at 10 pbf.

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[1] However, the levels of oocyst infection in *An. stephensi* did differ significantly, within time points, between the four experimental feeds (prevalence and intensity of early oocyst infection: Chi-square test, $\chi^2_{0.05,\,7.815} = 2.805$, d.f. = 3, $P = 0.423$ and Kruskal-Wallis test, $\chi^2_{0.05,\,7.815} = 14.127$, d.f. = 3, $P = 0.0027$ respectively; and prevalence and intensity of late oocyst infection: Chi-square test, $\chi^2_{0.05,\,7.815} = 12.039$, d.f. = 3, $P = 0.0072$ and $\chi^2_{0.05,\,7.815} = 35.902$, d.f. = 3, $P < 0.0001$, respectively).

Table 3.5 Comparison of the prevalence of early and late *P. falciparum* 3D7A oocyst infection within *An. stephensi*.

	Time (pbf)		*An. stephensi*		
Experimental feed	**Hours**	**Days**	***n*[a]**	**Prevalence[b]**	***P* value[c]**
5	76	3	9	77.8	0.570
	-	10	64	73.4	
6	56 & 62	2	20	95.0	0.708
	-	10	24	95.8	
7	56 & 62	2	20	95.0	0.694
	-	10	34	94.1	
8	56 & 62	2	22	81.8	0.660
	-	10	40	90.0	

[a] The number of midguts examined.
[b] The percentage of midguts examined containing at least one oocyst stage malaria parasite.
[c] Comparison of the prevalence of oocyst infection between time points, within each experimental feed, using a one-tailed Fisher's Exact test, with $\alpha = 0.05$.
N.B. The data presented in this table are the same as those presented in Table 3.2.

Table 3.6 Comparison of the intensity of early and late *P. falciparum* 3D7A oocyst infection within *An. stephensi*.

	Time (pbf)		*An. stephensi*			
Experimental feed	**Hours**	**Days**	***n***	**Intensity**	***z* statistic[c]**	***P* value**
5	76	3	9	3.0	0.229	0.410
	-	10	64	5.5		
6	56 & 62	2	20	21.5	-0.248	0.598
	-	10	24	23.5		
7	56 & 62	2	20	38.5	-0.493	0.689
	-	10	34	45.5		
8	56 & 62	2	22	11.5	-1.304	0.904
	-	10	40	16.5		

[a] The number of midguts examined.
[b] Median number of oocyst stage malaria parasites observed per midgut (including uninfected midguts).
[c] Comparison of the intensity of oocyst infection between time points, within each experimental feed, using a one-tailed Mann-Whitney *U* test, with $\alpha = 0.05$, where the critical value of $z = 1.645$.
N.B. The data presented in this table are the same as those presented in Table 3.3.

3. 4 Discussion

Comparison of the development of *P. falciparum* 3D7A in *An. albimanus* and *An. stephensi* using direct immunofluorescence microscopy has enabled partial characterization of when this malaria parasite clone fails to infect *An. albimanus*. Additionally, *P. falciparum* 3D7A stage-specific survival rates during various developmental transitions within each of the two mosquito species were simultaneously estimated.

3.4.1 *P. falciparum* 3D7A development in *An. albimanus*

The observations presented in this Chapter demonstrate that the *P. falciparum* 3D7A clone is able to successfully undergo gametogenesis, fertilization and ookinete development within *An. albimanus*, as previously reported for the NF54 isolate from which the 3D7A clone is derived (Mendis *et al.*, 1994; Vaughan *et al.*, 1994b). Furthermore, the efficiency of gamete activation, zygote formation and ookinete formation are roughly comparable in *An. albimanus* and *An. stephensi* (and possibly even greater in the former mosquito species) as indicated by the similar densities of round forms, retort-form ookinetes and mature ookinetes observed in both mosquito species. However, although mature ookinetes were shown to form within the bloodmeal of *An. albimanus*, these malaria parasite stages subsequently failed to establish early oocyst infections on the basal surface of the midgut epithelium. In marked contrast, oocyst infections were observed in *An. stephensi* simultaneously fed upon the same gametocyte cultures demonstrating the infectivity of the malaria parasites fed to *An. albimanus*. Consequently, the absence of oocysts in *An. albimanus* results from differences in susceptibility to *P. falciparum* 3D7A infection between the two mosquito species investigated.

The observations made here demonstrate that the *P. falciparum* 3D7A clone must fail to infect *An. albimanus* at one, or more, of the following stages:

(1) ookinete migration through the bloodmeal periphery to the peritrophic matrix;
(2) ookinete penetration of the peritrophic matrix;
(3) ookinete migration across the ectoperitrophic space;
(4) ookinete migration through the microvilli-associated network (?);
(5) ookinete entry into the midgut epithelium;
(6) ookinete migration through the midgut epithelium; and/or

(7) establishment of young oocysts beneath the basal lamina of the midgut epithelium.

The significantly lower levels of different developmental stages of the malaria parasite within the bloodmeals of *An. albimanus* compared to *An. stephensi* between 30 and 48 hours pbf suggests that the failure of the *P. falciparum* 3D7A clone to establish oocyst infections in *An. albimanus* is due to the loss of immature retort-form and mature ookinetes within the midgut lumen prior to invasion of the midgut epithelium, as previously suggested for *P. cynomolgi bastianelli* in the same mosquito species (Omar, 1968b). Furthermore, the correlation observed between the rate of bloodmeal digestion and the timing of the disappearance of different malaria parasite stages from the midgut lumen of *An. albimanus* and *An. stephensi* suggests that differences in the former may account for the differences between these two mosquito species in susceptibility to *P. falciparum* 3D7A oocyst infection.

Unfortunately, exactly when *P. falciparum* 3D7A fails to infect *An. albimanus* could not be more precisely defined with the direct immunofluorescence technique used as attempts to identify ookinetes within whole mounts of the mosquito midgut epithelium, as previously reported for the rodent malaria *P. berghei* (Han *et al.*, 2000), were unsuccessful in *An. albimanus*. However, negative results were also found for *An. stephensi* simultaneously fed the same gametocyte cultures, which subsequently developed mature oocyst infections. This observation implies that ookinetes invading the midgut epithelium were present, at least within *An. stephensi*, in the specimens examined by immunofluorescence microscopy. As the levels of mature oocyst infection in *An. stephensi* were high, the mosquitoes examined by immunofluorescence microscopy were unlikely to *all* be uninfected.[1] Consequently, the failure to observe ookinetes within the midgut epithelium of *An. albimanus* is not necessarily due to the absence of these malaria parasite stages within this location in this mosquito species. Whether *P. falciparum* 3D7A ookinetes invade the midgut epithelium of *An. albimanus* cannot be decided from the results presented in this Chapter.

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[1] Furthermore, examination of the midgut epithelial sheets from *An. stephensi* prepared for immunofluorescence microscopy by white light bright-field illumination suggested the presence of clusters of midgut epithelial cells protruding from the plane of the midgut epithelium, a characteristic associated with malaria parasite invasion of the midgut epithelium (Chapter 4), while associated tight clusters of haemozoin pigment granules suggested the possible presence of ookinetes as well.

The reasons for the failure to detect ookinetes or parasite-invaded midgut epithelial cells within the midgut epithelium of challenged *An. stephensi* using the FITC-labelled α-Pfs25 mAb are unknown. Previous work on *P. berghei* ookinete invasion of the midgut epithelium of *An. stephensi* used a mAb specific to Pbs21 (P28) (Winger *et al.*, 1988; Han *et al.*, 2000), the *P. berghei* paralogue of Pfs25 (P25) (Paton *et al.*, 1993; Tsuboi *et al.*, 1997). The P25 and P28 homologues have similar levels and patterns of protein expression (Vermeulen *et al.*, 1985a; Vermeulen *et al.*, 1986; Sinden *et al.*, 1987; Fries *et al.*, 1990; Paton *et al.*, 1993; Duffy & Kaslow, 1997; Alejo-Blanco *et al.*, 1999), and are apparently functionally equivalent (Tomas *et al.*, 2001). Therefore, the failure to detect *P. falciparum* ookinetes invading the midgut epithelium is unlikely, *per se*, to be due to using a mAb specific to the P25 rather than P28 homologue. Although P25 homologues have not previously been reported to be secreted into invaded midgut epithelial cells, there is evidence of P25 secretion during ookinete migration across the midgut epithelium (Meis & Ponnudurai, 1987b; Gouagna *et al.*, 1998). The failure to detect Pfs25-positive invaded midgut epithelial cells might be because *P. falciparum* ookinetes invade the midgut epithelium of *An. stephensi via* an intercellular route (Meis & Ponnudurai, 1987b; Meis *et al.*, 1989), although this would not explain why the ookinetes themselves were not detected. A possible explanation for the failure to detect *P. falciparum* ookinetes within the midgut epithelium of *An. stephensi* is inadequate permeabilization of the mosquito tissue during sample processing, preventing access of the mAb to its target antigen: Vernick *et al.* (1995) previously showed that mAb specific to P28 orthologue, Pgs28, was unable to recognise *P. gallinaceum* ookinetes within the midgut epithelium of *An. gambiae* in the absence of permeabilization. The inability of the α-Pfs25 mAb 32F72 to recognise bloodmeal ookinetes after paraformaldehyde fixation probably explains the failure to detect ookinetes within the midgut epithelium of *An. stephensi*. Previous studies have shown that another α-Pfs25 mAb, 32F71, recognises a conformation-dependent epitope that is destroyed by denaturation and reduction (Fries *et al.*, 1989). The α-Pfs25 mAb 32F71 binds to the same epitope of the Pfs25 antigen as the 32F72 mAb used in the work presented here (Vermeulen *et al.*, 1985b). Consequently, paraformaldehyde fixation might destroy the Pfs25 epitope recognised by the 32F72 mAb and explain the otherwise surprising absence of FITC-labelled α-Pfs25 mAb-positive malaria parasites within the midgut epithelium of *An. stephensi*.

3. 5 Summary

The development of the *P. falciparum* 3D7A clone was compared in *An. albimanus* and *An. stephensi* using FITC-labelled α-Pfs25 mAb to monitor the development of different malaria parasite stages within these two mosquito species.

Initially, malaria parasite development was similar in both mosquito species with similar densities of mature ookinetes forming within the bloodmeal of both *An. albimanus* and *An. stephensi*. However, the developmental stages of the malaria parasite present within the bloodmeal disappeared earlier from the midgut lumen of *An. albimanus* compared to *An. stephensi*, a difference which correlated with the more rapid rate of bloodmeal digestion in the former mosquito species. Subsequently, early oocysts were not observed in *An. albimanus*, although abundant oocysts were observed in *An. stephensi* simultaneously fed on the same gametocyte cultures. Attempts to identify malaria parasites invading midgut epithelium using the FITC-labelled α-Pfs25 mAb were unsuccessful for both mosquito species, probably because the mode of fixation used destroyed the epitope recognised by the mAb.

The *P. falciparum* 3D7A clone, therefore, fails to infect *An. albimanus* sometime between ookinete migration from the bloodmeal and establishment of early oocysts on the basal surface of the midgut epithelium.

Chapter 4. Histological investigation of the development of the *P. falciparum* 3D7A clone in *An. albimanus* and *An. stephensi*

4. 1 Introduction

The results presented in the previous Chapter demonstrated that the *P. falciparum* 3D7A clone is able to form mature ookinetes within the bloodmeal of *An. albimanus.* However, these malaria parasite stages are apparently unable subsequently to transform into oocysts on the basal surface of the midgut epithelium in this particular mosquito species. The direct immunofluorescence technique employed was unable to resolve precisely when the *P. falciparum* 3D7A clone failed to infect *An. albimanus.* As listed in Chapter 3, Section 3.4.1, *P. falciparum* 3D7A infection within *An. albimanus* could have failed during one of several distinct stages occurring between the formation of mature ookinetes within the bloodmeal and the establishment of oocysts on the basal surface of the midgut epithelium.

In order to clarify further why the *P. falciparum* 3D7A clone is unable to establish oocyst infections in *An. albimanus*, histological sections were prepared from blood-fed midguts of mosquitoes offered blood containing infectious gametocytes of this malaria parasite clone. For comparison, and as a positive control, histological sections were also prepared, at the same time, from the midguts of *An. stephensi* simultaneously challenged with the same parasite cultures. By examination of the histological sections, it was hoped to identify the precise time at which the *P. falciparum* 3D7A clone failed to infect *An. albimanus*, regardless of when this event happened.

Furthermore, examination of the histological sections would simultaneously provide an opportunity to investigate the route of *P. falciparum* ookinete invasion across the midgut epithelia of *An. albimanus* and *An. stephensi.* As discussed in Chapter 1, Section 1.4.7, the route of ookinete migration across the midgut epithelium has long been controversial. Initial controversies, still unresolved for *P. falciparum*, concentrated on whether the ookinete took a solely intra- or intercellular route across the midgut epithelium (Sinden & Billingsley, 2001). Numerous studies using light or electron microscopy of sectioned material have investigated ookinete migration across the midgut epithelium for various malaria species in diverse mosquito hosts (for a summary see Table 1.1) (Reichenow, 1932; Huff, 1934; Indacochea, 1935; Stohler,

1957; Garnham *et al.*, 1962; Howard, 1962; Omar, 1968b; Garnham *et al.*, 1969; Canning & Sinden, 1973; Maier, 1973; Davies, 1974; Mehlhorn *et al.*, 1980; Becker-Feldman *et al.*, 1985; Maier *et al.*, 1987; Meis & Ponnudurai, 1987b; Paskewitz *et al.*, 1988; Meis *et al.*, 1989; Syafruddin *et al.*, 1991; Torii *et al.*, 1992; Vernick *et al.*, 1995; Vernick *et al.*, 1999; Limviroj *et al.*, 2002). Some studies claimed that the route of ookinete migration was solely intercellular between midgut epithelial cells, whereas other researchers argued that malaria parasite invasion was solely intracellular. Ambivalent or contradictory observations were often reported (Omar, 1968b; Canning & Sinden, 1973; Syafruddin *et al.*, 1991), even when the same malaria parasite and mosquito species were examined. Studies claiming that ookinete migration was solely intercellular reported ookinetes on the apical side of the midgut epithelium beside, or entering between, the lateral plasma membranes of adjacent midgut epithelial cells (Stohler, 1957; Meis & Ponnudurai, 1987b; Syafruddin *et al.*, 1991). Furthermore, ookinetes and oocysts on the basal side of the midgut epithelium were found in extracellular positions between, or above, the lateral plasma membranes of adjacent healthy midgut epithelial cells (Omar, 1968b; Mehlhorn *et al.*, 1980; Meis & Ponnudurai, 1987b; Meis *et al.*, 1989; Meis & Ponnudurai, 1989; Syafruddin *et al.*, 1991). However, these studies did not observe ookinetes, in intercellular locations between adjacent midgut epithelial cells, *within* the apical region of the midgut epithelium. Regardless, as no unambiguously intracellular malaria parasites were observed, the previous observations were interpreted as conclusive evidence of a solely intercellular route of ookinete migration from the apical to the basal surface of the midgut epithelium (Stohler, 1957; Meis & Ponnudurai, 1987b; Meis *et al.*, 1989). Other studies reported the occurrence of intracellular ookinetes, and often noted that invaded midgut epithelial cells appeared to be damaged (Maier, 1973; Becker-Feldman *et al.*, 1985; Maier *et al.*, 1987; Paskewitz *et al.*, 1988; Meis *et al.*, 1989). Therefore, the route of ookinete migration was proposed to depend on the malaria parasite-mosquito vector combination, with "well-adapted" malaria parasites migrating *via* a solely intercellular route to reduce the harm caused to the mosquito vector (Maier, 1987; Meis & Ponnudurai, 1987a; Meis *et al.*, 1989). However, this tidy resolution ignored some remaining inconsistencies such as different investigators, using the same malaria parasite-mosquito vector species, observing ookinetes in different locations within the midgut epithelium. For example, Huff (1934) reported that *P. cathemerium* ookinetes migrated across the midgut epithelium of *Culex pipiens via* an intercellular route while

Maier (1973) observed intracellular malaria parasites and pathological changes in invaded midgut epithelial cells using the same parasite-vector combination. Subsequently, a single study found, within the same malaria parasite-mosquito vector combination, *P. gallinaceum* ookinetes in both intra- and intercellular locations within the midgut epithelium of *Ae. aegypti* (Torii *et al.*, 1992). Significantly, intra- and intercellular ookinetes were primarily observed in the apical and basal regions of the midgut epithelium, respectively, implying that during invasion of the midgut epithelium ookinetes enter into midgut epithelial cells and exit midgut epithelial cells to the extracellular basolateral space between adjacent midgut epithelial cells. Consequently, a model was proposed whereby malaria parasites initially enter the midgut epithelium *via* an intracellular route and then subsequently leave the midgut epithelium *via* an intercellular route (Torii *et al.*, 1992). As alluded to above, malaria parasite species supposedly taking a solely intercellular route had also been found in intracellular locations in different studies (also see Table 1.1). Surprisingly, the interpretation that ookinetes invade *via* both intra- and intercellular routes, albeit in different regions of the midgut epithelium, did not become widely accepted despite a second study that also found *P. gallinaceum* ookinetes in both locations within the midgut epithelium of the unnatural vector *An. gambiae* (Vernick *et al.*, 1995). However, some of the apparently conflicting studies reporting ookinetes of the same malaria parasite species in different locations within the midgut epithelium did not use the same mosquito species. Consequently, the observed differences could have been due to differences between the mosquito species used. Furthermore, the midgut epithelial cells invaded by *P. gallinaceum* ookinetes were morphologically normal in *Ae. aegypti* (Torii *et al.*, 1992) but exhibited signs of pathology in *An. gambiae* (Vernick *et al.*, 1995). Therefore, a number of issues were left unresolved by the "first intra- and then intercellular" model of ookinete invasion:

(1) the failure of researchers reporting intercellular migration to observe intracellular parasites (and *vice versa*);
(2) the occurrence of ookinetes apparently entering the midgut epithelium *between* adjacent midgut epithelial cells (i.e. intercellularly);
(3) the presence of ookinetes/oocysts in locations consistent with intercellular migration across the midgut epithelium; and
(4) the effect of intracellular ookinete invasion on midgut epithelial cells.

More recently, two conflicting models of intracellular ookinete invasion of the

mosquito midgut epithelium have also been proposed (Han & Barillas-Mury, 2002; Shahabuddin, 2002). The first model is based upon an *in vitro* system using cultured *P. gallinaceum* ookinetes and isolated midgut sheets from *Ae. aegypti*. This model claims that ookinetes preferentially invade a specific sub-population of midgut epithelial cells, referred to as Ross cells, that are morphologically and biochemically distinct (Shahabuddin & Pimenta, 1998; Cociancich *et al.*, 1999). Ross cells are characterised by several features, including light staining by toluidine blue, highly vacuolated cytoplasm, and an apical surface lacking, or relatively denuded of, microvilli. The second model is based upon both *in vitro* and *ex vivo* systems using *P. berghei* and *An. stephensi* (Han *et al.*, 2000; Zieler & Dvorak, 2000). This model claims that midgut epithelial cells are morphologically indistinguishable and that ookinete invasion induces pathological changes in midgut epithelial cells similar to the morphological and biochemical characteristics reported for Ross cells (Han *et al.*, 2000; Zieler & Dvorak, 2000). This second model has been termed the "Time Bomb model" as malaria parasite invasion appears to trigger a cascade of physical and chemical events within invaded midgut epithelial cells (the 'time bomb') that are presumed to lead to the eventual death and ejection of invaded midgut epithelial cells from the midgut epithelium (Han & Barillas-Mury, 2002; Kumar & Barillas-Mury, 2005). Consequently, malaria parasites may only have a limited time window in which to successfully traverse and exit invaded midgut epithelial cells before being killed by the mosquito responses induced by ookinetes invasion or ejected into the midgut lumen along with the invaded midgut epithelial cell. Ookinetes were further proposed to move both laterally within the midgut epithelium, through adjacent midgut epithelial cells, and basally over the haemocoelic surface of the midgut epithelium, away from the initial site of ookinete invasion possibly as a means of avoiding deleterious host responses triggered by cell invasion. Observations consistent with the Time Bomb model have also been made using the *in vitro P. gallinaceum-Ae. aegypti* system suggesting that ookinete midgut invasion may proceed by a mechanism conserved across evolutionary-distant malaria parasite and mosquito vector species (Zieler & Dvorak, 2000).

The studies supporting the Ross cell and Time Bomb models effectively ignored the previous controversy over whether the route of ookinete migration across the midgut epithelium was intra- or intercellular: intercellular ookinetes were not reported and little attempt was made to integrate the previous observations of intercellular ookinetes within the framework of these new models (Shahabuddin & Pimenta, 1998; Cociancich

et al., 1999; Han *et al.*, 2000; Zieler & Dvorak, 2000; Kumar *et al.*, 2004). Interpretation of these recent studies is also complicated by the use of *in vitro* or *ex vivo* systems and unnatural malaria parasite-mosquito vector combinations. The observations made in these investigations could be artefacts of the laboratory models used (Han *et al.*, 2000; Zieler & Dvorak, 2000). Furthermore, the relevance of these studies to human malaria parasites is unknown (Shahabuddin & Pimenta, 1998; Cociancich *et al.*, 1999; Han & Barillas-Mury, 2002). Previous studies of *P. falciparum* in *An. stephensi* only observed ookinetes in intercellular locations within the midgut epithelium (Meis & Ponnudurai, 1987b; Meis *et al.*, 1989). Consequently, observing the route of ookinete migration across the midgut epithelium for the *P. falciparum* 3D7A clone in *An. albimanus* and *An. stephensi* would be of considerable additional interest.

4. 2 Materials & Methods

4.2.1 Parasite culture and mosquito infection

Malaria parasite culture and mosquito infections were performed as previously described in Chapter 2, Section 2. 2. As before, for each infectious experimental feed undertaken, *An. albimanus* and *An. stephensi* were simultaneously fed gametocytes derived from the same cultures to enable direct comparisons to be made between the two mosquito species. Separate cohorts of *An. stephensi* were also offered either uninfected blood, or infected but non-infectious blood containing asexual erythrocytic stage malaria parasites only, using the same membrane-feeding protocol.

4.2.2 Preparation and examination of histological sections

At various times pbf (24, 28, 32, 36, 40, 44 and 48 hours), mosquitoes were anaesthetised with chloroform and kept on ice until dissection. Intact midguts, including bloodmeal contents, were dissected into phosphate-buffered saline at pH 7.2 and immediately fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (Na_2HPO_4/KH_2PO_4) at pH 7.2 and kept at 4 °C for at least 24 hours. Subsequently, midguts were washed three times for 10 minutes each in phosphate buffer and dehydrated through 10 minute rinses in 30, 50, 70 and 90% ethanol in phosphate buffer and then absolute ethanol for 30 minutes. Midguts were then embedded in Technovit 7100 (Heraeus Kulzer, Germany) (TAAB Laboratory Equipment, Cat. No. T218), a glycol methacrylate-based resin, according to the manufacturer's instructions.

Longitudinal serial sections 2 μm thick were dry-cut on an LKB-2 ultramicrotome using either a glass or diamond knife, stretched on water and attached to glass slides over a hot plate at 60°C for 10 minutes. Sections were stained with 5% Giemsa's solution for 15 to 20 minutes, washed with water, air dried and mounted with a coverslip using Gurr® DePex mounting medium (BDH Laboratory Supplies, Cat. No. 361254D). Each midgut was completely sectioned and all resulting sections successfully processed were examined using light microscopy (1000X magnification).

Images were captured using a Photometrics CoolSnap™ digital camera and Improvision® Openlab™ version 2 software. Further processing of the images (resizing, colour adjustment and labelling) was performed using Adobe® Photoshop® version 5.5.

4.2.3 Statistical analysis

Statistical tests were performed using either XLStat© version 7.5.2 (Addinsoft, 1995-2004) or SAS version 8.2 (SAS Institute Incorporated, Cary, NC, USA 1999-2001), except for the Dunn test which was performed manually.

4.2.3.1 Comparison of the levels of *P. falciparum* 3D7A infection

The prevalence of *P. falciparum* 3D7A infection observed in *An. albimanus* and *An. stephensi*, and at different times pbf within *An. stephensi*, were compared using either one- or two-tailed Fisher's Exact tests because of the small sample sizes for some of the categories (less than 5 observations).

The intensity of malaria parasite infection observed in *An. albimanus* and *An. stephensi*, and at different times pbf within *An. stephensi*, was compared using the non-parametric tests of the median because of the (often) overdispersed (i.e. non-normal) distribution of malaria parasites in the mosquito population. When the intensity of malaria parasite infection between two groups of mosquitoes was being compared, either a one- or two-tailed Mann-Whitney *U* test was employed. When the intensity of malaria parasite infection between three or more groups of mosquitoes was being compared, a Kruskal-Wallis test was initially performed and then multiple pairwise comparisons made using the Dunn test for samples of unequal size (Zar, 1984), to identify which particular groups of mosquitoes differed significantly from one another.

4.2.3.2 Comparison of morphologically-abnormal midgut epithelial cells

For analysis of the relationships between the occurrence of morphologically-abnormal midgut epithelial cells and malaria parasite infection of the midgut epithelium,

the absolute number of morphologically-abnormal midgut epithelial cells observed within each midgut was considered a biased parameter as individual malaria parasites infecting the midgut epithelium were frequently associated with more than one morphologically-abnormal midgut epithelial cell. Instead, the number of morphologically-abnormal midgut epithelial cell *events* observed within each midgut was considered a more reliable parameter (i.e. clusters of multiple adjacent morphologically-abnormal midgut epithelial cells were counted as "single events" and, therefore, given equal weighting to isolated morphologically-abnormal midgut epithelial cells observed singly within the midgut epithelium).

The median number of morphologically-abnormal midgut epithelial cell events observed in the midguts of mosquitoes fed different types of blood (i.e. uninfected, infected and infectious) were initially compared using the Kruskal-Wallis test and then the multiple pairwise comparison Dunn test for samples of unequal size when appropriate. The median number of morphologically-abnormal midgut epithelial cell events observed at different time points pbf were similarly compared *within* each group of mosquitoes fed different types of blood.

Simple (bivariate) linear regression analysis was performed to investigate the relationship between the number of morphologically-abnormal midgut epithelial cell events, and the number of malaria parasites infecting the midgut epithelium, observed within each mosquito. For the regression analysis, data from all four experimental (uninfected, infected and the two infectious) bloodfeeds were pooled and analysed simultaneously.

In order to maintain comparability between the sample groups, and the assumption of a linear relationship between different variables in the regression analysis, only observations made between 24 and 36 hours pbf inclusive were statistically analysed (i.e. the observations made at 40 and 44 hours pbf for the second group of *An. stephensi* fed infectious gametocyte stage malaria parasites were excluded when making comparisons *between* the sample groups).

4. 3 Results

Histological sections were prepared on day 1 pbf from the midguts of *An. albimanus* and *An. stephensi* fed either uninfected blood, infected but non-infectious blood containing *P. falciparum* 3D7A asexual erythrocytic stage malaria parasites alone, or infectious blood containing both *P. falciparum* 3D7A gametocytes and asexual

erythrocytic malaria parasite stages. Details of the number of midguts/sections examined, the time from which the midguts were sampled, and the type of bloodmeal given to the mosquitoes, are given for both mosquito species in Table 4.1. Despite the best laid schemes of this man, a prolonged power cut to the building housing the insectaries meant things went a'gley and prevented a complete time course being obtained for the first infectious experimental feed. Consequently, a second infectious experimental feed was undertaken.

4.3.1 Histological description of the mosquito midgut during bloodmeal digestion

4.3.1.1 Structure of the mosquito midgut

Figure 4.1[1] presents a schematic diagram of the architecture of the mosquito midgut, illustrating and labelling the major features observable in the Giemsa-stained histological sections.

4.3.1.1.1 The bloodmeal

The bloodmeal was located within the endoperitrophic space of the midgut lumen separated from the surrounding midgut epithelium by the peritrophic matrix. There were two distinct, differentially-staining regions that apparently corresponded to the different stages in the process of bloodmeal digestion: a central mass of undigested morphologically normal erythrocytes and a peripheral zone of digested morphologically abnormal erythrocytes (Figure 4.5, Figure 4.6 and Figure 4.27A2) (Huff, 1934; Omar, 1968b). The narrow peripheral zone of digested erythrocytes apparent at 24 hours pbf gradually increased over time, moving inwards while the inner central mass of undigested erythrocytes concomitantly diminished. In the posterior-most region of the midgut, the peripheral zone of digested erythrocytes was more extensive, and encroached relatively rapidly on the inner central mass of undigested erythrocytes. In contrast, in the anterior-most region of the midgut, the peripheral zone of digested erythrocytes remained a thin perimeter around the unchanging inner mass of undigested erythrocytes until the final stages of bloodmeal digestion. Consequently, at later time points, a large ball of undigested erythrocytes was present in the anterior half of the endoperitrophic space while the bloodmeal within the posterior half of the midgut lumen was completely digested.

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[1] Please note Figures are located at the end of the main text of this Chapter (from page 167).

Table 4.1 Summary of the histological sections from *An. albimanus* and *An. stephensi* midguts examined.

		An. albimanus		*An. stephensi*	
Experimental feed[a]	**Time (hours pbf)**	***n*[b]**	**Number of sections examined**	***n***	**Number of sections examined**
Uninfected:	24	-	-	2	628
no malaria	28	-	-	2	686
parasites	32	-	-	2	629
	36	-	-	2	587
	Sub-total			8	2530
Infected:	24	-	-	2	847
asexual stages	28	-	-	2	755
only	32	-	-	2	630
	36	-	-	2	744
	Sub-total			8	2976
1st Infectious:	28	1	429	2	790
gametocytes +	32	5	1180	3	766
asexual stages	Sub-total	6	1609	5	1556
2nd Infectious:	24	2	853	3	1263
gametocytes +	28	2	539	2	789
asexual stages	32	2	411	2	590
	36	2	601	2	672
	40	1	302	2	577
	44	2	460	2	516
	Sub-total	11	3166	13	4407
Overall	Total	17	4775	34	11469

[a] Indicates the infection status of the blood fed to the mosquitoes and what malaria parasite stages were present in the blood. [b] The number of midguts examined.

In mosquitoes given bloodmeals containing infectious gametocytes, asexual and sexual intra-erythrocytic stage malaria parasites, as well as extracellular round-forms and immature and mature ookinetes, were frequently apparent within the central region of undigested erythrocytes throughout the period of bloodmeal digestion. The different stages of asexual and sexual intra-erythrocytic development could be distinguished in the histological sections including ring-forms, trophozoites, schizonts and the various stages of gametocyte development (although mature gametocytes were sometimes difficult to discriminate from retort-forms and mature ookinetes). However, intra-erythrocytic malaria parasites, and extracellular round forms, were only rarely observed within the peripheral digested region of the bloodmeal presumably implying rapid digestion by midgut proteases. In contrast, immature and mature ookinetes were more frequently observed within the peripheral digested region of the bloodmeal, implying a relatively greater level of resistance to digestion than the intra-erythrocytic stage malaria parasites.

4.3.1.1.2 The peritrophic matrix

The peritrophic matrix surrounding the bloodmeal was more or less distinguishable from the ectoperitrophic space, depending on the specimen and time after bloodfeeding, and varied in thickness from approximately 2 to 10 μm (e.g. Figure 4.2 and Figure 4.17C2). There were occasionally regions where the peritrophic matrix was either ruptured or apparently absent, such that the apical surface of the midgut epithelium was in direct contact with the bloodmeal. However, no midguts were observed where the peritrophic matrix was absent throughout the entire specimen. Freyvogel & Stäubli (1965) stated that incomplete peritrophic matrix formation was a normal and frequent occurrence. In the current work, the localised absence of the peritrophic matrix beneath the midgut epithelium was probably an artefact of sample preparation caused by tearing of the peritrophic matrix during dissection of the mosquito, as lengths of intact peritrophic matrix were usually seen extending down into the bloodmeal, away from the apical surface of the midgut epithelium, in such instances. In general, the peritrophic matrix persisted until approximately 32 to 36 hours pbf. From this time onwards, the peritrophic matrix began to disappear, first from the posterior-most, and finally from the anterior-most, region of the midgut, paralleling the pattern of change in the peripheral digested, and central undigested, regions of the bloodmeal observed in the posterior and anterior regions of the midgut lumen (Section 4.3.1.1.1). Consequently, at later time points, the peritrophic matrix was absent from

the posterior, but was still fully formed in the anterior, region of the midgut lumen.

4.3.1.1.3 The midgut epithelium

The midgut epithelium comprised a simple monolayer of polarised columnar epithelial cells arranged in a "honeycomb" fashion (Figure 4.2 and Figure 4.3). Smaller, basally-located, triangular cells of variable shape, size and intensity of staining were sparsely scattered between the columnar midgut epithelial cells (Figure 4.4 and Chapter 5). Circular and longitudinal muscle fibres were frequently observed over the outer (basal) surface of the midgut epithelium, forming a regular latticework (e.g. Figure 4.2 and Figure 4.4). Occasionally, tracheoles were also apparent over the basal surface of the midgut epithelium. Although previously observed in histological sections (Pal, 1943; Freyvogel & Stäubli, 1965), the basal lamina surrounding the midgut epithelium was not usually apparent in the specimens examined here (but see Section 4.3.3.1.2).

4.3.1.1.3.1 Midgut epithelial cells

Midgut epithelial cells were approximately 10 to 20 μm wide and 20 to 30 μm high, although their size and shape varied from squamous through cuboidal to columnar depending on location within the midgut and the stage of bloodmeal digestion. Midgut epithelial cells were otherwise morphologically indistinguishable from one another possessing large centrally-located nuclei containing a single prominent darkly staining nucleolus and a "striated" apical border approximately 5 μm deep corresponding to microvilli (Figure 4.2). As previously reported, other organelles observable in midgut epithelial cells using electron microscopy, such as mitochondria, endoplasmic reticulum, Golgi apparatus, and various secretory granules, were not apparent in the histological sections examined (Freyvogel & Stäubli, 1965). The lateral plasma membranes of midgut epithelial cells could often be discriminated in the histological sections, but the extensive in-foldings of the basal and lateral plasma membranes, known collectively as the basal labyrinth, were not readily apparent (Freyvogel & Stäubli, 1965; Hecker, 1977).

The cytoplasm exhibited granular staining of similar intensity in different midgut epithelial cells, and typically contained one or several, often quite extensive, unstained regions of irregular shape and size (Figure 4.2). The nature and identity of these entities is unknown. Freyvogel & Stäubli (1965) observed similar structures in histological sections, but were unable to identify corresponding structures in electron micrographs.

These authors concluded that these entities were probably not membrane-bound vacuoles but protoplasm of "varying stainability".

In general, the microvillar brush border of the apical surface of the midgut epithelium was continuous throughout the posterior midgut. The microvilli typically extended perpendicularly, in an ordered array, from the apical surface of the midgut epithelial cells towards the bloodmeal within the midgut lumen. Clear narrow "channels" or partings (less than 1 μm wide) were often apparent within the microvillar brush border, immediately beneath where the lateral plasma membranes of the adjacent overlying midgut epithelial cells converged (Figure 4.3B). Occasionally, the microvilli appeared to be disorganised and dishevelled extending in multiple directions from the surface of the midgut epithelial cells (Figure 4.8D). There was no evidence of the microvilli-associated network reported in aedine mosquitoes (Zieler & Dvorak, 2000; Zieler *et al.*, 2000).

"Blebbing" of the apical surface of midgut epithelial cells was also occasionally observed (Figure 4.11B and Figure 4.37A), except for three midguts where relatively extensive regions of the midgut epithelium exhibited marked apical blebbing. Previous authors have reported similar observations and have regarded such structures either as evidence of apocrine secretion (Omar, 1968b), or as artefacts of sample preparation (Freyvogel & Stäubli, 1965; Brunings & de Priester, 1971).

4.3.1.1.3.2 Regenerative cells

Small, dark and homogeneously-staining triangular cells of variable size that lacked apical extensions were sparsely scattered throughout the basal region of the midgut epithelium, between adjacent midgut epithelial cells. These dark-staining triangular cells are believed to be regenerative cells (e.g. Hecker, 1977) and were sometimes observed immediately adjacent to other midgut cells possessing various unusual (but not necessarily abnormal) morphologies apparently intermediate between regenerative cells and normal columnar midgut epithelial cells. These putative regenerative cells, and the potential significance of the morphologically unusual midgut cells associated with them, are discussed in further detail in Chapter 5.

4.3.1.1.3.3 Endocrine cells

Lightly-staining triangular cells of similar size and morphology to the dark-staining triangular cells were also observed, sparsely scattered throughout the basal region of the midgut epithelium between adjacent midgut epithelial cells (Figure 4.4).

The lightly-staining triangular cells possessed long and narrow apical extensions, possibly lined by microvilli-like structures, that opened into the midgut lumen through the microvillar brush border of the midgut epithelium. The apical surface of the light staining triangular cells sometimes appeared above, or, more typically, beneath, the plane of the microvillar brush border of the surrounding midgut epithelial cells, and occasionally exhibited "blebbing" (Figure 4.4D). The nuclei of these cells were basally-located, relatively large, and occupied much of the cytoplasm. Typically, the apical extension and basal body of the light staining triangular cells were not present within the same section making initial identification of these cells difficult. Furthermore, although these cells were readily apparent in heavily-stained preparations, they were often difficult to discern in lightly-stained sections. Unlike the dark-staining triangular cells, the light staining triangular cells were not observed in adjacent pairs. These light-staining triangular cells also always seemed to be located immediately beneath the muscle fibres encircling the basal surface of the midgut epithelium. Although no formal attempt at quantification was attempted, there were approximately several hundred of the light staining triangular cells within each midgut (one or two of these being observed within each section).

4.3.1.2 Comparison of the midguts of *An. albimanus* and *An. stephensi*

Few morphological differences were apparent in the midguts of *An. albimanus* and *An. stephensi* examined in histological sections; both conformed to the description given above (Section 4.3.1.1). The structure of the midgut epithelium was indistinguishable, except that, in general, all types of midgut cell appeared smaller in *An. albimanus* compared to those of *An. stephensi*. The region of the peritrophic matrix exhibited some differences between the two mosquito species, varying in structure and intensity of staining. In *An. albimanus*, the peritrophic matrix (or, at least, the endoperitrophic region immediately adjacent to peritrophic matrix) was often distinctively laminated and, although poorly stained, easily distinguished from the ectoperitrophic space (Figure 4.5C). In contrast, the peritrophic matrix of *An. stephensi* stained deeply and homogeneously, lacking any evident internal structure, and was often difficult to discriminate from the ectoperitrophic space (e.g. Figure 4.6 and Figure 4.7).

The two most singular differences between *An. albimanus* and *An. stephensi* related to the bloodmeal. First, the rate of bloodmeal digestion differed appreciably between the two mosquito species, being markedly more rapid in *An. albimanus*: the

peripheral region of digested erythrocytes was more extensive, and the central undigested erythrocytic mass considerably smaller, in *An. albimanus* compared to *An. stephensi* at equivalent times after bloodfeeding. Second, there was a marked difference with regard to the presence of bacterial microorganisms within the bloodmeal. The midguts of most *An. stephensi* were apparently free of microorganisms with only a couple of the midguts examined containing scant bacteria (Figure 4.6C). In contrast, in *An. albimanus* large, dense bacterial aggregates were present within the midgut lumen of all the mosquitoes examined. The bacteria appeared to be mainly bacilli; cocci-like microorganisms were only rarely observed and, if present, only in small clusters of a handful of organisms. Typically, the bacterial aggregates were located in the periphery of the bloodmeal within the zone of digested erythrocytes and within the endoperitrophic space created by the peritrophic matrix. However, extremely dense aggregates of bacteria, many tens of micrometres in diameter and probably consisting of many thousands of bacilli, were often observed in the anterior-most region of the midgut lumen, where the posterior midgut joined the anterior midgut. These dense bacterial aggregates were sometimes located within the ectoperitrophic space and/or sandwiched within the anterior plug of peritrophic matrix, as well as the more common bloodmeal location. Infrequently, very low densities of yeast and/or fungi-like microorganisms were also present within the bloodmeals of both mosquito species.

4.3.2 Comparison of *P. falciparum* 3D7A development in *An. albimanus* and *An. stephensi*

4.3.2.1 *An. albimanus* fed *P. falciparum* 3D7A gametocytes

4.3.2.1.1 Histological sections from midguts sampled on day 1 post-bloodfeeding

In histological sections derived from the midguts of *An. albimanus* fed infectious *P. falciparum* 3D7A gametocytes, ookinetes could be found within the central undigested regions of the bloodmeal between 24 to 32 hours pbf (Figure 4.5A). One ookinete observed with the central undigested region of the bloodmeal appeared morphologically abnormal possessing two large non-staining "vacuolated" areas (Figure 4.5B). Due to the large size of the bloodmeal relative to the midgut epithelium, and, depending on the plane of sectioning, the difficulty in discriminating ookinetes from the other erythrocytic malaria parasite stages present within the ingested blood, no attempt was made to quantify the number of ookinetes present within the central undigested region of the bloodmeal.

Ookinetes were only rarely seen within the peripheral digested regions of the bloodmeal ($n = 5$) and even less frequently observed adjacent to the endoperitrophic surface of the peritrophic matrix ($n = 3$) (Figure 4.5C) (Table 4.2). The few ookinetes associated with endoperitrophic surface of the peritrophic matrix appeared to be morphologically abnormal possessing uncharacteristic "rounded" or "tear-drop" form (Figure 4.5D). In *An. albimanus*, ookinetes were not seen within the peritrophic matrix, the ectoperitrophic space or the midgut epithelium at any of the time points examined in either of the infectious experimental feeds undertaken (Table 4.2). Similarly, ookinetes and oocysts were not observed on the basal surface of the midgut epithelium (Table 4.2). However, morphologically-abnormal midgut epithelial cells protruding from the midgut epithelium into the midgut lumen were very rarely observed in *An. albimanus* fed *P. falciparum* 3D7A gametocytes.

4.3.2.1.2 Levels of oocyst infection on day 10 post-bloodfeeding

As reported in Chapter 2, at day 10 pbf no oocyst infection was observed, in either of the two infectious experimental feeds, on the midguts of other mosquitoes from the same cohort of *An. albimanus* challenged with *P. falciparum* 3D7A and prepared for histology at day 1 pbf (Table 4.3).

4.3.2.2 *An. stephensi* fed *P. falciparum* 3D7A gametocytes

4.3.2.2.1 Histological sections from midguts sampled on day 1 post-bloodfeeding

In histological sections derived from the midguts of *An. stephensi* fed gametocytes of the *P. falciparum* 3D7A clone, all stages of malaria parasite migration from the bloodmeal of the endoperitrophic space to the basal surface of the midgut epithelium were observed between 24 and 44 hours pbf (Figure 4.6 to Figure 4.38). The number of malaria parasite stages observed at different times in different locations within the midguts examined is shown for both infectious experimental feeds in Table 4.2. The overall percentage of malaria parasites observed in different locations associated with the midgut epithelium, and the change in these percentages over time, are shown in Figure 4.44.

Ookinetes were frequently found within both the central undigested, and peripheral digested, regions of the bloodmeal (Figure 4.6). Ookinetes were also often seen adjacent to, and, markedly more frequently, within, the peritrophic matrix (Figure 4.7). Less often, ookinetes were observed within the ectoperitrophic space, either adjacent to, or within, the apical microvillar brush border surface of the midgut

Table 4.2 Number of *P. falciparum* 3D7A parasites observed in histological sections from *An. albimanus* and *An. stephensi*.

		An. albimanus				*An. stephensi*			
Experimental feed	**Time (hours pbf)**	***n*[a]**	**OOK PM/EPS/MV[b]**	**OOK ME[b]**	**OOC[b]**	***n***	**OOK PM/EPS/MV**	**OOK ME**	**OOC**
1st Infectious:	28	1	0	0	0	2	89	36	22
gametocytes +	32	5	1	0	0	3	23	39	38
asexual stages	Sub-total	6	1	0	0	5	112	75	60
2nd Infectious:	24	2	1	0	0	3	39	20	2
gametocytes +	28	2	1	0	0	2	40	12	9
asexual stages	32	2	0	0	0	2	13	9	22
	36	2	0	0	0	2	4	3	30
	40	1	0	0	0	2	18	9	53
	44	2	0	0	0	2	0	1	16
	Sub-total	11	2	0	0	13	114	54	132
Overall	Total	17	3	0	0	18	226	129	192

[a] The number of midguts examined.

[b] OOK PM/EPS/MV = all ookinetes associated with the peritrophic matrix and the ectoperitrophic space, including malaria parasites within the microvillar brush border prior to entry into the midgut epithelium. OOK ME = all ookinetes entering or within the midgut epithelium, including intracellular malaria parasites within midgut epithelial cells completely separated from the midgut epithelium. OOC = oocysts.

Table 4.3 Levels of mature oocyst infection observed at day 10 post-bloodfeeding in the cohorts of *An. albimanus* and *An. stephensi* fed gametocytes of *P. falciparum* 3D7A and used to prepare the histological sections.

Experimental feed	Mosquito species	n[a]	Prevalence[b]	P value[c]	Intensity[d]	z statistic[e]	P value[f]
1st Infectious	***An. albimanus***	28	0.0	<0.0001*	0.0	-6.440	<0.0001*
	An. stephensi[g]	27	92.6		19.0		
2nd Infectious	***An. albimanus***	42	0.0	<0.0001*	0.0	-6.557	<0.0001*
	An. stephensi[g]	42	71.4		6.5		

[a] The number of midguts examined at day 10 pbf.

[b] The percentage of midguts examined harbouring at least one oocyst.

[c] Comparison of the prevalence of oocyst infection in *An. albimanus* and *An. stephensi* using a two-tailed Fisher's Exact test, with $\alpha = 0.05$.

[d] Median number of oocysts observed per midgut (including uninfected midguts).

[e, f] Comparison of the intensity of oocyst infection in *An. albimanus* and *An. stephensi* using a two-tailed Mann-Whitney U test, with $\alpha = 0.05$, where the critical value of $z = \pm 1.960$.

[g] The prevalence and intensity of oocyst infection at day 10 pbf also differed significantly within *An. stephensi* between the two experimental feeds. See main text for results of statistical analyses (Section 4.2.3).

* Indicates statistically significant differences between *An. albimanus* and *An. stephensi*.

epithelial epithelium (Figure 4.8 and Figure 4.9). Overall, 226 ookinetes were observed within either the peritrophic matrix and/or the ectoperitrophic space. As the peritrophic matrix and the ectoperitrophic space were often difficult to discriminate in the Giemsa-stained histological sections, a precise quantification of the exact number of ookinetes observed within each of these two distinct locations within the midgut lumen was not possible. Ookinetes were most abundant within these locations between 24 and 28 hours pbf. The number of malaria parasites observed within the peritrophic matrix and/or ectoperitrophic space fell markedly from 32 hours pbf onwards (Table 4.2 and Figure 4.44). In *An. stephensi*, none of the ookinetes observed within the peritrophic matrix and/or ectoperitrophic space exhibited unusual or abnormal morphology.

Although ookinete entry into the midgut epithelium was only rarely seen, ookinetes were frequently observed within the midgut epithelium ($n = 129$) (Figure 4.10 to Figure 4.33). Ookinete migration across the midgut epithelium occurred throughout the time period investigated (Table 4.2 and Figure 4.44). However, most of the observed ookinetes (84%, 109 of 129) invaded the midgut epithelium prior to 36 hours pbf. Oocysts were also frequently found on the basal surface of the midgut epithelium ($n = 192$) (Figure 4.34 to Figure 4.36). Oocysts were observed throughout the time period investigated and could be seen as early as 24 hours pbf (Table 4.2 and Figure 4.44). However, oocysts were relatively rare before 32 hours pbf, increasing markedly in number at later time points (from 32 hours pbf onwards). In *An. stephensi*, malaria parasite infection of the midgut epithelium was also associated with an increase in the occurrence of morphologically-abnormal midgut epithelial cells (see Section 4.3.6 below). A detailed description of ookinete migration across the mosquito midgut epithelium, and the morphological changes in the latter associated with malaria parasite infection, derived from examination of the histological sections, is given later in this Chapter (Sections 4.3.3 and 4.3.4).

4.3.2.2.2 Levels of oocyst infection on day 10 post-bloodfeeding

As reported in Chapter 2, at day 10 pbf heavy oocyst infections were observed, in both infectious experimental feeds, on the midguts of other mosquitoes from the same cohort of *An. stephensi* fed *P. falciparum* 3D7A gametocytes and prepared for histology (Table 4.3). However, the level of oocyst infection observed in *An. stephensi* at day 10 pbf differed significantly between the two infectious experimental feeds (prevalence: two-tailed Fisher's Exact test, $P = 0.037$; and intensity: two-tailed Mann-Whitney U test, $z_{0.05,\pm1.960} = 3.447$, $P = 0.0006$).

4.3.2.3 Quantitative comparison of the level of *P. falciparum* 3D7A infection in *An. albimanus* and *An. stephensi*

4.3.2.3.1 Histological sections from midguts sampled on day 1 post-bloodfeeding

The levels of *P. falciparum* 3D7A ookinete and oocyst infection observed within the histological sections from *An. albimanus* and *An. stephensi* were compared separately for each experimental feed. The prevalence and intensity of ookinetes, both those associated with the peritrophic matrix/ectoperitrophic space, and invading the midgut epithelium, were significantly different between *An. albimanus* and *An. stephensi* (Table 4.4 and Table 4.5). Similarly, the prevalence and intensity of oocyst infection of the midgut epithelium observed within the histological sections also differed significantly between *An. albimanus* and *An. stephensi* (Table 4.4 and Table 4.5).

4.3.2.3.2 Levels of oocyst infection on day 10 post-bloodfeeding

The levels of oocyst infection observed at day 10 pbf in the same cohort of *An. albimanus* and *An. stephensi* challenged with the *P. falciparum* 3D7A clone and prepared for histology were also compared separately for each experimental feed. As reported in Chapter 2, both the prevalence and intensity of oocyst infection at day 10 pbf differed significantly between *An. albimanus* and *An. stephensi* (Table 4.3).

4.3.2.4 Population dynamics of *P. falciparum* 3D7A development within *An. albimanus* and *An. stephensi*

The levels of *P. falciparum* 3D7A infection observed at day 1 pbf in the histological sections and on midguts from the same cohort of challenged mosquitoes dissected at day 10 pbf were also compared *within* each mosquito species. The aim was to provide estimates of the stage-specific survival rates for ookinetes during migration between different locations within the midgut, and during the developmental transition from ookinetes into mature oocysts on the basal surface of the midgut epithelium.

4.3.2.4.1 P. falciparum *3D7A stage-specific survival in* An. albimanus

The population dynamics of *P. falciparum* 3D7A development within *An. albimanus*, from ookinete penetration of the peritrophic matrix to subsequent development into mature oocysts on the basal surface of the midgut epithelium, could not be characterised for this mosquito species due to the apparent absence of infection during this period of malaria parasite development. Presumably, none of the few

Table 4.4 Comparison of the prevalence of *P. falciparum* 3D7A parasites observed in histological sections in different locations within the midguts of *An. albimanus* and *An. stephensi*.

		An. albimanus		*An. stephensi*		
Experimental feed	**Stage / Location[a]**	***n*[b]**	**Prevalence[c]**	***n***	**Prevalence**	***P* value[d]**
1st Infectious	OOK PM/EPS/MV	6	16.7	5	100.0	0.015*
	OOK ME		0.0		100.0	0.0022*
	OOC		0.0		100.0	0.0022*
2nd Infectious	OOK PM/EPS/MV	11	18.2	13	76.9	0.012*
	OOK ME		0.0		92.0	<0.0001*
	OOC		0.0		76.9	0.00015*

[a] OOK PM/EPS/MV = all ookinetes associated with the peritrophic matrix and the ectoperitrophic space, including malaria parasites within the microvillar brush border prior to entry into the midgut epithelium. OOK ME = all ookinetes entering or within the midgut epithelium, including intracellular malaria parasites within midgut epithelial cells completely separated from the midgut epithelium. OOC = oocysts.
[b] The number of midguts examined.
[c] The percentage of midguts containing at least one of the relevant malaria parasite stages.
[d] Two-tailed Fisher's Exact test, with $\alpha = 0.05$.
* Indicates statistically significant differences between *An. albimanus* and *An. stephensi*.
N.B. Midguts from different time points were pooled and simultaneously compared. See Table 4.1 and Table 4.2 for details.

Table 4.5 Comparison of the intensity of *P. falciparum* 3D7A parasites observed in histological sections in different locations within the midguts of *An. albimanus* and *An. stephensi*.

		An. albimanus		*An. stephensi*			
Experimental feed	**Stage / Location[a]**	***n*[b]**	**Intensity[c]**	***n***	**Intensity**	***z* statistic[d]**	***P* value**
1st Infectious	OOK PM/EPS/MV	6	0.16	5	22.4	-2.694	0.0071*
	OOK ME		0.0		15.0	-2.795	0.0052*
	OOC		0.0		12.0	-2.795	0.0052*
2nd Infectious	OOK PM/EPS/MV	11	0.18	13	8.8	-3.463	0.00053*
	OOK ME		0.0		11.7	-4.358	<0.0001*
	OOC		0.0		10.2	-3.806	0.00014*

[a] OOK PM/EPS/MV = all ookinetes associated with the peritrophic matrix and the ectoperitrophic space, including malaria parasites within the microvillar brush border prior to entry into the midgut epithelium. OOK ME = all ookinetes entering or within the midgut epithelium, including intracellular malaria parasites within midgut epithelial cells completely separated from the midgut epithelium. OOC = oocysts.

[b] The number of midguts examined.

[c] Median number of malaria parasite stages observed per midgut (including uninfected midguts).

[d] Two-tailed Mann-Whitney *U* test, with $\alpha = 0.05$, where the critical value of $z = \pm 1.960$.

* Indicates statistically significant differences between *An. albimanus* and *An. stephensi*.

N.B. Midguts from different time points were pooled and simultaneously compared. See Table 4.1 and Table 4.2 for details.

ookinetes attaining the peritrophic matrix subsequently entered and invaded the midgut epithelium (Section 4.3.2.1).

4.3.2.4.2 P. falciparum *3D7A stage-specific survival in* An. stephensi

In *An. stephensi*, the numbers of *P. falciparum* 3D7A parasites observed in the histological sections attaining the peritrophic matrix and infecting the midgut epithelium on day 1 pbf were compared, within each cohort of challenged mosquitoes, to the level of oocyst infection seen at day 10 pbf. In the first infectious experimental feed, the prevalence of malaria parasite infection was not significantly different between days 1 and 10 pbf (Table 4.6). In contrast, in the second infectious experimental feed, the prevalence of malaria parasite infection was significantly lower at day 10 compared to day 1 pbf (Table 4.6). However, overall, in both experimental feeds, there were significant differences between the intensities of malaria parasites attaining the peritrophic matrix, and infecting the midgut epithelium, at day 1pbf, and the number of mature oocysts observed at day 10 pbf (Kruskal-Wallis test, $\chi^2_{0.05,\,5.991} = 13.48$, d.f. = 2, $P = 0.0012$ and $\chi^2_{0.05,\,5.991} = 13.34$, d.f. = 2, $P = 0.0013$, respectively, for each experimental feed). Subsequent pairwise comparisons revealed significant differences, in both experimental feeds, between the intensities of ookinetes attaining the peritrophic matrix and mature oocysts at day 10 pbf (Dunn test, $Q_{0.05,\,2.394} = 3.41$, $P < 0.002$ and $Q_{0.05,\,2.394} = 4.11$, $P < 0.001$, respectively) (Table 4.6). However, in neither of the experimental feeds undertaken did the intensity of ookinetes entering the midgut epithelium differ significantly from the intensity of either ookinetes attaining the peritrophic matrix or mature oocysts observed at day 10 pbf (Dunn test, $Q_{0.05,\,2.394} < 2.33$, $P > 0.05$ for all comparisons in both experimental feeds) (Table 4.6).

The values for the median intensity of malaria parasite infection obtained from the histological sections were used to estimate the stage-specific survival rates ("yields") for *P. falciparum* 3D7A within *An. stephensi* between ookinete penetration of the peritrophic matrix, infection of the midgut epithelium and subsequent development into mature oocysts (Table 4.7). Overall, on average, approximately 40% of the ookinetes attaining the peritrophic matrix on day 1 pbf subsequently developed into mature oocysts on the midgut epithelium at day 10 pbf. Approximately one third of the ookinetes attaining the peritrophic matrix apparently failed to infect the midgut epithelium, while a similar proportion of those ookinetes infecting the midgut epithelium subsequently failed to develop into mature oocysts (Table 4.7).

Table 4.6 Comparison of the levels of *P. falciparum* 3D7A infection within *An. stephensi* at days 1 and 10 post-bloodfeeding.

Experimental feed	Stage / Location / Time (days pbf)[a]	*n*[b]	Prevalence[c]	*P* value[d]	Intensity[e]	*P* value[f]
1st Infectious	OOK PM+ D1	5	100.0	0.708	39.0[y]	0.0012*
	OOK/OOC ME D1		100.0		27.0[z]	
	OOC D10	27	92.6		19.0[z]	
2nd Infectious	OOK ME+ D1	13	100.0	0.025*	20.0[y]	0.0013*
	OOK/OOC ME D1		100.0		11.0[z]	
	OOC D10	42	71.4		6.5[z]	

[a] OOK PM+ D1 = all ookinetes within the histological sections associated with the peritrophic matrix, the ectoperitrophic space, the microvillar brush border, and the midgut epithelium including intracellular malaria parasites within midgut epithelial cells completely separated from the midgut epithelium, together with all oocysts observed on day 1 pbf. OOK/OOC ME D1 = all ookinetes entering or within the midgut epithelium, including intracellular malaria parasites within midgut epithelial cells completely separated from the midgut epithelium, and oocysts observed on day 1 pbf (i.e. OOK PM+ D1 excluding all malaria parasites associated with the peritrophic matrix, the ectoperitrophic space and the microvillar brush border). OOC D10 = oocysts observed day 10 pbf on the midguts of other mosquitoes from the same cohort prepared for histology.

[b] The number of midguts examined.

[c] The percentage of midguts examined containing at least one of the relevant malaria parasite stages.

[d] Comparison of the prevalence of infection between malaria parasite stages/locations using a one-tailed Fisher's Exact test, with $\alpha = 0.05$.

[e] Median number of malaria parasite stages observed per midgut (including uninfected midguts).

[f] Simultaneous comparison of the intensity of infection between all three malaria parasite stages/locations using Kruskal-Wallis test. [y, z] indicate significant differences, within each experimental feed, between malaria parasite stages/locations in subsequent pairwise comparison tests. Different letter indicates significant, while same letter indicates no significant, difference between malaria parasite stages/locations. See main text for details of the statistical analyses (Section 4.2.3). * Indicates statistically significant differences between malaria parasite stages/locations.

Table 4.7 Estimated survival rates of *P. falciparum* 3D7A within *An. stephensi* between ookinete penetration of the peritrophic matrix and development into mature oocysts on the basal surface of the midgut epithelium.

Experimental feed	% Survival (OOK PM+ D1 to OOK/OOC ME D1)[a]	95% CI	% Survival (OOK/OOC ME D1 to OOC D10)[b]	95% CI	Overall % Survival (OOK PM+ D1 to OOC D10)[c]	95% CI
1st Infectious	69.2	-	70.4	-	48.7	-
2nd Infectious	55.0	-	59.1	-	32.5	-
Overall[d]	62.1	(48.2 to 76.1)	64.7	(53.7 to 75.8)	40.6	(24.7 to 56.5)

[a] Percentage of ookinetes attaining the peritrophic matrix that enter the midgut epithelium based upon the median numbers of malaria parasites observed in histological sections reported in Table 4.6. See footnotes to Table 4.6 for a full definition of OOK PM+ D1 and OOK/OOC ME D1.
[b] Percentage of ookinetes infecting the midgut epithelium that develop into mature oocysts at day 10 pbf based upon the median numbers of malaria parasites observed in histological sections reported in Table 4.6. See footnotes to Table 4.6 for a full definition of OOC D10.
[c] Percentage of ookinetes attaining the peritrophic matrix that develop into mature oocysts at day 10 pbf based upon the median numbers of malaria parasites observed in histological sections reported in Table 4.6 (equal to the product of the first and the second columns).
[d] The average of the survival rates from the first and second experiments together with their respective 95% confidence intervals (95% CI).
N.B. Midguts from different time points were pooled and simultaneously compared. See Table 4.1 and Table 4.2 for details.

4.3.3 Detailed histological description of *P. falciparum* 3D7A infection of the *An. stephensi* midgut epithelium

4.3.3.1 Morphological description of *P. falciparum* 3D7A parasites

4.3.3.1.1 Ookinetes observed in histological sections

Mature ookinetes possessed a distinctive morphology enabling their identification in the Giemsa-stained histological sections (Figure 4.6 to Figure 4.29). Although the overall shape of the ookinete was generally banana-like, approximately 2-3 by 10-15 μm, there was some variation in the morphology of these malaria parasite stages. Some ookinetes were sausage-like, possessing an even diameter throughout their length while others were more rhomboidal possessing one relatively short, blunt and broad region that gradually tapered to the narrow point of the other region of the malaria parasite. The curvature of the ookinetes also varied from straight to semi-circular: most ookinetes exhibited an intermediate degree of curvature while a small number of intracellular malaria parasites appeared to have "rounded up" completely (e.g. Figure 4.14C1 and Figure 4.32A). Some ookinetes were relatively narrow and elongated while others were apparently "stumpy" seeming short and fat, although this may have been an artefact of sectioning. Some of the variation in the shape, length and breadth of the ookinetes seemed to result from interactions with specific mosquito structures, especially the midgut epithelium, as ookinetes within the bloodmeal generally exhibited a relatively homogenous appearance of intermediate length and uniform width. In several instances, ookinetes interacting with mosquito structures exhibited a distinct "kink" (Figure 4.8D) while other ookinetes associated with the midgut epithelium possessed distinct narrow and elongated "stalks" (Section 4.3.3.3.2 and 4.3.3.4.4).

The cytoplasm of ookinetes stained the homogenous purple colour typical of erythrocytic malaria parasite stages more commonly stained with Giemsa. Generally, this enabled easy differentiation of ookinetes from other elements present in the histological sections (e.g. Figure 4.6). A single and darker staining purple oval structure, located approximately midway along the length of the malaria parasite, was apparent in many ookinetes (Figure 4.6A and Figure 4.13C). Previous electron microscopy studies suggest that this structure is the nucleus of the malaria parasite (Chapter 1, Section 1.4.3). Occasionally, one or two pale staining oval bodies, similar in size and shape to the nucleus, were also seen within the ookinete cytoplasm anterior and/or posterior to the nucleus (Figure 4.6A). Although no direct evidence is given

here, these structures probably correspond to the crystalloid inclusions previously described in ookinetes of various haemosporidia using light and electron microscopy (Garnham *et al.*, 1962; Trefiak & Desser, 1973). Typically, an extremely prominent, single, discrete and relatively large cluster of haemozoin pigment granules was present at one end of the ookinete (Figure 4.6A). Occasionally, single isolated grains of haemozoin were observed elsewhere within the malaria parasite cytoplasm (Figure 4.6B). The unmistakable, intensely dark, haemozoin pigment granules frequently possessed a characteristic golden aura, when viewed *via* the light microscope. Assuming that the pole of the ookinete orientated towards the basal surface of the midgut epithelium represents the apical complex, most observations were consistent with the cluster of haemozoin pigment granules being located within the anterior third of the ookinete, in front of the nucleus of the malaria parasite (e.g. Figure 4.10 and Figure 4.13C). However, in a small number of instances, it was unclear whether the haemozoin pigment was located in the anterior, rather than posterior, third of the ookinete, as the former interpretation implied ookinete movement in an unexpected direction (Figure 4.6F and I, and Figure 4.11C1). Therefore, haemozoin pigment may also rarely be located in the posterior third of the ookinete and/or the ookinete movement may not always follow a direct route towards the basal surface of the midgut epithelium. No other internal structures (i.e. apical complex, micronemes, subpellicular microtubules) were discernable by light microscopy in the histological sections examined, although the pellicle of the ookinetes may sometimes have been apparent (Figure 4.13C and Figure 4.32A).

Although ookinetes could be clearly identified within the bloodmeal, peritrophic matrix, ectoperitrophic space and midgut epithelium, these malaria parasite stages were not always easily identified, especially when present within midgut epithelial cells that exhibited abnormal dark staining (Figure 4.16D, Figure 4.18, Figure 4.19A and E, and Figure 4.33A). In such midgut epithelial cells, the colour of the ookinete cytoplasm was similar to that of the invaded midgut epithelial cell making discrimination difficult. However, the presence of a tight cluster of haemozoin pigment granules frequently provided confirmation of the presence of a malaria parasite. Although the haemozoin granules seemed to be an accurate diagnostic indicator of the presence of an ookinete, scattered haemozoin granules were not infrequently found "within" the midgut epithelium. However, most of the latter haemozoin granules seemed to be artefactual, clearly differing in distribution and density from the clusters present within ookinetes,

and were possibly "dragged" into the midgut epithelium during sectioning of the embedded midgut samples. In a small number of instances, haemozoin pigment clusters, resembling those present within ookinetes, were observed within morphologically-normal midgut epithelial cells without any other evidence of the presence of a malaria parasite.

4.3.3.1.2 Oocysts observed in histological sections

Like ookinetes, oocysts also possessed a distinctive morphology that enabled their identification in the Giemsa-stained histological sections (Figure 4.36). Oocysts were morphologically similar to mature ookinetes in terms of cytoplasmic staining, and the presence of a single dark staining nucleus and numerous haemozoin pigment granules. There were nevertheless some distinct morphological differences between these two different stages in the malaria parasite life cycle. First, the oocysts were no longer banana-shaped but irregularly-shaped ellipsoids approximately 4 to 8μm in diameter. Second, the crystalloids observed in ookinetes were no longer clearly present, although the cytoplasm of oocysts did occasionally exhibit some variation in the intensity of staining (e.g. Figure 4.36J). Third, the haemozoin pigment granules were no longer found in a single tight cluster but dispersed throughout the malaria parasite often in distinctive arcs (Figure 4.36D). Most malaria parasites observed within the midgut epithelium could be clearly designated as either ookinetes or oocysts. However, occasionally, malaria parasites on the basal surface of the midgut epithelium were observed that appeared to be intermediate forms partly spherical but still possessing either an apical prominence (Figure 4.26C, Figure 4.37A and B) or a posterior "tail" (Figure 4.29B).

Rarely, a faint, pink staining "halo" was also apparent around the haemocoelic surface of some oocysts (Figure 4.36B and I), presumably corresponding to the basal lamina surrounding the basal surface of the midgut epithelium, which was otherwise difficult to discriminate from the underlying midgut epithelial cells.

4.3.3.2 Ookinete penetration of the peritrophic matrix

Ookinetes were most frequently observed in the process of penetrating the peritrophic matrix (Figure 4.6 and Figure 4.7). These malaria parasite stages could be observed entering the endoperitrophic surface of the peritrophic matrix (Figure 4.6I and Figure 4.7A), entirely within peritrophic matrix (Figure 4.7H and J), and emerging from the ectoperitrophic surface of the peritrophic matrix (Figure 4.7B and Figure 4.8A).

Ookinetes in the process of penetrating the peritrophic matrix were often parallel, rather than orthogonal, to this mosquito structure (Figure 4.7A, B and H to J). Other ookinetes, apparently within the peritrophic matrix, curved upwards away from the longitudinal, and towards the perpendicular, plane of the peritrophic matrix (Figure 4.7D to G). Ookinetes crossing the peritrophic matrix generally appeared morphologically normal, with no evidence of constriction, although some of these malaria parasite stages were more "rhomboidal" than other ookinetes observed within the bloodmeal (cf. Figure 4.7B, C, F, H and I with Figure 4.6A, C, F and G). Ookinetes were frequently observed penetrating the peritrophic matrix, but there was little evidence of disruption to the peritrophic matrix where the ookinetes had invaded. In a few instances, the peritrophic matrix beneath ookinetes invading the midgut epithelium appeared to be slightly outwardly evaginated towards the ectoperitrophic space, while yellow-staining material reminiscent of the peripheral digested region of the bloodmeal appeared to be present within the latter (Figure 4.10, Figure 4.13, Figure 4.19B and Figure 4.23C). The vast majority of malaria parasites penetrating the peritrophic matrix were single and apparently isolated. However, in several instances, groups of two or three ookinetes were observed entering the peritrophic matrix in close proximity to one another (Figure 4.7K).

4.3.3.3 Ookinete entry into the midgut epithelium

4.3.3.3.1 Ookinetes within the microvillar brush border of the midgut epithelium

Several ookinetes were observed within the microvillar brush border of the midgut epithelium apparently immediately before entry into midgut epithelial cells (Figure 4.8 and Figure 4.9). No ookinetes were observed within the dense mounds of microvilli covering the central apical surface of individual midgut epithelial cells. Rather, the ookinetes often appeared to be aligned with the "partings" present within the microvillar brush border, beneath the lateral membranes of the overlying midgut epithelial cells (Figure 4.9). In most instances, the ookinetes were clearly apposed to sites where the lateral membranes of adjacent midgut epithelial cells converged. Specifically, ookinetes were frequently apposed to sites where *three* adjacent midgut epithelial cells converged. In regions where ookinetes were found within the microvillar brush border of the midgut epithelium, before entry into the latter, the midgut epithelium was morphologically normal and exhibited no signs of invagination.

4.3.3.3.2 Ookinete entry into the apical surface of the midgut epithelium

The initial moment of ookinete entry into the apical surface of the midgut epithelium was unambiguously observed five times (four of these five "entry" events are shown; Figure 4.10 and Figure 4.11). Ookinetes appeared to enter the midgut epithelium at sites where the apicolateral plasma membranes of adjacent midgut epithelial cells converged. In three instances, ookinetes clearly entered the midgut epithelium at sites where the lateral membranes of *three* adjacent midgut epithelial cells converged (Figure 4.10, Figure 4.11A and C). In the two remaining entry events, the lateral plasma membranes and the number of midgut epithelial cells converging around the invading ookinete could not be clearly identified (Figure 4.11B and not shown). The ookinetes observed in these invasion events appeared to enter the midgut epithelium at an angle which curved upwards from the longitudinal plane of the microvillar brush border of the apical surface of the midgut epithelium, such that as these malaria parasites entered into the midgut epithelium they became increasingly orthogonal to the longitudinal plane of the midgut epithelium.

With the exception of one invasion event described below (Figure 4.11C), the midgut epithelial cells at the site of ookinete entry into the midgut epithelium appeared morphologically normal, possessing typical centrally-located nuclei, abundant microvilli and staining with an intensity equivalent to other morphologically-normal midgut epithelial cells not associated with invading malaria parasites. However, the midgut epithelium exhibited significant invagination centred around, and localised to, the site of ookinete entry into the midgut epithelium (Figure 4.10, Figure 4.11A and B). The localised invagination of the midgut epithelium was sometimes extensive: up to one half or more of the depth of the midgut epithelium. Similar invaginations were also occasionally associated with other malaria parasites further in the process of invading the midgut epithelium (Figure 4.13, Figure 4.25, Figure 4.37, Figure 4.38B and C) and were not observed in the absence of invading malaria parasites, either within *P. falciparum* infected or uninfected midguts. In the one exceptional instance, the midgut epithelium did not exhibit any invagination but the midgut epithelial cell apparently being entered by the ookinete exhibited markedly darker, morphologically abnormal, staining compared to the surrounding uninvaded midgut epithelial cells (Figure 4.11C).

In four of the five observed entry events, the invading ookinetes appeared to be entirely extracellular, within the ectoperitrophic space of the midgut lumen, but embedded against the apicolateral surface of the midgut epithelium (Figure 4.10, Figure

4.11A and C, and not shown). In the remaining instance, although the ookinete was predominantly extracellular within the microvillar brush border, the anterior-most region of the malaria parasite appeared to reside within the midgut epithelium (Figure 4.11B). Furthermore, this anterior region of the ookinete seemed constricted into a narrow and elongated protuberance reminiscent of the "stalk-form" morphology previously described of ookinetes crossing between or exiting midgut epithelial cells (Vernick *et al.*, 1999; Han *et al.*, 2000) (see Section 4.3.3.4.4 below).

One further entry event was probably observed, which was initially difficult to interpret because the plane of sectioning through the midgut epithelium meant that the location of the malaria parasite was ambiguous (Figure 4.12). However, the previously described entry events (e.g. Figure 4.10 and Figure 4.11A), which were sectioned though the orthogonal plane of the midgut epithelium, provided a template for interpreting this otherwise ambiguous instance (Figure 4.12A). The invading ookinete again appeared to enter the midgut epithelium where multiple adjacent midgut epithelial cells converged, although the exact number of midgut epithelial cells converging at the precise site of ookinete entry could not be unambiguously determined. Furthermore, the midgut epithelium surrounding the ookinete was again morphologically normal except for an apparent marked invagination of the apical surface of the midgut epithelium centred around, and localised to, the invading malaria parasite, such that the microvillar brush border was present in the same plane as the nuclei of the midgut epithelial cells.

If it is accepted that the latter ambiguously located ookinete is extracellular within the ectoperitrophic space but embedded against the invaginated apical surface of the midgut epithelium, then overall only 5% (6 of 129) ookinetes associated with the midgut epithelium (excluding those ookinetes within the microvillar brush border, Section 4.3.3.3.1) were observed in the process of entering the midgut epithelium.

4.3.3.4 Ookinete migration through the midgut epithelium

Overall, 123 ookinetes were observed in various locations *within* the midgut epithelium (excluding the 6 ookinetes described in Section 4.3.3.3.2 *entering* the midgut epithelium)[1]. Ookinetes were frequently observed in both intra- and intercellular locations, although a substantial proportion of the ookinetes observed could not be

[1] All intracellular ookinetes located within midgut epithelial cells protruding from the midgut epithelium into the midgut lumen, even when these midgut epithelial cells were completely separated from the overlying midgut epithelium, were counted as being "within the midgut epithelium".

unambiguously assigned to either one of these positions within the midgut epithelium. However, regardless of their location within the midgut epithelium, 93% (114 of 123) of the ookinetes observed within the midgut epithelium were associated with morphologically-abnormal midgut epithelial cells. The remaining ookinetes were either intracellular (3%, 4 of 123) within morphologically-normal midgut epithelial cells, or intercellular (4%, 5 of 123) situated between morphologically-normal midgut epithelial cells in the central and basal regions of the midgut epithelium.

Most ookinetes observed within the midgut epithelium were orientated orthogonally to the longitudinal plane of the latter (i.e. the anterior pole of ookinete was directed towards the basal surface of the midgut epithelium while posterior pole of the malaria parasite was directed towards the apical surface of the midgut epithelium) (e.g. Figure 4.13, Figure 4.17 and Figure 4.19B to D). However, some ookinetes within the midgut epithelium were apparently parallel to the longitudinal plane of the midgut epithelium (Figure 4.16 and Figure 4.20).

4.3.3.4.1 Intracellular ookinetes within midgut epithelial cells

Forty-four per cent (54 of 123) of all ookinetes observed within the midgut epithelium were unambiguously located *within* midgut epithelial cells (Figure 4.13 to Figure 4.21 and Figure 4.32). In all these instances, the intracellular location of the malaria parasites was demonstrated by following the relevant infected midgut epithelial cells through multiple consecutive serial sections (Figure 4.13, Figure 4.15 and Figure 4.17). Ookinetes were never seen within any other type of cell within the midgut epithelium, including the pale "open" endocrine cells, dark regenerative cells and the morphologically unusual intermediate forms associated with regenerative cells described in Chapter 5. Furthermore, intracellular ookinetes were only observed singly; no more than one ookinete was ever observed within a single midgut epithelial cell.

The midgut epithelial cells containing malaria parasites exhibited considerable morphological variation. A small number of the ookinete-infected midgut epithelial cells observed (9.3%, 5 of 54) were morphologically normal possessing typical centrally-located nuclei, dense microvilli, and an intensity of staining similar to that of morphologically-normal uninfected midgut epithelial cells (Figure 4.13 and Figure 4.14). Two of the ookinetes observed on these occasions had apparently just entered into the midgut epithelial cells (Figure 4.13; other event not shown). These malaria parasites were located in the corners of the midgut epithelial cells created by the convergence of the lateral plasma membranes of three adjacent midgut epithelial cells

(Figure 4.13A). In both of these instances, the invaded midgut epithelial cells were morphologically indistinguishable from the surrounding uninvaded midgut epithelial cells (Figure 4.13D), with the exception that the midgut epithelium again exhibited marked invagination immediately beneath the invading malaria parasites (Figure 4.13B).

The majority of midgut epithelial cells containing malaria parasites (90.7%, 49 of 54) exhibited a range of morphological characteristics not typically observed of normal uninfected midgut epithelial cells, including: differential staining of the cytoplasm; apparent changes in turgidity; various degrees of protrusion from the midgut epithelium into the midgut lumen; condensation, fragmentation and/or apical translocation of the nucleus; shortening and loss of microvilli from the apical surface of the midgut epithelial cell; and, sometimes, extensive cytoplasmic vacuolisation (Figure 4.15 to Figure 4.21). The abnormal morphological characteristics of the midgut epithelial cells associated with malaria parasites invading the midgut epithelium are described in detail in Section 4.3.4 below.

There was considerable variation in each of the atypical characteristics of the morphologically-abnormal midgut epithelial cells containing ookinetes, especially with regard to the degree of protrusion from the midgut epithelium. A few ookinetes were observed within midgut epithelial cells that exhibited only minor swelling or bulging from (or within) the midgut epithelium (Figure 4.15, Figure 4.16A and Figure 4.18B). However, malaria parasites were more frequently found within midgut epithelial cells exhibiting marked protrusion from the midgut epithelium into the midgut lumen (e.g. Figure 4.17, Figure 4.19 and Figure 4.32). In several instances, malaria parasites were even apparently present within midgut epithelial cells completely separated from the midgut epithelium (Figure 4.33).

Although ookinetes were occasionally seen within the apical region of midgut epithelial cells (Figure 4.14C1 and Figure 4.16D) most intracellular malaria parasites were observed in the basal region of invaded midgut epithelial cells (Figure 4.14A and B, Figure 4.15, Figure 4.16A and C, Figure 4.17). Intracellular ookinetes were often observed lying immediately adjacent and parallel to the basal region of the lateral plasma membrane of the invaded midgut epithelial cells (Figure 4.19B and C). In contrast, other ookinetes occupied a more central location within the cytoplasm apparently unassociated with the lateral plasma membranes of the invaded midgut epithelial cells (Figure 4.16A and B, Figure 4.17 and Figure 4.20).

Typically, intracellular ookinetes appeared to be in direct contact with the cytoplasm of invaded midgut epithelial cells (Figure 4.14, Figure 4.16C, Figure 4.17, Figure 4.19B and C, Figure 4.20 and Figure 4.21). However, an irregular unstained region was apparent around six ookinetes observed within the midgut epithelium. Two of these ookinetes were probably extracellular parasites present within the basolateral space between adjacent midgut epithelial cells (not shown). However, the remaining four ookinetes surrounded by an unstained region were clearly intracellular (Figure 4.15, Figure 4.16A and B, and Figure 4.32B). A few other ookinetes were also surrounded by a regular, narrow, and sometimes barely visible, unstained region of uniform width (Figure 4.13C, Figure 4.29A and Figure 4.32A). In these instances, where visible, borders of the associated midgut epithelial cells exhibited a similar aspect (Figure 4.32A).

Most ookinetes observed within midgut epithelial cells appeared intact and morphologically normal, possessing distinct nuclei, characteristic haemozoin pigment granules, and crystalloid bodies (e.g. Figure 4.16B to D, Figure 4.17 and Figure 4.19B to D). There was little obvious evidence that intracellular ookinetes were undergoing lysis or other forms of degenerative change within midgut epithelial cells. However, the morphology of some of the malaria parasites observed within midgut epithelial cells, especially midgut epithelial cells exhibiting marked protrusion from the midgut epithelium, was often poorly defined and suggestive of morphologically abnormality (Figure 4.32 and Figure 4.33).

4.3.3.4.2 Ookinetes associated with multiple morphologically-abnormal midgut epithelial cells

Clusters of adjacent midgut epithelial cells exhibiting various degrees of morphological abnormality were frequently associated with malaria parasites invading the midgut epithelium (Figure 4.19 to Figure 4.21, Figure 4.24, Figure 4.33A and B). Overall, including both ookinetes and oocysts, 34% (69 of 204) of all malaria parasites found in morphologically-abnormal regions of the midgut epithelium were associated with more than one abnormal midgut epithelial cell. Such clusters of morphologically-abnormal midgut epithelial cells typically consisted of two or three adjacent midgut epithelial cells. In several instances, greater numbers of midgut epithelial cells protruding from the midgut epithelium were associated with invading malaria parasites (Figure 4.21). These abnormal midgut epithelial cells either protruded as a single mass from the midgut epithelium (Figure 4.19D Figure 4.41A, B, C and E) or, more

commonly, formed a sequential trail of associated midgut epithelial cells exhibiting increasingly greater degrees of protrusion from the midgut epithelium (Figure 4.19A to C, Figure 4.20, Figure 4.21, Figure 4.24 and Figure 4.41D).

4.3.3.4.3 Ookinete egress from midgut epithelial cells

Overall, 25% (31 of 123) of the ookinetes observed within the midgut epithelium were ambiguously located and could not be clearly assigned as either wholly within or outside midgut epithelial cells, as it was not possible to discriminate the lateral plasma membranes of the surrounding midgut epithelial cells (Figure 4.19D, Figure 4.24, Figure 4.22, Figure 4.23 and Figure 4.38A). These malaria parasites were generally located within the central and basal regions of the midgut epithelium and often appeared to be in the process of exiting invaded midgut epithelial cells. However, whether these ookinetes were exiting from midgut epithelial cells into adjacent midgut epithelial cells or the extracellular basolateral intercellular space between adjacent midgut epithelial cells was impossible to ascertain. Ambiguously located ookinetes were typically situated above morphologically-abnormal midgut epithelial cells, which frequently exhibited marked protrusion from the midgut epithelium into the midgut lumen. In a few instances, malaria parasites were observed apparently emerging from the basal surface of morphologically-abnormal midgut epithelial cells extruded from the midgut epithelium and entering the apicolateral membrane of overlying healthy midgut epithelial cells (Figure 4.19E and Figure 4.25). One ookinete was also observed apparently perpendicularly spanning the plasma membranes of two adjacent morphologically abnormal epithelial cells exhibiting different degrees of protrusion into the midgut lumen (Figure 4.19A). The changes in the shape of the protruding morphologically-abnormal midgut epithelial cells associated with this ookinete prevented unambiguous classification of the exact midgut epithelial cell plasma membranes that this malaria parasite crossed.

In a few rare instances, extracellular ookinetes, either within the bloodmeal or the ectoperitrophic space, were also observed adjacent to, or apparently partly within, midgut epithelial cells exhibiting substantial or complete extrusion from the midgut epithelium (Figure 4.43). No other invading malaria parasites (either ookinetes or oocysts) were obviously associated with these particular morphologically-abnormal midgut epithelial cells. However, it was not possible to determine from the histological sections examined whether these particular malaria parasites were always causally related to such abnormal midgut epithelial cells or had just coincidentally migrated into

a region where such midgut epithelial cells were already present.

4.3.3.4.4 "Stalk-form" ookinetes within the midgut epithelium

Three morphologically unusual ookinetes, previously described as "stalk-forms" (Vernick *et al.*, 1999), were also observed within the midgut epithelium (2%, $n = 123$) (Figure 4.30 and Figure 4.31). These ookinetes possessed extremely narrow and elongated regions ("stalks") at either, or both, ends of which were larger and wider bulbous regions of the malaria parasites. In two of the instances, the malaria parasites were apparently emerging from the contracted basal surface of morphologically-abnormal midgut epithelial cells exhibiting marked protrusion from the midgut epithelium into the midgut lumen (Figure 4.30A and B). It was unclear if one of these ookinetes was exiting from the infected protruding midgut epithelial cell into the basolateral extracellular space between adjacent midgut epithelial cells, or entering another neighbouring midgut epithelial cell (Figure 4.30A). However, the contracted basal surface of the invaded protruding midgut epithelial cell clearly converged with the narrow elongated stalk region of this ookinete. The other of these two ookinetes was observed in an apparently intercellular location within the apical region of the midgut epithelium situated between two morphologically-normal midgut epithelial cells (Figure 4.30B). In adjacent sections, a protruding midgut epithelial cell was apparent immediately beneath this malaria parasite. The narrow elongated stalk of this ookinete appeared to extend from this protruding midgut epithelial cell. However, the distance between the sections was too great for accurate determination of the exact relationship between the malaria parasite and the protruding midgut epithelial cell. The third stalk-form ookinete, and the precise location of this malaria parasite within the midgut epithelium, were somewhat difficult to discern (Figure 4.31). The apical surface of the midgut epithelial cell immediately below this ookinete was unusually concave, but there were no obviously protruding or other morphologically-abnormal midgut epithelial cells associated with this ookinete.

4.3.3.4.5 Intercellular ookinetes situated between morphologically-normal midgut epithelial cells

The remaining 28% (35 of 123) ookinetes observed within the midgut epithelium were unambiguously situated in intercellular locations, within the central and basal regions of the midgut epithelium, between morphologically-normal midgut epithelial cells (Figure 4.26 to Figure 4.29 and Figure 4.30B). The intercellular location of these

malaria parasites was inferred through identification of the lateral plasma membranes of the midgut epithelial cells surrounding, or beneath, the ookinetes. In most instances, intercellular ookinetes were in close proximity to the basal surface of the midgut epithelium. With one exception (Figure 4.30B), ookinetes were not observed in intercellular locations in the apical region of the midgut epithelium. Most intercellular ookinetes were located between two adjacent midgut epithelial cells (Figure 4.26A to C, Figure 4.27 and Figure 4.29A). However, some intercellular ookinetes appeared to be situated where three adjacent midgut epithelial cells converged (Figure 4.29C and Figure 4.38A). Other intercellular ookinetes were not obviously associated with any midgut epithelial cells displaying morphological abnormality (Figure 4.26), although some were situated above marked invaginations of the apical surface of the midgut epithelium (Figure 4.37) similar to those observed during ookinete entry into the midgut epithelium (Section 4.3.3.3.2). The majority of the intercellular ookinetes (86%, 30 of 35) were associated with midgut epithelial cells exhibiting the atypical morphological characteristics associated with most intracellular ookinetes (Section 4.3.3.4.1). Usually, these abnormal midgut epithelial cells were in the immediate vicinity of the intercellular parasites and were often observed in the same sections (Figure 4.28, Figure 4.29A and B). In several instances, though, intercellular malaria parasites were found in sections some distance (approximately 10 to 30 μm) from those containing the morphologically-abnormal midgut epithelial cells (Figure 4.27, Figure 4.29C and Figure 4.30B). Typically, the abnormal midgut epithelial cells associated with intercellular parasites exhibited marked protrusion from the midgut epithelium into the midgut lumen.

4.3.3.5 Oocysts on the basal surface of the midgut epithelium

Malaria parasites that had reached the basal surface of the midgut epithelium assumed the rounded, spherical form of early oocysts (n = 192). No malaria parasites on the basal surface of the midgut epithelium, entirely outside the midgut epithelium, were observed that obviously possessed the typical elongated banana-shape of mature ookinetes. However, some malaria parasite forms apparently intermediate between ookinetes and oocysts were occasionally observed (Figure 4.26C, Figure 4.29B, Figure 4.37A and B). With one exception (Figure 4.26D), all oocyst stage malaria parasites were located on the basal surface of the midgut epithelium. In the exceptional case, the oocyst appeared to be located intercellularly within the central region of the midgut epithelium between two morphologically-normal midgut epithelial cells (Figure 4.26D). In numerous instances, oocysts on the basal surface of the midgut epithelium were

clearly located immediately above the lateral plasma membranes of two adjacent underlying midgut epithelial cells (Figure 4.34A, C, D, Figure 4.35A to F, H and I). In other instances, oocysts appeared to be similarly located above the junction between two adjacent midgut epithelial cells, although the lateral plasma membranes of the underlying midgut epithelial cells could not be identified (Figure 4.34B and Figure 4.36G). Occasionally, oocysts appeared to be located immediately above sites in the midgut epithelium where three adjacent underlying midgut epithelial cells converged (Figure 4.35, Figure 4.38B, C and D). An oocyst was also observed which appeared to be situated immediately above the centre of a "rosette" of multiple converging adjacent midgut epithelial cells (Figure 4.36K). However, the plane of sectioning through the midgut epithelium prevented an unambiguous determination of exactly how many of these adjacent midgut epithelial cells converged beneath this malaria parasite. Overall, 47% (91 of 192) of oocysts were obviously associated with morphologically-abnormal midgut epithelial cells displaying various degrees of extrusion from the midgut epithelium (Figure 4.34 and Figure 4.35). Sometimes, in the same section, morphologically-abnormal midgut epithelial cells were observed protruding into the midgut lumen from beneath the lateral plasma membranes of the morphologically-normal midgut epithelial cells over which the malaria parasites were positioned (Figure 4.34B). On other occasions, oocysts were in the same section as midgut epithelial cells entirely separated from the midgut epithelium (Figure 4.34C and D). However, in most instances, oocysts and their associated morphologically-abnormal midgut epithelial cells were not present within the same histological section (Figure 4.35). Consequently, oocysts associated with morphologically-abnormal midgut epithelial cells often appeared to reside within morphologically-normal midgut epithelium. The remaining oocysts (53%, 101 of 192) were not obviously associated with any morphologically-abnormal midgut epithelial cells and appeared to be genuinely surrounded by morphologically-normal midgut epithelium, with no evidence of any disruption, especially at later time points (Figure 4.36). However, in a few instances, oocysts were observed on the basal surface of the midgut epithelium immediately above marked invaginations of the apical surface of the midgut epithelium similar to those observed during ookinete entry into the midgut epithelium (Figure 4.37B and C, and Figure 4.38B and C) (Section 4.3.3.3.2). None of these oocysts exhibited any obvious association with morphologically-abnormal midgut epithelial cells.

4.3.4 Detailed histological description of the morphologically-abnormal midgut epithelial cells

4.3.4.1 *An. stephensi* fed *P. falciparum* 3D7A gametocytes

The characteristics of the morphologically-abnormal midgut epithelial cells observed within the midguts of *An. stephensi* fed infectious blood containing *P. falciparum* 3D7A gametocytes were varied. In general, three distinct "types" of morphologically-abnormal midgut epithelial cell could be identified: "turgid", "flaccid" and "lysing" midgut epithelial cells (Figure 4.39 to Figure 4.42). As described below, there was some overlap in the characteristics of the turgid and flaccid abnormal midgut epithelial cells. In contrast, the morphological characteristics of the allegedly lysing abnormal midgut epithelial cells appeared to be unique and were not observed in the other types of abnormal midgut epithelial cells.

4.3.4.1.1 "Turgid" morphologically-abnormal midgut epithelial cells

Turgid abnormal midgut epithelial cells were the most common type of morphologically-abnormal midgut epithelial cell observed (Figure 4.39 to Figure 4.41). These abnormal midgut epithelial cells exhibited various degrees of protrusion, from relatively minor swelling towards the midgut lumen, to complete separation from the midgut epithelium. Complete separation of turgid abnormal midgut epithelial cells was demonstrated by following the relevant midgut epithelial cells through multiple consecutive serial sections. In marked contrast to morphologically-normal midgut epithelial cells located within the midgut epithelium, turgid midgut epithelial cells, lying within the midgut lumen and completely separated from the midgut epithelium, appeared to be relatively formless spheres or ellipsoids lacking any obvious polarity or surface structure (Figure 4.39H and Figure 4.40D). Other turgid midgut epithelial cells that appeared to be separated from the midgut epithelium in some sections were in fact connected to the latter *via* thin prominences observable in other sections (Figure 4.39E and G). Regardless of the degree of protrusion into the midgut lumen, these abnormal midgut epithelial cells remained "inflated", with no apparent loss in volume, even when completely separated from the midgut epithelium (Figure 4.33C, Figure 4.34D, Figure 4.39, Figure 4.40B to C and Figure 4.41). Turgid midgut epithelial cells appeared to posses appreciable resistance as the peritrophic matrix underlying these abnormal midgut epithelial cells was often locally depressed, as if forced inwardly, towards the bloodmeal (Figure 4.17, Figure 4.19C, Figure 4.30, Figure 4.39C and D, and Figure

4.41A), or more broadly separated from the apical surface of the midgut epithelium substantially increasing the depth of the ectoperitrophic space (Figure 4.19B, Figure 4.20, Figure 4.35, Figure 4.39E, and Figure 4.40D). Sometimes, the peritrophic matrix around protruding turgid midgut epithelial cells appeared to have been disrupted such that these abnormal midgut epithelial cells were in direct contact with the bloodmeal within the endoperitrophic space (Figure 4.19D, Figure 4.23, Figure 4.24, Figure 4.35, Figure 4.39G, Figure 4.40B, Figure 4.41B, C and E). However, turgid midgut epithelial cells were also apparently partly deformable sometimes conforming to the confines of the ectoperitrophic space (e.g. Figure 4.40D). Relative to morphologically normal uninfected midgut epithelial cells, the cytoplasm of turgid midgut epithelial cells exhibited light, equivalent (i.e. normal) or dark staining (e.g. Figure 4.24, Figure 4.41A and Figure 4.40B to C, respectively). Rarely, the cytoplasm of individual turgid midgut epithelial cells also varied in intensity, staining normally in some areas/sections and lighter and/or darker in others (Figure 4.23A). The nuclei of turgid midgut epithelial cells were no longer centrally-located but apically-situated (Figure 4.39A to E). The nuclei often remained relatively morphologically normal despite extensive protrusion, or even complete separation, from the midgut epithelium of the turgid midgut epithelial cells (Figure 4.17A, Figure 4.19B and C, Figure 4.39A to F and H, and Figure 4.41B). However, the nuclei of some turgid midgut epithelial cells exhibiting marked protrusion from the midgut epithelium possessed lighter and less granular staining together with intense dark staining of the nuclear periphery (Figure 4.19B, Figure 4.24 and Figure 4.39F and H). Less frequently, the nuclei of other protruding turgid midgut epithelial cells were markedly altered, containing a single, or sometimes several discrete, condensed areas that stained intensely (Figure 4.19E, Figure 4.34D and Figure 4.39G1). The nuclei of dark-staining turgid midgut epithelial cells were often very difficult to discriminate but did not appear to be markedly condensed, unlike the nuclei of the dark-staining flaccid abnormal midgut epithelial cells described below (Section 4.3.4.1.2) (Figure 4.40, Figure 4.41C and D). The density of microvilli on the apical surface of turgid midgut epithelial cells varied, more or less, according to the degree of protrusion from the midgut epithelium, such that turgid midgut epithelial cells exhibiting marked protrusion into the midgut lumen had few or no discernable microvilli (Figure 4.39). Occasionally, turgid midgut epithelial cells, especially those that were either nearly or completely extruded from the midgut epithelium, appeared to contain numerous, and extensive, regularly-shaped vacuoles (Figure 4.39G). Turgid midgut epithelial cells

were often observed in clusters of multiple adjacent morphologically-abnormal midgut epithelial cells (e.g. Figure 4.21 and Figure 4.41).

4.3.4.1.2 "Flaccid" morphologically-abnormal midgut epithelial cells

Flaccid abnormal midgut epithelial cells were the second most common type of morphologically-abnormal midgut epithelial cell observed, although these abnormal midgut epithelial cells were seen considerably less frequently than the turgid abnormal midgut epithelial cells. In contrast to the turgid midgut epithelial cells, flaccid midgut epithelial cells were predominantly found within the midgut epithelium and seemed to have undergone major morphological changes prior to any appreciable extrusion into the midgut lumen (Figure 4.16D, Figure 4.23B and C, Figure 4.28, Figure 4.29 and Figure 4.40A). Occasionally, small and irregularly shaped dark staining midgut epithelial cells, presumed to correspond to the flaccid cells seen within the midgut epithelium, were observed against the apical surface of the midgut epithelium (Figure 4.32E). Flaccid midgut epithelial cells appeared to have either collapsed or shrunk inwardly, like a deflated balloon, and were frequently shrivelled and reduced in size. The cytoplasm of these abnormal midgut epithelial cells always stained darkly while the nuclei were a single highly-condensed and intensely-staining mass. Typically, the nuclei did not exhibit the marked apical location characteristic of turgid and lysing abnormal midgut epithelial cells, presumably reflecting the less-marked protrusion of the flaccid abnormal midgut epithelial cells from the midgut epithelium. Some flaccid midgut epithelial cells possessed microvilli of varying length (Figure 4.16D, Figure 4.23B, Figure 4.29A and Figure 4.40) while others apparently lacked such structures (Figure 4.23C, Figure 4.28, Figure 4.29B and C, and Figure 4.32C). Flaccid midgut epithelial cells also did not exhibit the vacuolisation occasionally observed within turgid midgut epithelial cells located within the midgut lumen. Some of the dark-staining "turgid" midgut epithelial cells located within the midgut epithelium (Section 4.3.4.1.1) may have been "flaccid" midgut epithelial cells observed prior to "collapsing" and "shrivelling" (e.g. Figure 4.11C and Figure 4.40A). The flaccid abnormal midgut epithelial cells were only observed singly within the midgut epithelium and, with one possible exception (Figure 4.40A), were associated with invading malaria parasites. Ookinetes were either observed in the apical region of flaccid midgut epithelial cells (Figure 4.16D) or emerging from the basal region of these abnormal midgut epithelial cells apparently into the basolateral intercellular space between the surrounding uninvaded midgut epithelial cells (Figure 4.23B and C, Figure 4.28 and Figure 4.29). In

one instance, an apparently morphologically-abnormal ookinete was observed within in a small and irregularly-shaped flaccid midgut epithelial cell located against the apical surface of the midgut epithelium (Figure 4.32E).

4.3.4.1.3 "Dissolving" morphologically-abnormal midgut epithelial cells

Dissolving abnormal midgut epithelial cells were the least common type of morphologically-abnormal midgut epithelial cell, being observed on a few occasions only. These abnormal midgut epithelial cells were observed both within the midgut epithelium (Figure 4.18B and Figure 4.40F) and protruding from the latter into the ectoperitrophic space of the midgut lumen (Figure 4.25, Figure 4.27 and Figure 4.42). Unlike turgid and flaccid abnormal midgut epithelial cells, dissolving abnormal midgut epithelial cells, especially those exhibiting marked protrusion from the midgut epithelium, appeared diffuse lacking a defined cell boundary and definite structure (Figure 4.18A and Figure 4.25, Figure 4.27 and Figure 4.42). In these latter instances, the dissolving midgut epithelial cells seemed to be "pouring" from the midgut epithelium into the ectoperitrophic space of the midgut lumen. No inward depression of the peritrophic matrix towards the bloodmeal was apparent beneath these abnormal midgut epithelial cells. The cytoplasm of dissolving abnormal midgut epithelial cells, which stained a unique pale blue, was often difficult to discriminate from the ectoperitrophic space (Figure 4.25, Figure 4.27 and Figure 4.42). Although the nuclei stained a similar, if darker, blue to the cytoplasm, the size, shape and internal morphology of the nuclei of dissolving midgut epithelial cells did not differ markedly from those of morphologically-normal midgut epithelial cells (Figure 4.40F and Figure 4.42A and B). Some of the dissolving midgut epithelial cells possessed microvilli (Figure 4.18) while others apparently lacked these foldings of the apical plasma membrane (Figure 4.25 Figure 4.27, Figure 4.40 and Figure 4.42). Although the presence of microvilli generally varied according to the degree of protrusion into the midgut lumen, a dissolving midgut epithelial cell almost completely separated from the midgut epithelium was observed that possessed abundant normal microvilli (Figure 4.18A). Like flaccid abnormal midgut epithelial cells, dissolving midgut epithelial cells were only observed singly and, with one exception (Figure 4.40F), were associated with malaria parasites invading the midgut epithelium.

4.3.4.2 *An. stephensi* fed uninfected or non-infectious *P. falciparum* 3D7A-infected blood

Midgut epithelial cells exhibiting the various degrees of morphological abnormality were also observed very rarely in the histological sections from the midguts of *An .stephensi* fed either uninfected blood, or infected but non-infectious blood containing asexual erythrocytic stage malaria parasites alone (not shown). The characteristics of these morphologically-abnormal midgut epithelial cells were similar to those observed in *An. stephensi* fed infectious *P. falciparum* 3D7A gametocytes (described in Section 4.3.4.1 above). However, clusters of multiple adjacent morphologically-abnormal midgut epithelial cells were not observed in the histological sections from *An. stephensi* fed uninfected or non-infectious *P. falciparum*-infected blood; only single isolated morphologically-abnormal midgut epithelial cells were observed.

4.3.5 Temporal dynamics of morphologically-abnormal midgut epithelial cells

4.3.5.1 *An. stephensi* fed *P. falciparum* 3D7A gametocytes

Morphologically-abnormal midgut epithelial cells were observed in histological sections from the midguts of *An. stephensi* fed *P. falciparum* 3D7A gametocytes throughout the time period investigated (Table 4.8). For the second infectious experimental feed, for which a broader range of time points was available, the median number of morphologically-abnormal midgut epithelial cells observed within each midgut was highest between 32 and 40 hours pbf and lowest at 44 hours pbf (Table 4.8). The number of morphologically-abnormal midgut epithelial cells did not differ significantly between the time points (Kruskal-Wallis test, $\chi^2_{0.05,\,11.070} = 5.591$, d.f. = 5, $P = 0.348$).

The *degree* of protrusion of morphologically-abnormal midgut epithelial cells from the midgut epithelium also did not appear to be strongly dependent on time pbf, as midgut epithelial cells exhibiting marked protrusion could be observed as early as 24 and 28 hours pbf (e.g. Figure 4.17, Figure 4.19 and Figure 4.39E). However, at later time points (36 hours pbf onwards), most of the morphologically-abnormal midgut epithelial cells observed were entirely separated from the midgut epithelium and/or exhibited marked deterioration (Figure 4.33, and Figure 4.34C and D).

4.3.5.2 *An. stephensi* fed uninfected or non-infectious *P. falciparum* 3D7A-infected blood

Morphologically-abnormal midgut epithelial cells were also observed throughout the time period investigated in histological sections from the midguts of *An. stephensi* fed either uninfected blood or non-infectious *P. falciparum* 3D7A-infected blood containing asexual erythrocytic stage malaria parasites only (Table 4.8). However, the median number of morphologically-abnormal midgut epithelial cells per midgut did not differ appreciably, or significantly, between time points (Kruskal-Wallis test, $\chi^2_{0.05,7.815} = 2.604$, d.f. = 5, $P = 0.457$, and $\chi^2_{0.05,7.815} = 3.057$, d.f. = 5, $P = 0.383$, respectively, for uninfected and infected non-infectious bloodfeeds).

4.3.6 Quantitative relationships between malaria parasites invading the midgut epithelium and the occurrence of morphologically-abnormal midgut epithelial cells

4.3.6.1 *An. stephensi* fed *P. falciparum* 3D7A gametocytes

Overall, 65% (204 of 315) of malaria parasites infecting the midgut epithelium were spatially associated with morphologically-abnormal midgut epithelial cells in the histological sections. Forty per cent (204 of 508) of morphologically-abnormal midgut epithelial cell events were similarly associated with malaria parasites infecting the midgut epithelium. Consequently, an appreciable proportion of both malaria parasites infecting the midgut epithelium (primarily oocysts) and morphologically-abnormal midgut epithelial cells did not exhibit any obvious spatial association with one another.

4.3.6.2 Comparison of *An. stephensi* fed uninfected and *P. falciparum* 3D7A-infected, non-infectious and infectious, blood

The number of morphologically-abnormal midgut epithelial cells differed significantly between *An. stephensi* mosquitoes fed different types of blood (Kruskal-Wallis test, $\chi^2_{0.05,7.815} = 23.17$, d.f. = 3, $P < 0.0001$) (Table 4.8). In subsequent pairwise comparisons, the number of morphologically-abnormal midgut epithelial cells was not significantly different between mosquitoes fed uninfected blood and non-infectious infected blood containing asexual erythrocytic stage malaria parasites alone (Dunn test, $Q_{0.05,2.639} = 0.01$, $P > 0.500$) (Table 4.8). However, the number of morphologically-abnormal midgut epithelial cells differed significantly between mosquitoes given an infectious bloodmeal containing both gametocytes and asexual erythrocytic stage malaria parasites compared to mosquitoes given either uninfected blood or non-

Table 4.8 Comparison of the number of morphologically abnormal midgut epithelial cells observed in histological sections from *An. stephensi* fed uninfected or *P. falciparum* 3D7A-infected blood.

Experimental feed	Time (hours pbf)	*n*[a]	Total number of abnormal midgut epithelial cell events	Median number of abnormal midgut epithelial cell events per midgut
Uninfected:	24	2	8	4.0
no malaria	28	2	4	2.0
parasites	32	2	7	3.5
	36	2	9	4.5
	Overall[b]	8	28	3.5[y]
Infected:	24	2	7	3.5
asexual stages	28	2	10	5.0
only	32	2	10	5.0
	36	2	4	2.0
	Overall	8	31	3.9[y]
1st Infectious:	28	2	92	46.0
gametocytes +	32	3	121	42.0
asexual stages	Overall	5	213	42.0[z]
2nd Infectious:	24	3	53	21.0
gametocytes +	28	2	42	21.0
asexual stages	32	2	58	29.0
	36	2	54	27.0
	(Sub-total	9	207	24.0)[z]
	40	2	63	31.5
	44	2	25	12.5
	Overall	13	295	21.0

[a] The number of midguts examined. [b] Grey boxes highlight summaries of midguts used in statistical analysis; to maintain comparability between experimental feeds, only midguts sampled between 24 and 36 hrs pbf were included. [y, z] indicate significant differences, identified in pairwise comparison tests, between experimental feeds, in the overall number of morphologically abnormal midgut epithelial cell events observed. Different letter indicates significant, while same letter indicates no significant, difference between experimental feeds in the overall number of morphologically abnormal midgut epithelial cell events observed. See main text for details of the statistical analyses (Section 4.2.3).

infectious infected blood containing asexual erythrocytic stage malaria parasites alone (Dunn test, $Q_{0.05,\,2.639} > 2.92$, $P < 0.050$ in all relevant pairwise comparisons) (Table 4.8). (As asexual erythrocytic stage malaria parasites are present in gametocyte cultures, a group of mosquitoes were also fed the former malaria parasite stages alone to demonstrate that the increase in morphologically-abnormal midgut epithelial cells was not a consequence of the presence of these parasite stages within the infectious bloodmeal.) However, the number of morphologically-abnormal midgut epithelial cells was not significantly different between the two experimental feeds in which *An. stephensi* were fed infectious blood containing both gametocytes and asexual erythrocytic stage malaria parasites (Dunn test, $Q_{0.05,\,2.639} = 1.44$, $P > 0.500$) (Table 4.8).

The number of morphologically-abnormal midgut epithelial cells observed within *An. stephensi* fed different types of blood also showed a highly significant positive linear correlation with the overall number of malaria parasites infecting the midgut epithelium, with latter accounting for almost 85% of the observed variation in the former ($F_{1,28} = 144.78$, $P = 1.40 \times 10^{-12}$, $r^2 = 0.838$) (Figure 4.45). A more detail regression analysis, relating numbers of malaria parasites infecting the midgut epithelium to the occurrence of morphologically-abnormal midgut epithelial cells, is presented in Chapter 5.

4. 4 Discussion

Examination of histological sections prepared from the midguts of mosquitoes challenged with infectious gametocytes of the *P. falciparum* 3D7A clone has enabled the stage at which this malaria parasite fails to infect *An. albimanus* to be further clarified. This work has also provided novel observations of ookinete invasion of the midgut epithelium for this malaria parasite species in *An. stephensi*.

4.4.1 *P. falciparum* 3D7A infection in *An. albimanus*

As shown in Chapter 3, mature ookinetes of the *P. falciparum* 3D7A clone form within the bloodmeal of *An. albimanus* but subsequently fail to transform into oocysts in this mosquito species. The work presented in this Chapter further demonstrates that *P. falciparum* 3D7A ookinetes fail to invade the midgut epithelium of *An. albimanus*. Although ookinetes were rarely associated with the endoperitrophic surface of the peritrophic matrix, no malaria parasites were seen within any region of the midgut epithelium in this mosquito species. As a positive control, *An. stephensi* mosquitoes

were simultaneously challenged with the same gametocyte cultures. All stages of malaria parasite migration from the bloodmeal and invasion of the midgut epithelium were observed in *An. stephensi*, demonstrating the infectivity of the gametocytes used to challenge *An. albimanus*. Consequently, the absence of ookinetes within the midgut epithelium of *An. albimanus* results from differences in susceptibility to malaria parasite infection between the two mosquito species examined.

4.4.2 *P. falciparum* 3D7A infection in *An. stephensi*

In contrast to *An. albimanus*, ookinete invasion of the midgut epithelium and oocyst formation were observed in histological sections from *An. stephensi* fed gametocytes of the *P. falciparum* 3D7A clone.

4.4.2.1 The route of ookinete invasion across the midgut epithelium

The observations of ookinete invasion of the mosquito midgut epithelium presented in this Chapter suggests that *P. falciparum* uses *both* intra- and intercellular routes to migrate across the *An. stephensi* midgut epithelium, albeit in different regions of the midgut epithelium. Furthermore, *P. falciparum* ookinetes also appear to cause pathological changes to invaded midgut epithelial cells similar to those previously reported for avian and rodent malaria parasites (Han *et al.*, 2000; Zieler & Dvorak, 2000; Vlachou *et al.*, 2004; Gupta *et al.*, 2005).

Although *P. falciparum* ookinetes have been found in intracellular locations in the midgut epithelium of *Anopheles maculipennis* (Indacochea, 1935), previous electron microscopy studies of *P. falciparum* ookinete invasion of the midgut epithelium of *An. stephensi* observed malaria parasites in three other locations (Meis & Ponnudurai, 1987b; Meis *et al.*, 1989):

(1) extracellular ookinetes within the midgut lumen entering the midgut epithelium between adjacent midgut epithelial cells;
(2) intercellular ookinetes within the basal region of the midgut epithelium located between morphologically-normal midgut epithelial cells;
(3) oocysts on the basal surface of the midgut epithelium located immediately above the lateral plasma membranes of the underlying midgut epithelial cells.

No intracellular ookinetes or morphologically-abnormal midgut epithelial cells were observed in this parasite-vector combination during these earlier studies. Consequently, although ookinetes were *not* observed in intercellular locations within the apical region of the midgut epithelium, the route of ookinete invasion was proposed to be solely

intercellular from the apical to the basal surface of the midgut epithelium (Meis & Ponnudurai, 1987b; Meis *et al.*, 1989).

In the current study, *P. falciparum* ookinetes and oocysts were again observed in similar locations within the *An. stephensi* midgut epithelium, consistent with solely intercellular migration. However, in contrast to the previous electron microscopy studies, ookinetes were also observed within midgut epithelial cells. Furthermore, invading malaria parasites were often associated with midgut epithelial cells exhibiting various morphological abnormalities. These differing observations are unlikely to be due to differences between the malaria parasites or mosquito strains investigated: the *P. falciparum* NF54 isolate, from which the 3D7A clone is derived (Chapter 2, Section 2.2.1.1), and the same strain of *An. stephensi* (Sind-Kasur) were used in the earlier electron microscopy studies (Meis & Ponnudurai, 1987b; Meis *et al.*, 1989). Therefore, the route of ookinete migration across the midgut epithelium would not be expected to differ between the laboratory models used. The most likely explanation is that intracellular ookinetes and morphologically-abnormal midgut epithelial cells were present in the samples examined in the earlier studies, but not observed.

In the earlier electron microscopy studies, Meis *et al.* (1987b; 1989) sought ookinetes between 29 and 36 hours pbf. Subsequently, after observing *P. berghei* ookinetes in intracellular locations within the midgut epithelium of *An. stephensi*, Han *et al.* (2000) suggested that *P. falciparum* ookinetes might initially also enter the midgut epithelium *via* an intracellular route, at approximately 24 hours pbf, but, over the following hours, move to an intercellular location within the basolateral space between adjacent midgut epithelial cells. In general, the observations reported here support this interpretation, as both intra- and intercellular ookinetes were observed within, and between, midgut epithelial cells, respectively. Significantly, morphologically-abnormal midgut epithelial cells were also associated with most intercellular *P. falciparum* ookinetes, providing strong evidence that these malaria parasites had initially entered the midgut epithelium by an intracellular route. However, the apparent absence of intracellular ookinetes in the earlier electron microscopy studies of *P. falciparum* was probably not, as suggested by Han *et al.* (2000), due to the time at which ookinetes invading the midgut epithelium were sought, as intracellular malaria parasites were observed here between 28 and 36 hours pbf, while ookinetes were seen entering the

midgut epithelium as late as 40 hours pbf.[1] Similarly, Meis & Ponnudurai (1987b) also observed ookinete entry into the midgut epithelium at 32 hours pbf. As previously noted (Omar, 1968b), the quantitative data presented here (Figure 4.44) demonstrate that ookinete invasion of the midgut epithelium is an asynchronous process spread over a protracted period of at least 12 hours: some malaria parasites reach the basal surface of the midgut epithelium within 24 hours pbf while others only begin entry into the midgut epithelium after 32 hours pbf. The asynchrony of malaria parasite invasion of the midgut epithelium is presumably related to differences in the rate of ookinete development and/or the location of these malaria parasite stages within the endoperitrophic space, such that ookinetes situated in the bloodmeal periphery reach the midgut epithelium before the ookinetes located in more central regions of the bloodmeal (Omar, 1968b). However, although at the level of individual ookinetes invasion of the midgut epithelium was asynchronous, at the level of the malaria parasite population there was a distinct window during which ookinete invasion of the midgut epithelium occurred (Figure 4.44).

Although oocysts were frequently observed immediately above the lateral plasma membranes of the midgut epithelial cells underlying them – a location consistent with a solely intercellular route of migration across the midgut epithelium – approximately half of these malaria parasite stages were associated with morphologically-abnormal midgut epithelial cells, again providing evidence that entry into the midgut epithelium was initially intracellular. However, the remaining 50 per cent of oocysts were not obviously associated with any morphologically-abnormal midgut epithelial cells. Conceivably, two distinct sub-populations of malaria parasites may exist, which invade the midgut epithelium by different routes: one sub-population initially entering, and then exiting, the midgut epithelium *via* an intra- and then intercellular route, respectively, while another sub-population migrates across the entire midgut epithelium

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[1] The rate of ookinete development within the bloodmeal, and, hence, the timing of ookinete invasion of the midgut epithelium, are temperature-dependent processes, occurring earlier with increasing temperature. Consequently, the differences between the electron microscopy studies of Meis *et al.* and the worked presented here could, conceivably, be due to differences in the temperature at which the infected mosquitoes were maintained. However, in the previous electron microscopy studies, the mosquitoes were kept at 25°C (Meis *et al.*, 1989), one degree lower than mosquitoes examined here. Consequently, if the "time of observation" argument of Han *et al.* were applicable, Meis *et al.* should have been *more*, not less, likely to observe intracellular ookinetes, as ookinete development would have been marginally slower during their investigations compared to the current work.

via a solely intercellular route. However, this interpretation would not explain why over 95% of *ookinetes* were either intracellular and/or associated with morphologically-abnormal midgut epithelial cells. Although a small minority of ookinetes (no more than approximately 4%) may migrate across the midgut epithelium *via* a solely intercellular route, such a route of migration cannot account for the high proportion of *oocysts* not associated with morphologically-abnormal midgut epithelial cells.[2]

An alternative interpretation is that the problematic unexplained oocysts *did* initially migrate across the midgut epithelium *via* an intracellular route but, for some reason, were not (or could not be) associated with morphologically-abnormal midgut epithelial cells in histological sections. Fortunately, a number of more or less plausible reasons, both biological and technical, present themselves.

First, intracellular migration by ookinetes may not always result in morphological changes to invaded midgut epithelial cells (Mota & Rodriguez, 2001). A similar argument is that the rate of induction of the morphological abnormalities in invaded midgut epithelial cells following malaria parasite penetration is variable, such that morphological abnormalities may only become manifest some time after ookinete invasion (e.g. Figure 4.34A). However, although ookinete penetration may sometimes not have any, or, at least, an immediate, effect on the morphology of invaded midgut epithelial cells, these arguments also fail to explain why the proportion of ookinetes is so much greater than the proportion of oocysts associated with morphologically-abnormal midgut epithelial cells.

Second, ookinete invasion might always (or nearly always) result in morphological changes to invaded midgut epithelial cells, but detecting associations with the latter, especially after malaria parasite transformation into oocysts, might not always be possible. Indeed, a sufficient number of morphologically-abnormal midgut

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[2] A solely intercellular route of ookinete migration for a sub-population of malaria parasites could still explain the occurrence of such a high proportion of oocysts not associated with morphologically-abnormal midgut epithelial cells, *if* the numbers of ookinetes invading the midgut epithelium and the numbers of oocysts observed were markedly different, such that ~4% of the ookinetes were numerically equivalent to ~50% of the oocysts in terms of absolute numbers of malaria parasites. However, this is clearly not the case in this instance, as the median numbers of ookinetes and oocysts per midgut are approximately equivalent – see Table 4.5. Furthermore, this explanation would additionally require that ~96% of the ookinetes that were either intracellular and/or associated with morphologically-abnormal midgut epithelial cells should miraculously disappear and fail to transform into oocysts, which is again unlikely in this instance – see Table 4.7.

epithelial cell events, apparently unassociated with invading malaria parasites and otherwise unexplained, were observed in the histological sections for this interpretation to be possible. Malaria parasites infecting the midgut epithelium and their associated morphologically-abnormal midgut epithelial cells were often present in different, and sometimes distant, sections (e.g. Figure 4.27 and Figure 4.29C). Han *et al.* (2000) also reported that a minority of malaria parasites on the basal surface of the midgut epithelium were located several cell diameters from morphologically-abnormal midgut epithelial cells suggesting movement of ookinetes away from their initial site of intracellular entry into the midgut epithelium. Consequently, the increasing physical distance between malaria parasites on the basal surface of the midgut epithelium and invaded midgut epithelial cells as the latter protrude into the midgut lumen can make it difficult to discern spatial associations in histological sections, especially when the sections are taken through the longitudinal plane of the midgut epithelium (rather than orthogonally to the latter). This problem is particularly acute for oocysts where morphologically-abnormal midgut epithelial cells are frequently nearly, or completely, separated from the midgut epithelium. Indeed, in some instances, extruded midgut epithelial cells completely separated from the midgut epithelium appeared to have "drifted" marked distances into the endoperitrophic space of the midgut lumen (Figure 4.24, Figure 4.34D and Figure 4.39H).[3] However, whether these midgut epithelial cells were actually causally related to malaria parasites invading the midgut epithelium was impossible to determine from Giemsa-stained histological sections (e.g. Figure 4.24).

Third, morphologically-abnormal midgut epithelial cells protruding from the midgut epithelium into the midgut lumen appear to undergo progressive deterioration. Although the number of morphologically-abnormal midgut epithelial cells did not change significantly over time (probably due to small sample sizes and the low power of the non-parametric statistical test employed), the apparent morphological deterioration of these midgut epithelial cells suggests that there is a real decline in their numbers at later time points. Consequently, the morphologically-abnormal midgut epithelial cells generated during ookinete invasion may have completely disintegrated, or been voided into the hindgut along with the digested bloodmeal, preventing their association with

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[3] A sentence of caution: The apparent "drifting" within the midgut lumen of midgut epithelial cells completely separated from the midgut epithelium might be an artefact of manipulation of the midgut during mosquito dissection, and subsequent fixation, causing movement of the bloodmeal within the midgut lumen.

malaria parasites invading the midgut epithelium. Similarly, some morphologically-abnormal midgut epithelial cells associated with invading parasites, especially the apparently "lysing" abnormal midgut epithelial cells (e.g. Figure 4.25 and Figure 4.27), were either difficult to discern and/or seemingly ephemeral relatively to other types of morphologically abnormal (i.e. "turgid") midgut epithelial cells.

4.4.2.2 Effect of ookinete invasion on midgut epithelial cells

The morphological abnormalities observed in midgut epithelial cells associated with invading *P. falciparum* 3D7A ookinetes were indicative of significant pathology and similar to changes in ookinete-invaded midgut epithelial cells described in previous studies using rodent and avian malaria parasites (Han *et al.*, 2000; Zieler & Dvorak, 2000). These morphological abnormalities included translocation of the nucleus from a central to an apical position within the midgut epithelial cell, and loss of microvilli from the apical surface of the midgut epithelial cell. Furthermore, in the work presented here, some of these midgut epithelial cells were within the midgut lumen, completely separated from the midgut epithelium. Some previous studies did not directly observe ookinete-invaded midgut epithelial cells completely extruded from the midgut epithelium (Han *et al.*, 2000; Shahabuddin, 2002). Han *et al.* (2000) used an *ex vivo* technique whereby infected midguts were cut open and washed before examination to remove the luminal bloodmeal contents. This procedure presumably removed extruded midgut epithelial cells free within the midgut lumen and completely separated from the midgut epithelium. Consequently, Han *et al.* (2000) were unable to formally demonstrate that ookinete-invaded midgut epithelial cells protruding from the midgut epithelium were eventually expelled into the midgut epithelium. The observations made here with *P. falciparum* in *An. stephensi* provide direct evidence supporting the hypothesis that ookinete-invaded midgut epithelial cells are extruded into the midgut lumen. Maier and colleagues (Becker-Feldman *et al.*, 1985; Maier *et al.*, 1987) also reported complete extrusion of ookinete-invaded midgut epithelial cells into the midgut lumen. However, the studies describing these observations have apparently not been published in detail and some researchers (e.g. Han *et al.*, 2000; Shahabuddin, 2002) appear to be unaware of the work by Maier and colleagues.

Morphologically-abnormal midgut epithelial cells were observed in histological sections from the midguts of both *An. albimanus* and *An. stephensi* fed uninfected and *P. falciparum*-infected blood. However, there were significantly more morphologically-abnormal midgut epithelial cells observed in *An. stephensi* fed infectious gametocytes.

This observation, taken with the correlation of these midgut epithelial cells with the number of malaria parasites infecting the midgut epithelium, strongly suggests that the morphological abnormalities observed in many midgut epithelial cells are a consequence (rather than a cause) of ookinete invasion of the midgut epithelium. Furthermore, this quantitative evidence is consistent with the detailed observations of ookinete invasion of the midgut epithelium made from the histological sections. These suggest that the midgut epithelium is morphologically normal prior to ookinete invasion and subsequently undergoes morphological changes following malaria parasite infection.

The overall number of malaria parasites infecting the midgut epithelium explained nearly 85% of the observed variation in the number of morphologically-abnormal midgut epithelial cells. However, an appreciable proportion of the morphologically-abnormal midgut epithelial cells observed in *An. stephensi* fed infectious gametocytes (60%; see Section 4.3.6.1) did not have any obvious *spatial* association with malaria parasites invading the midgut epithelium. As discussed in Section 4.4.2.1 above, both biological and technical factors might prevent morphologically-abnormal midgut epithelial cells generated by ookinete invasion being associated with their respective malaria parasites in sectioned material. Even if all malaria parasites observed invading the midgut epithelium are assumed to enter the midgut epithelium *via* an intracellular route and to always cause morphological abnormalities to invaded midgut epithelial cells, 38% of observed morphologically-abnormal midgut epithelial cells are still unaccounted. The presence of morphologically-abnormal midgut epithelial cells in *An. stephensi* fed uninfected or non-infectious *P. falciparum*-infected blood implies that some of the morphologically-abnormal midgut epithelial cells observed in *An. stephensi* fed infectious gametocytes are causally unrelated to malaria parasites invading the midgut epithelium. Extrapolating from *An. stephensi* fed uninfected or non-infectious *P. falciparum*-infected blood, a further 13% of morphologically-abnormal midgut epithelial cells in *An. stephensi* fed infectious gametocytes can be regarded as background, malaria parasite-independent events, leaving approximately 25% of the observed morphologically-abnormal midgut epithelial cells unexplained. If these unexplained morphologically-abnormal midgut epithelial cells are assumed to have been invaded by ookinetes, they would account for the approximately one third (28.6%) of the total number of morphologically-abnormal midgut epithelial cells presumed to have been invaded by malaria parasites. Interestingly, a similar proportion of malaria

parasites are lost during ookinete invasion of the midgut epithelium (35.3%) (Table 4.7) suggesting that these lost ookinetes may have been destroyed during infection of the otherwise unexplained morphologically-abnormal midgut epithelial cells.

Several technical limitations inherent with non-specific staining of sectioned material could account for the remaining unexplained morphologically-abnormal midgut epithelial cells observed in *An. stephensi* fed infectious gametocytes.

First, Han *et al.* (2000) found morphologically-abnormal midgut epithelial cells in mosquitoes fed infectious *P. berghei* gametocytes that were not directly associated with ookinetes invading the midgut epithelium. A stage-specific malaria parasite surface molecule released during ookinete gliding motility and invasion of midgut epithelial cells was detected in these morphologically-abnormal midgut cells suggesting intracellular lysis of the invading ookinetes (Han *et al.*, 2000). Some of the unexplained morphologically-abnormal midgut epithelial cells present in *An. stephensi* fed infectious *P. falciparum* gametocytes might therefore have been caused by invading ookinetes which were subsequently destroyed by mosquito lytic immune defence mechanisms (Vernick *et al.*, 1995; Blandin *et al.*, 2004). Although some intracellular *P. falciparum* ookinetes did exhibit dubious morphology (Figure 4.32 and Figure 4.33), there was little morphological evidence of ookinete destruction within the midgut epithelium. However, the quantitative data provided evidence that an appreciable proportion of ookinetes were lost between penetration of the peritrophic matrix and the formation of oocysts on the basal surface of the midgut epithelium (Table 4.6 and Table 4.7).

Second, when examining Giemsa-stained histological sections, the number of malaria parasites invading the midgut epithelium is likely to be underestimated relative to the number of morphologically-abnormal midgut epithelial cells because the smaller size and similar coloration of malaria parasites to midgut epithelial cells makes them harder to detect in non-specifically-stained sectioned material. Furthermore, some histological sections were inevitably lost or damaged or otherwise rendered unreadable during processing of the midgut samples. Consequently, as malaria parasites are typically only present in one or two sections, depending on the plane of sectioning, and midgut epithelial cells can persist through multiple (ten or more) sections, counts of the malaria parasites are likely to be disproportionately affected by sporadic loss of histological sections during sample preparation. Unfortunately, no record was kept of when histological sections were lost, so the importance of this phenomena cannot be estimated.

4.4.3 Significance of morphologically-abnormal midgut epithelial cells

As already noted, morphologically-abnormal midgut epithelial cells were also observed rarely in midguts from *An. stephensi* fed uninfected blood, as well as in *An. albimanus* fed infectious gametocyte stage malaria parasites. Consequently, the existence of these morphologically-abnormal midgut epithelial cells either cannot, or is unlikely to, be explained by ookinete invasion of the midgut epithelium. Han *et al.* (2000) did not previously observe protruding midgut epithelial cells in uninfected *An. stephensi*. The *ex vivo* technique used by Han and colleagues may partly explain their failure to observe morphologically-abnormal midgut epithelial cells in uninfected mosquitoes. However, midgut epithelial cells extruded from the midgut epithelium into the midgut lumen have been reported in uninfected and virus-infected midguts from several aedine and culicine mosquito species (Houk *et al.*, 1985; Weaver *et al.*, 1988; Weaver & Scott, 1990a; Weaver, Lorenz & Scott, 1992; Okuda *et al.*, 2002). Therefore, extrusion of midgut epithelial cells from the midgut epithelium into the midgut lumen may be a general mechanism of tissue repair, rather than an antimalarial parasite-specific response, that is utilised in the mosquito midgut in response to cell damage induced by senescence, stress or infection (Okuda *et al.*, 2002). Consequently, it should not be assumed that morphological and/or molecular changes occurring in midgut epithelial cells after ookinete invasion are a specific response to malaria parasites.

Three distinct types of morphologically-abnormal midgut epithelial cell were observed during the examination of histological sections: turgid, flaccid and dissolving. These different abnormal midgut epithelial cell morphologies may represent distinct modes of cell death, different stages of a common process, or may simply reflect differences in the initial physiological state of the midgut epithelial cells. Whether ookinete-invaded midgut epithelial cells undergo necrotic or programmed cell death, or both, in response to ookinete invasion is not clear (Han & Barillas-Mury, 2002; Shahabuddin, 2002). Zieler and Dvorak (2000) reported that occasionally some midgut epithelial cells appeared to explode rapidly upon ookinete invasion, releasing their cytoplasmic contents. Such "exploding" midgut epithelial cells might be equivalent to the flaccid and/or dissolving midgut epithelial cells observed in *An. stephensi* infected with *P. falciparum* 3D7A. In contrast, Han *et al.*, (2000) did not observe exploding midgut epithelial cells. Again, the *ex vivo* technique used by these researchers would presumably make detection of rapidly lysing midgut epithelial cells, if such were present, difficult. The majority of ookinete-invaded midgut epithelial cells have been

reported to exhibit molecular and morphological changes indicative of apoptosis (Han *et al.*, 2000; Zieler & Dvorak, 2000; Kumar *et al.*, 2004; Danielli *et al.*, 2005). The condensation and fragmentation of the nuclei of turgid midgut epithelial cells observed in the histological sections examined here are also consistent with programmed cell death.

4. 5 Summary

The migration of ookinetes of the *P. falciparum* 3D7A clone from the bloodmeal across the midgut epithelium was compared in *An. albimanus* and *An. stephensi* using light-microscopical examination of Giemsa-stained histological sections prepared from the midguts of mosquitoes that had ingested a bloodmeal containing infectious gametocytes.

In *An. albimanus*, ookinetes were seen within the central undigested region, and, occasionally, within the peripheral digested region, of the bloodmeal. However, ookinetes were only very rarely associated with the peritrophic matrix and were never observed within the ectoperitrophic space or the midgut epithelium. The absence of *P. falciparum* 3D7A oocyst infection in *An. albimanus*, therefore, apparently results from the failure of the ookinetes of this malaria parasite clone to egress from the bloodmeal and enter the midgut epithelium.

In *An. stephensi*, ookinetes were frequently observed in all stages of migration from the bloodmeal to the basal surface of the midgut epithelium. In contrast to earlier studies, ookinetes were found within midgut epithelial cells, demonstrating intracellular migration across the midgut epithelium, as well as within the intercellular locations previously reported. Ookinetes entered the midgut epithelium at sites where three adjacent midgut epithelial cells converged apparently passing into morphologically-normal midgut epithelial cells possessing centrally-located nuclei, dense microvilli and cytoplasmic staining indistinguishable from that of uninvaded midgut epithelial cells. Ookinete invasion apparently resulted in significant morphological changes to invaded midgut epithelial cells, including differential staining, condensation and fragmentation of the nucleus, various degrees of protrusion into the midgut lumen, loss of microvilli, and, sometimes, vacuolisation. Midgut epithelial cells that were completely separated from the midgut epithelium were also found within the midgut lumen, and were frequently associated with invading malaria parasites, implying that ookinete invasion resulted in complete ejection of midgut epithelial cells from the midgut epithelium.

Small clusters of morphologically-abnormal midgut epithelial cells, and invading ookinetes spanning the membranes of adjacent morphologically-abnormal midgut epithelial cells, were also observed consistent with intracellular movement of ookinetes between neighbouring midgut epithelial cells. Ookinetes and oocysts in the central and basal regions of the midgut epithelium were often situated between, or above, the lateral plasma membranes of morphologically-normal midgut epithelial cells consistent with intercellular migration in these regions of the midgut epithelium. However, many of these latter malaria parasites, especially ookinetes, were associated with morphologically-abnormal midgut epithelial cells.

Figure 4.1 Diagrammatic representation of the structure of the mosquito midgut wall and midgut lumen.

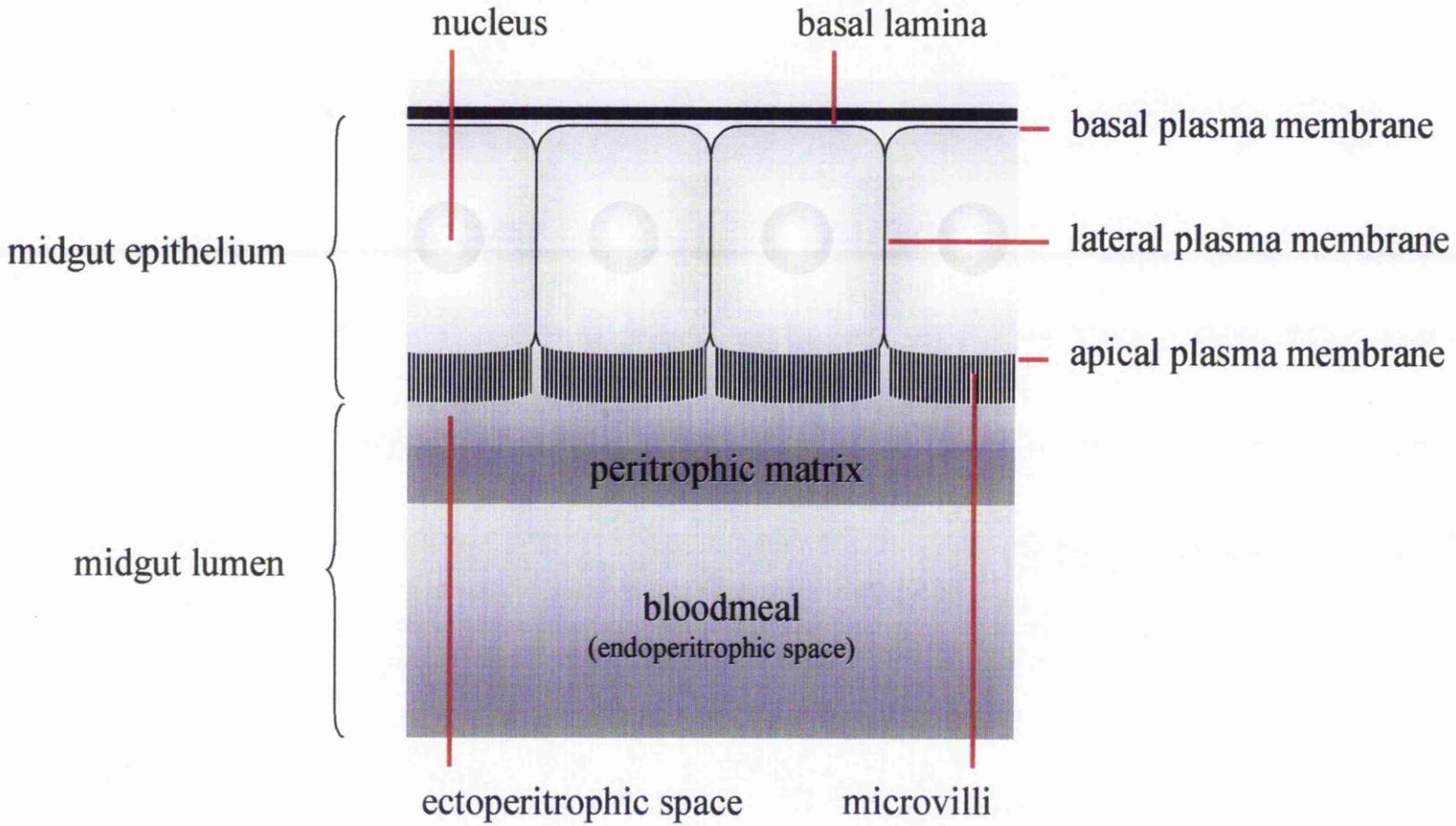


Figure 4.2 The midgut epithelium of *An. stephensi*.

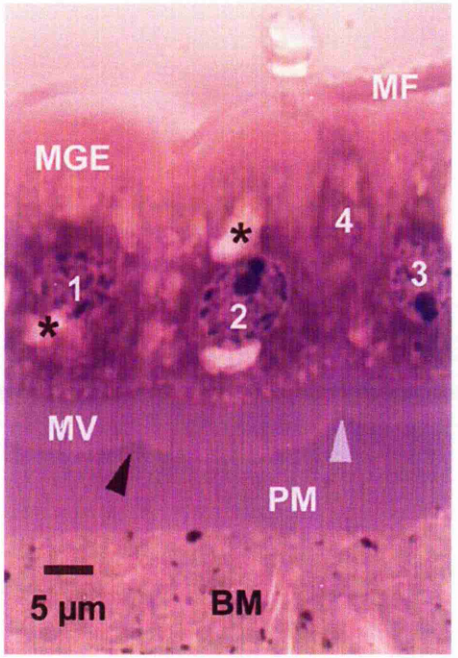


Image of a section taken orthogonally through the longitudinal plane of the midgut epithelium illustrating the major structural features of the midgut wall of a mature blood-fed adult female mosquito. The peripheral digested region of the bloodmeal (BM) is apparent at the bottom of the image shown. Surrounding the bloodmeal is the peritrophic matrix (PM), which varies in thickness from approximately 2 to 10 µm, and lies immediately beneath the midgut epithelium (MGE). The overlying midgut epithelium is primarily composed of a monolayer of polarised midgut epithelial cells. Four morphologically normal cuboidal midgut epithelial cells (labelled 1, 2, 3 and 4) are present in the section shown. Midgut epithelial cell 4 is only partially apparent and does not extend to the apical surface of the midgut epithelium in this section. The midgut epithelial cells possess large centrally-located nuclei (labels 1, 2, and 3 are located within the nuclei of their respective midgut epithelial cells). The apical plasma membranes of the midgut epithelial cells are folded into dense microvilli (MV) to form the apical brush border surface of the midgut epithelium. Regular "kinks" are apparent within the microvillar brush border of the midgut epithelium (white arrowhead) where the lateral plasma membranes of adjacent midgut epithelial cells meet. Between the microvillar brush border of the midgut epithelium and the underlying peritrophic matrix is the ectoperitrophic space (indicated by dark arrowhead), which is of variable size. In the section shown, the ectoperitrophic space is small such that the midgut epithelium and peritrophic matrix are closely apposed to one another. The cytoplasm of the midgut epithelial cells exhibits granular staining and frequently contains one or several relatively large unstained regions of irregular shape and size (asterisks). Muscle fibres (MF), and occasionally tracheoles (not present in the section shown), can be seen over the outer basal surface of the midgut epithelium. 28 hrs pbf.

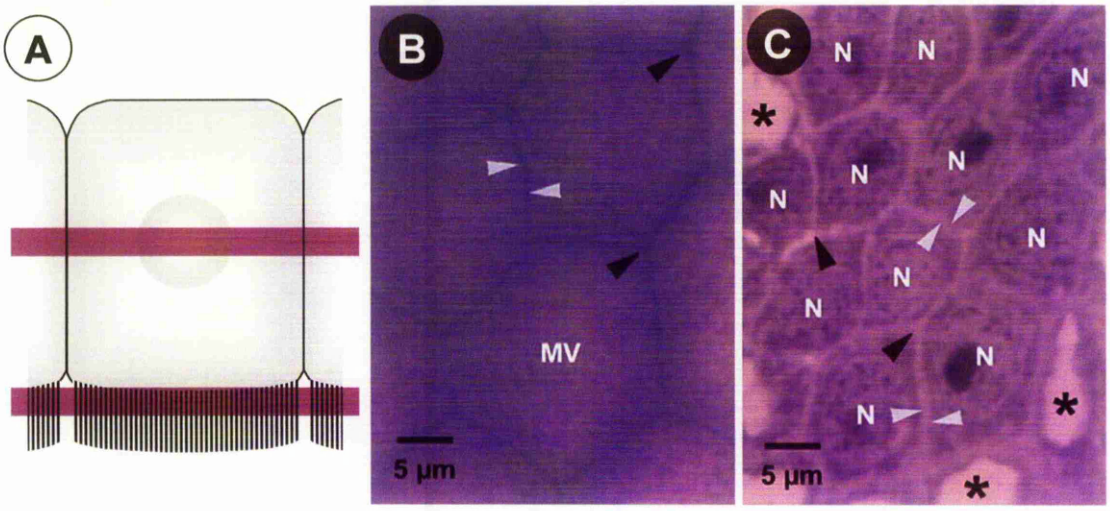


Figure 4.3 "Honeycomb" arrangement of the midgut epithelial cells within the mosquito midgut epithelium.

(A) Diagram illustrating the relative location of the sections shown in **B** and **C**. A section taken perpendicularly through the longitudinal plane of the midgut epithelium is illustrated. Purple lines indicate orthogonal planes through which the sections shown in **B** (lower line) and **C** (upper line) are taken.

(B) Section through the longitudinal plane of the microvillar brush border of the apical surface of the midgut epithelium, looking down into the midgut lumen. White arrowheads indicate partings within the microvilli (MV), immediately beneath the lateral membranes of the overlying midgut epithelial cells, between which is the ectoperitrophic space. Note "honeycomb" arrangement of the partings within the microvillar brush border and their convergence to create "three-cell" junctions (dark arrowheads). 28 hrs pbf.

(C) Section through the centre of the longitudinal plane of the midgut epithelium, looking down into the midgut lumen, showing the honeycomb arrangement of the midgut epithelial cells. White arrowheads indicate the lateral plasma membranes of the midgut epithelial cells and dark arrowheads indicate the "three-cell" junctions where adjacent midgut epithelial cells converge. The nuclei (N) and irregular unstained regions (asterisks) within the cytoplasm of the midgut epithelial cells are also shown. 32 hrs pbf.

Note the difference in the diameter of the midgut epithelial cells shown in **B** and **C**: the midgut epithelial cells in **B** are cuboidal while those in **C** have started to resume the columnar (and, hence, narrower) form of the pre-bloodfed midgut epithelium.

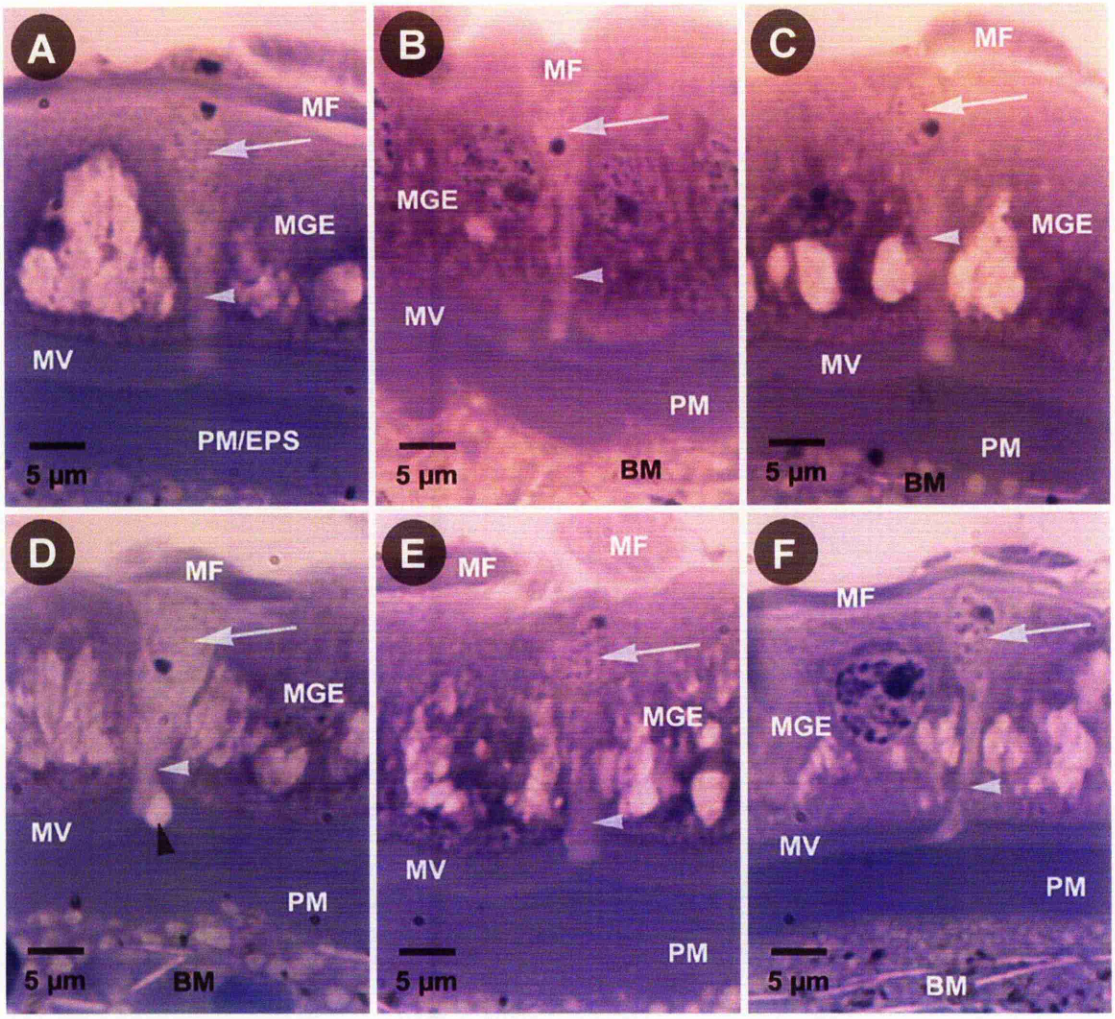


Figure 4.4 "Open" endocrine cells within the midgut epithelium of *An. stephensi*.

(A to F) Different examples of the lightly staining triangular cells observed within the midgut epithelium (MGE). Note the basally located nuclei (white arrows) of the lightly staining cells, which contain a single prominent darkly staining nucleolus and the long narrow apical extensions (white arrowheads), which pass through the microvillar brush border (MV) of the surrounding midgut epithelial cells. "Blebbing" of the anterior-most region of the apical extensions (dark arrowhead in **D**) was sometimes observed. Note also the muscle fibres (MF) lying over the outer basal surface of the midgut epithelium, which are located immediately above the lightly staining triangular cells. All images are taken from the midguts of mosquitoes given an infectious bloodmeal containing cultured *P. falciparum* 3D7A clone gametocytes. Similar cells were observed in the midguts of mosquitoes given uninfected or non-infectious *P. falciparum*-infected bloodmeals (images not shown). All images 28 hours pbf.

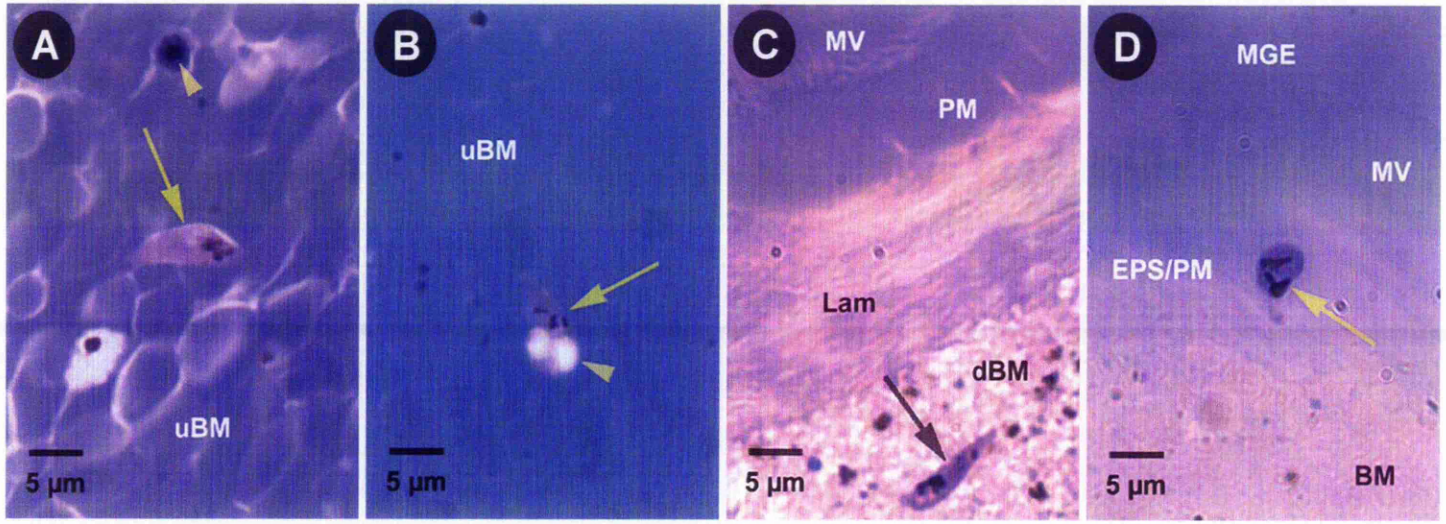


Figure 4.5 ***P. falciparum*** **3D7A ookinetes within the bloodmeal and peritrophic matrix of** ***An. albimanus*****.**

(A) Morphologically normal ookinete (yellow arrow) located within the central undigested region of the bloodmeal (uBM). An asexual intra-erythrocytic stage malaria parasite (yellow arrowhead) is also present in the section shown. 32 hrs pbf.

(B) Morphologically abnormal ookinete located within the central undigested region of the bloodmeal. The posterior region of the ookinete appears to be vacuolated (yellow arrowhead). 32 hrs pbf. **(C)** Ookinete located within the peripheral digested region of the bloodmeal (dBM). 28 hrs pbf. **(D)** "Tear-drop" shaped ookinete located within the peritrophic matrix (PM). 24 hrs pbf.

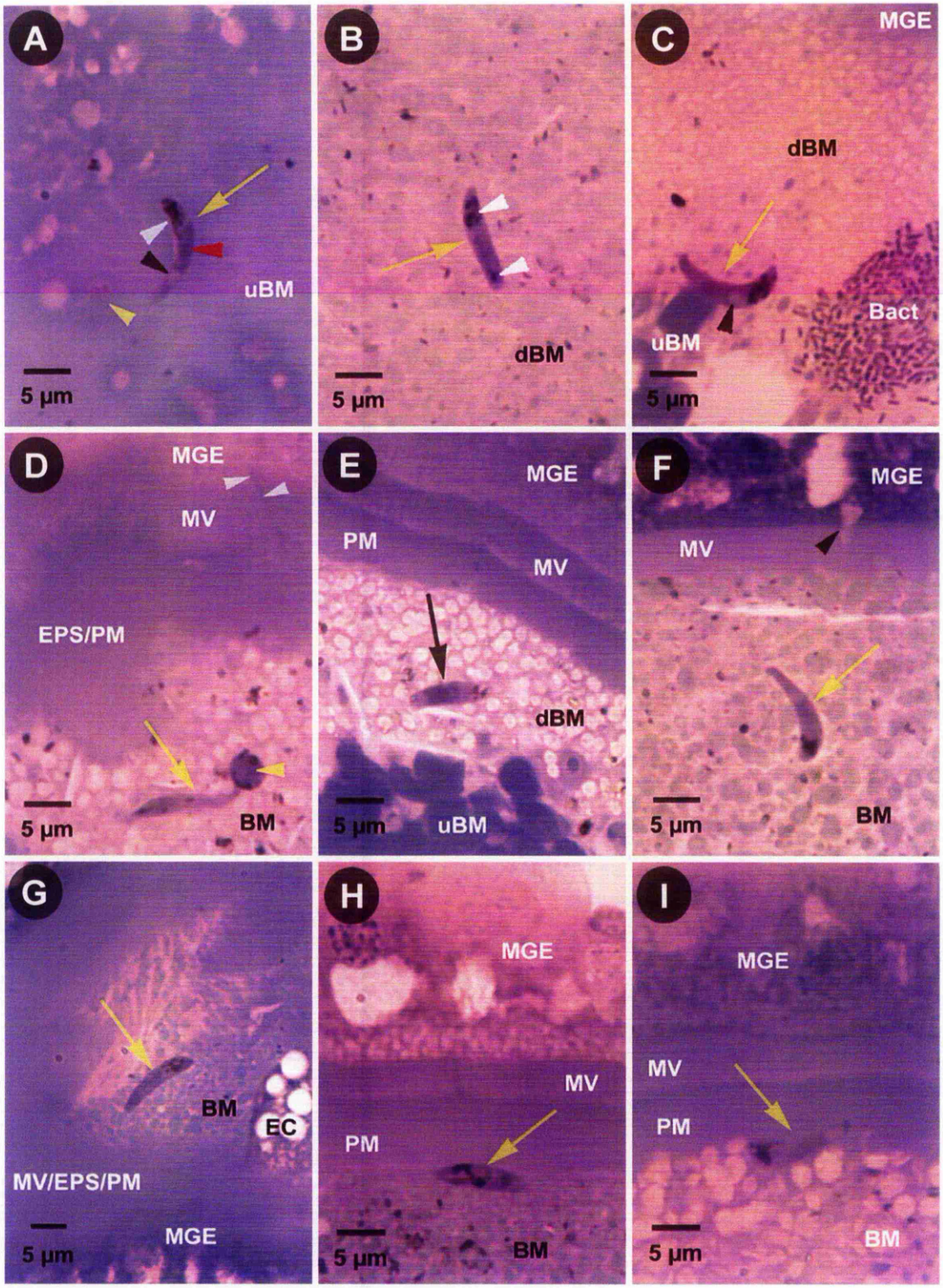


Figure 4.6 ***P. falciparum*** **3D7A ookinetes within the bloodmeal of** ***An. stephensi.***

Legend to images on following page.

Figure 4.6 *P. falciparum* 3D7A ookinetes within the bloodmeal of *An. stephensi*.

Legend to images on previous page.

(A) Ookinete (yellow arrow) within the central undigested region of the bloodmeal (uBM) embedded within the dense mass of intact and compacted erythrocytes that fills the midgut lumen after bloodfeeding. Individual erythrocytes cannot be easily distinguished. The nucleus (red arrowhead), a characteristic cluster of haemozoin granules (white arrowhead), and a crystalloid body (dark arrowhead) can be seen within the ookinete. Yellow arrowhead indicates an asexual intra-erythrocytic stage malaria parasite (probably a trophozoite) also present within the bloodmeal. 28 hrs pbf.
(B) Intact and morphologically normal ookinete located within completely digested bloodmeal (dBM). One large and one small cluster of haemozoin granules are present within each end of the ookinete (white arrowheads). Note the absence of any asexual intra-erythrocytic stage parasites and the presence of scattered haemozoin granules throughout the bloodmeal. 40 hrs pbf.
(C) Ookinete located in the interface between the central undigested and peripheral digested regions of the bloodmeal. A small cluster of bacilli (Bact) are also present within the peripheral digested region of the bloodmeal next to the ookinete. 24 hrs pbf.
(D) A retort-form ookinete located in the peripheral digested region of the bloodmeal. Yellow arrowhead indicates the spherical basal body of the original zygote and the yellow arrow the emerging ookinete. 28 hrs pbf.
(E to G) Different ookinetes (dark arrow in **E** and yellow arrows in **F** and **G**) located within the peripheral digested region of the bloodmeal. The peritrophic matrix (PM) is apparently absent in the section shown in **F**. The apical extension of an endocrine cell (dark arrowhead) can be seen emerging through the microvillar brush border (**F**). A vacuolated midgut epithelial cell (EC), protruding from the midgut epithelium into the midgut lumen, is visible in **G**. Note lower magnification of **G**. **E to G** are 32 hrs pbf.
(H) Ookinete within the peripheral digested region of the bloodmeal located immediately adjacent, and parallel, to the inner (endoperitrophic) surface of the peritrophic matrix. 32 hrs pbf.
(I) Ookinete in the initial stages of penetrating the inner surface of the peritrophic matrix. 24 hrs pbf.

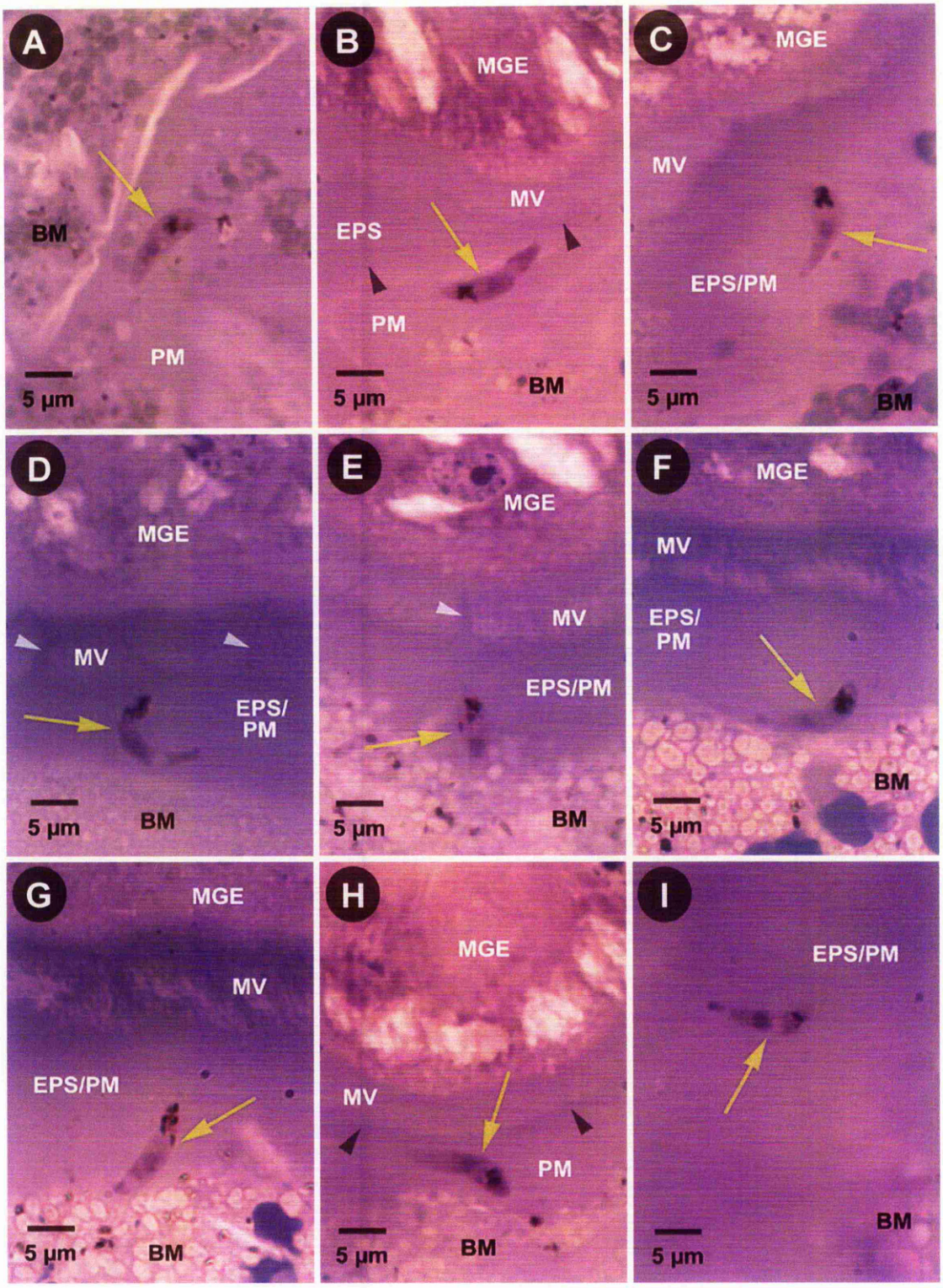


Figure 4.7 ***P. falciparum*** **3D7A ookinetes penetrating the peritrophic matrix in the midgut lumen of** ***An. stephensi.***

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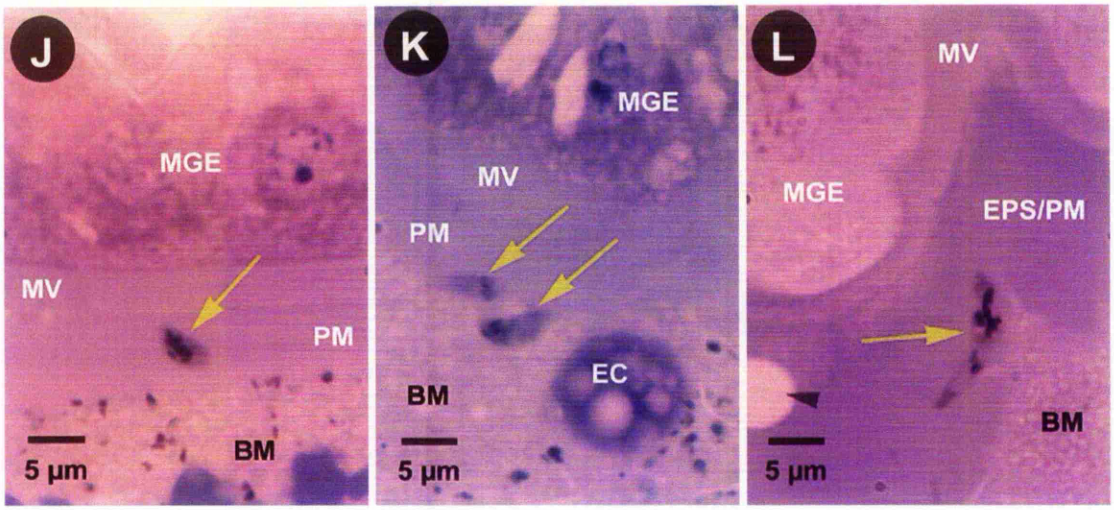


Figure 4.7 ***P. falciparum*** **3D7A ookinetes penetrating the peritrophic matrix in the midgut lumen of** ***An. stephensi.***

Legend to images on this and previous page.

(A to L) Examples of ookinetes (yellow arrows) associated with the peritrophic matrix (PM). Dark arrowheads indicate the ectoperitrophic space (EPS), where it is distinct from the peritrophic matrix. However, in most of the images shown, the ectoperitrophic space and the peritrophic matrix cannot be clearly distinguished from one another (labelled EPS/PM). White arrowheads indicate partings within the microvillar brush border (MV) of the apical surface of the midgut epithelium (MGE). **(A)** Section taken through the longitudinal plane of the peritrophic matrix, close to the inner (endoperitrophic surface) of the latter, showing an ookinete having just entered the peritrophic matrix. Partially digested erythrocytes within the bloodmeal (BM) are apparent through the peritrophic matrix. 28 hrs pbf. **(B and C)** Oblique sections through the planes of the peritrophic matrix and midgut epithelium. Again, partially digested erythrocytes within the bloodmeal (BM) are visible through the peritrophic matrix. **B** and **C** 28 and 32 hrs pbf, respectively. **(D to H)** Sections orthogonal to the longitudinal plane of the peritrophic matrix and midgut epithelium are shown. **D** 24 hrs pbf. **E to H** 28 hrs pbf. **(I)** Section taken through the longitudinal plane of the ectoperitrophic space/peritrophic matrix. 28 hrs pbf. **(J and K)** Sections orthogonal to the longitudinal plane of the peritrophic matrix and midgut epithelium are shown. Two ookinetes located closely to one another are present in **K**. A midgut epithelial cell (EC) separated from the midgut epithelium is also present. **(L)** Oblique section through the planes of the peritrophic matrix and midgut epithelium. **J to L** are 28 hrs pbf.

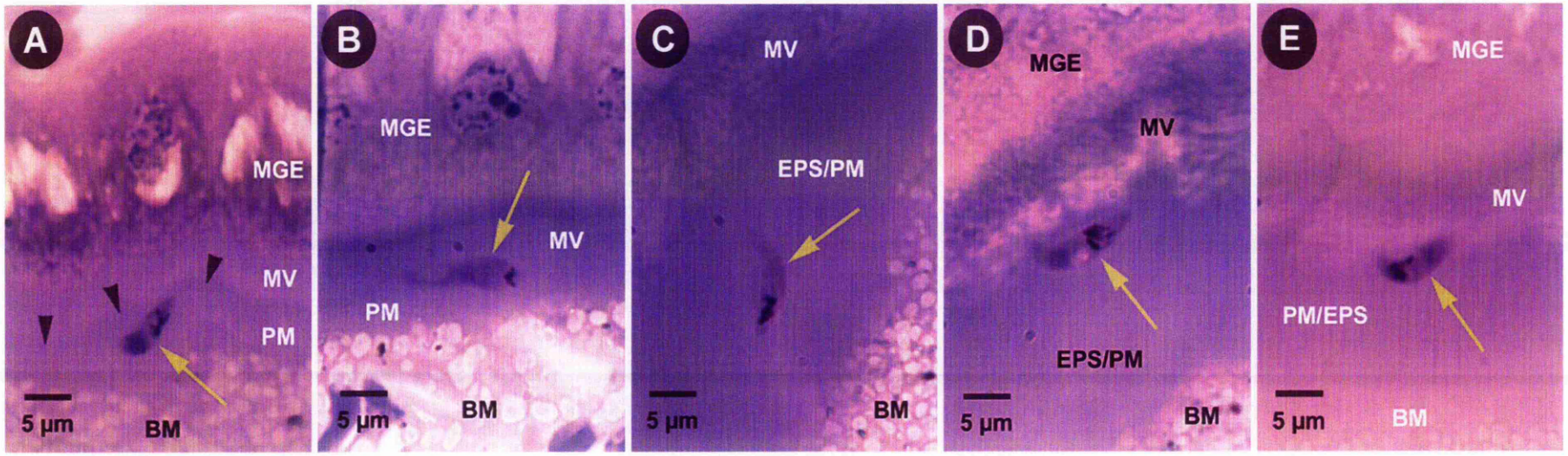


Figure 4.8 ***P. falciparum*** **3D7A ookinetes within the ectoperitrophic space of the midgut lumen of** ***An. stephensi.***

(A) Ookinete (yellow arrow) on the outer (ectoperitrophic) surface of the peritrophic matrix (PM). Dark arrowheads indicate the ectoperitrophic space (EPS) of the midgut lumen. 28 hrs pbf.

(B) Ookinete within the ectoperitrophic space sandwiched between the underlying peritrophic matrix and overlying microvillar brush border (MV) of the apical surface of the midgut epithelium (MGE). 28 hrs pbf.

(C) Ookinete located within the ectoperitrophic space/peritrophic matrix. 32 hrs pbf.

(D and E) Different ookinetes within the ectoperitrophic space of the midgut lumen immediately adjacent to the microvillar brush border of the apical surface of the midgut epithelium. Note the apparent "kink" in the left-hand side of the malaria parasite shown in **D**. **D** and **E** are 32 and 24 hrs pbf, respectively.

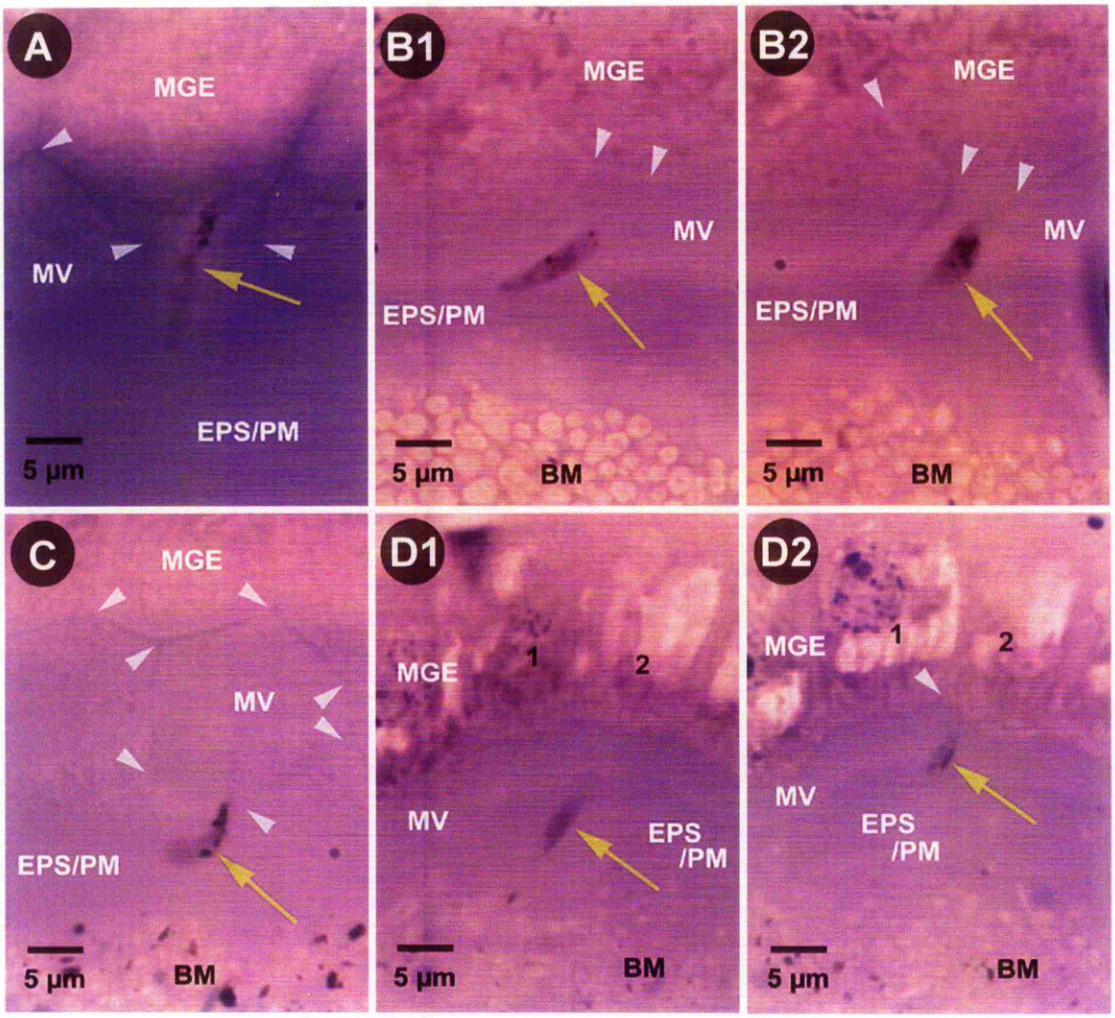


Figure 4.9 ***P. falciparum*** **3D7A ookinetes within the microvillar brush border of the apical surface of the midgut epithelium of** ***An. stephensi.***

(A to C) Oblique sections through the plane of the midgut epithelium (MGE) showing different ookinetes (yellow arrows) entering the microvillar brush border (MV) of the midgut epithelium. Partings within the microvillar brush border, present beneath the lateral membranes of the overlying midgut epithelial cells, are indicated by white arrowheads, which are specifically located at sites where three adjacent midgut epithelial cells converge. **(B1/B2)** Consecutive serial sections showing the same ookinete entering the microvillar brush border. **A** and **C** 28 and **B1/B2** 24 hrs pbf, respectively.

(D1/D2) Consecutive serial sections showing a single ookinete entering the microvillar brush border of the midgut epithelium close to a site where the lateral membranes of at least two adjacent midgut epithelial cells (labelled 1 and 2) meet (white arrowhead). 28 hrs pbf.

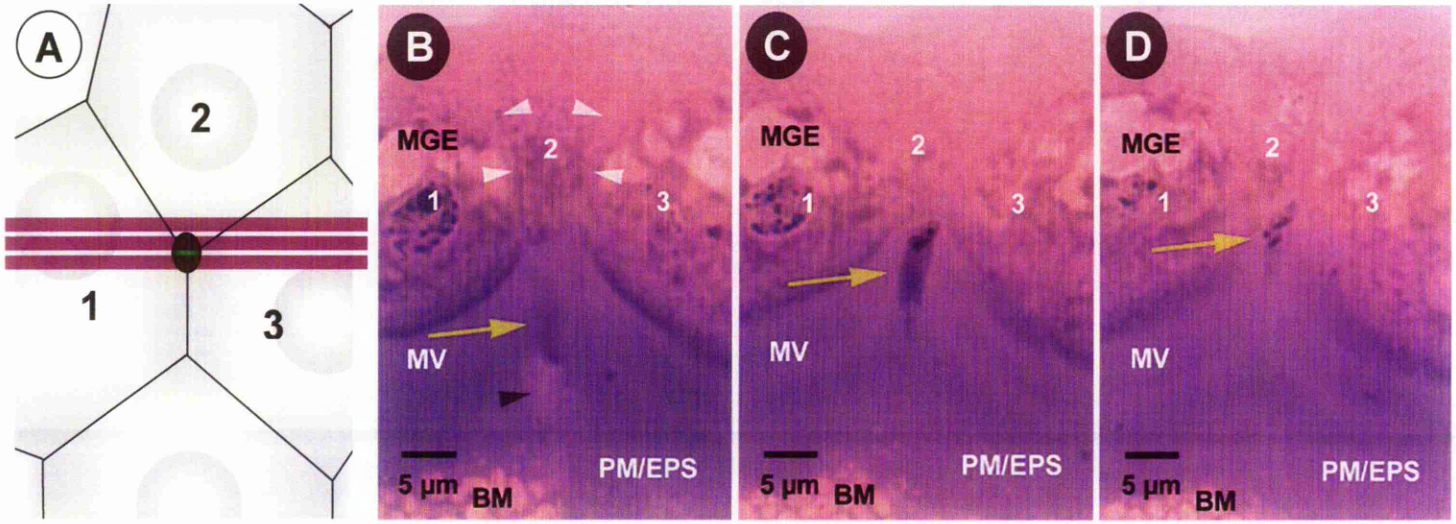


Figure 4.10 ***P. falciparum*** **3D7A ookinete entering the midgut epithelium of** ***An. stephensi.***

(A) Diagram illustrating the relative location of the sections shown in **B to D**. A section taken through the middle of the longitudinal plane of the midgut epithelium, which would be observed if looking down onto the midgut epithelium from above, is illustrated. Purple lines indicate orthogonal planes through which the sections are taken (top to bottom represent **B to D** respectively). The ookinete is shown in green. **(B to D)** Consecutive serial sections showing a single ookinete (yellow arrows) entering the midgut epithelium (MGE) where three adjacent midgut epithelial cells (labelled 1, 2 and 3) converge. Examination of these and other sections demonstrated that all three midgut epithelial cells were morphologically normal possessing centrally-located nuclei and dense microvilli (MV). The ookinete appears to be entering midgut epithelial cell 2 or 3 through the apicolateral plasma membrane. White arrowheads indicate lateral plasma membranes of the adjacent midgut epithelial cells **(B)**. Note localised invagination, at the site of ookinete entry, of the midgut epithelium. Disruption of the peritrophic matrix (PM) and bloodmeal (BM) material within the ectoperitrophic space (EPS)(dark arrowhead in **B**) are apparent beneath the ookinete. 24 hrs pbf.

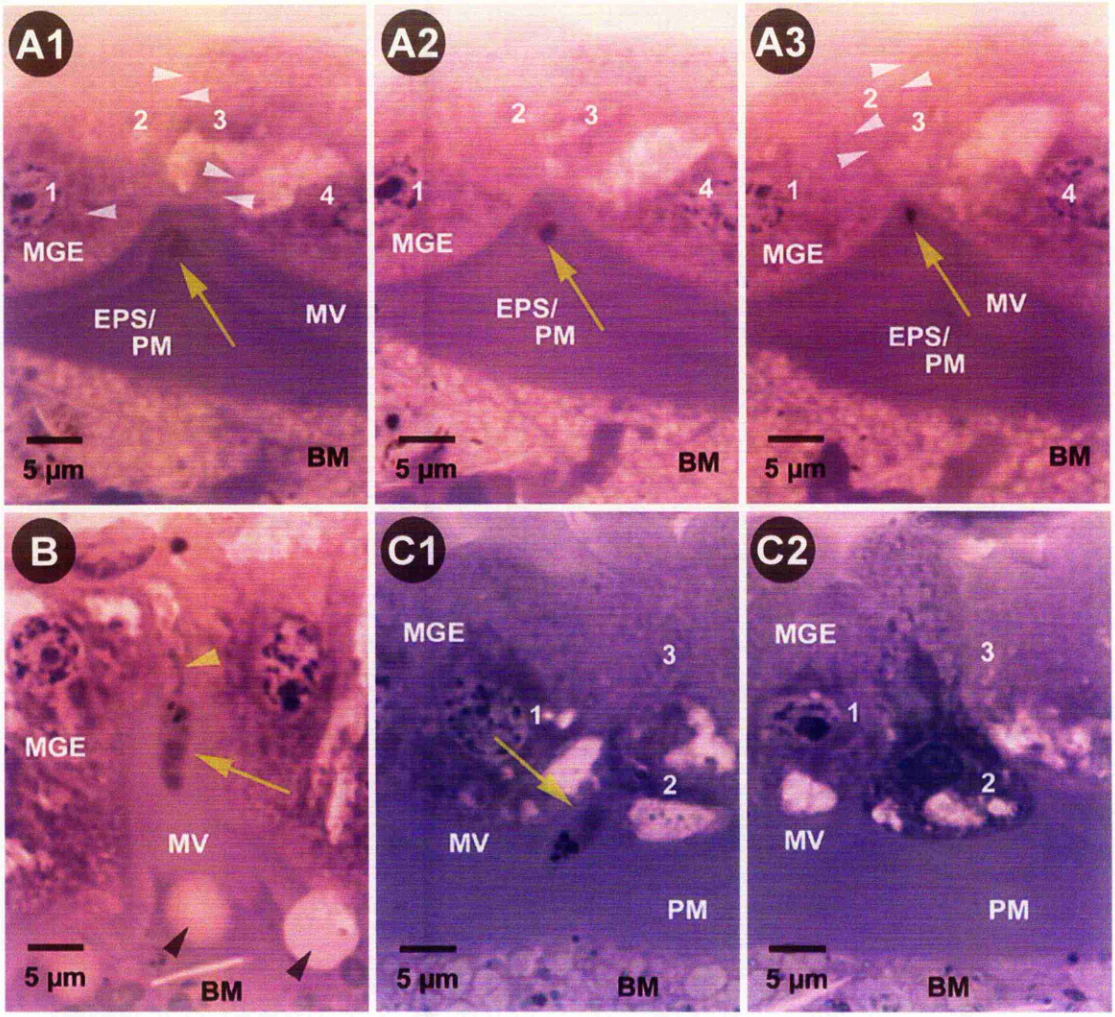


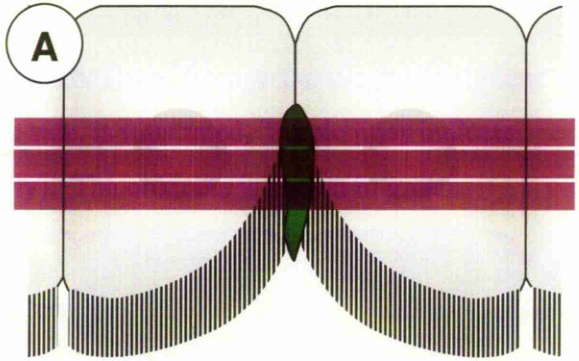
Figure 4.11 ***P. falciparum*** **3D7A ookinetes entering the midgut epithelium.**

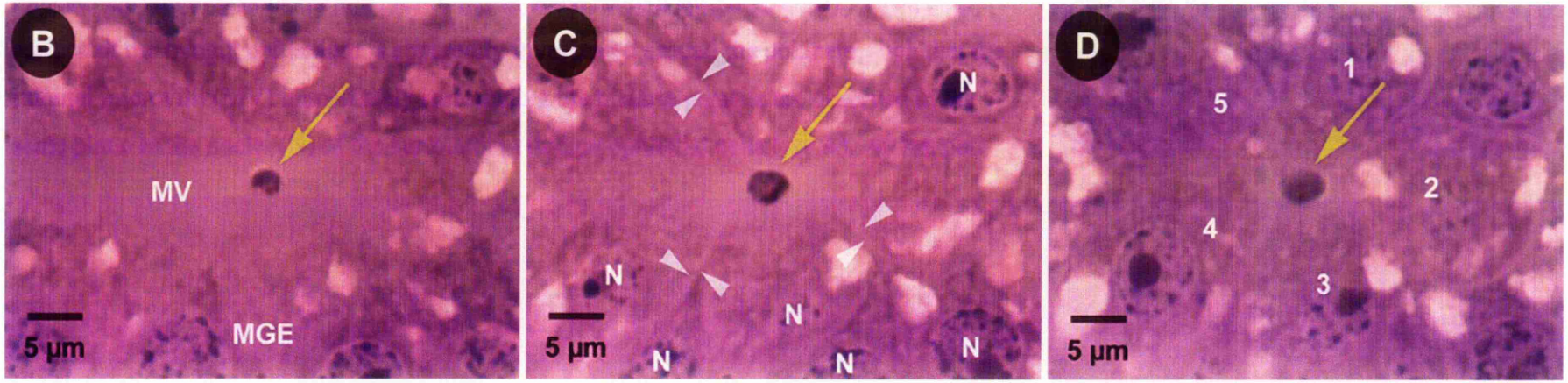
(A1 to A3) Consecutive serial sections showing a single ookinete (yellow arrows) entering the midgut epithelium (MGE) where three adjacent midgut epithelial cells (labelled 1, 2 and 3) converge. White arrowheads indicate lateral plasma membranes of the midgut epithelial cells. 24 hrs pbf. **(B)** "Stalk-form" ookinete: the anterior "stalk" region of the ookinete (yellow arrowhead) is apparently intracellular within the midgut epithelium while the posterior region is extracellular within the midgut lumen and surrounded by microvilli (MV). "Blebbing" of the apical surface of the midgut epithelial cells is apparent (dark arrowheads). 32 hrs pbf. Note localised invagination of the midgut epithelium surrounding the ookinetes shown in **A** and **B**. **(C1/C2)** Consecutive serial sections, 6 µm apart, showing extracellular ookinete entering the midgut epithelium where three adjacent midgut epithelial cells (1, 2 and 3) converge. Midgut epithelial cell 2, only partially apparent in **C1** and seen in fuller view in **C2**, exhibits abnormal dark staining and an apically situated nucleus but abundant microvilli. 28 hrs pbf.

Figure 4.12 ***P. falciparum*** **3D7A ookinete entering the midgut epithelium of** ***An. stephensi.***

(A) Diagram illustrating the relative location of the sections shown in **B to D**. A section taken perpendicularly through the longitudinal plane of the midgut epithelium, which would be observed if looking into the midgut epithelium from the side, is illustrated. Purple lines indicate orthogonal planes through which the sections are taken (bottom to top represent **B to D** respectively). The ookinete is shown in green.

(B to D) Consecutive serial sections apparently showing a single extracellular ookinete (yellow arrows) entering the midgut epithelium (MGE) at a site where multiple adjacent midgut epithelial cells (1, 2, 3, 4, and 5) converge towards one another (white arrowheads in **C** indicate the lateral plasma membranes of the adjacent midgut epithelial cells). The apical surface of the midgut epithelium is apparently invaginated around the ookinete, such that the luminal microvilli (MV) immediately surrounding the malaria parasite are observed in the same plane as the nuclei (N) of the surrounding midgut epithelial cells (see **A**). 40 hrs pbf.





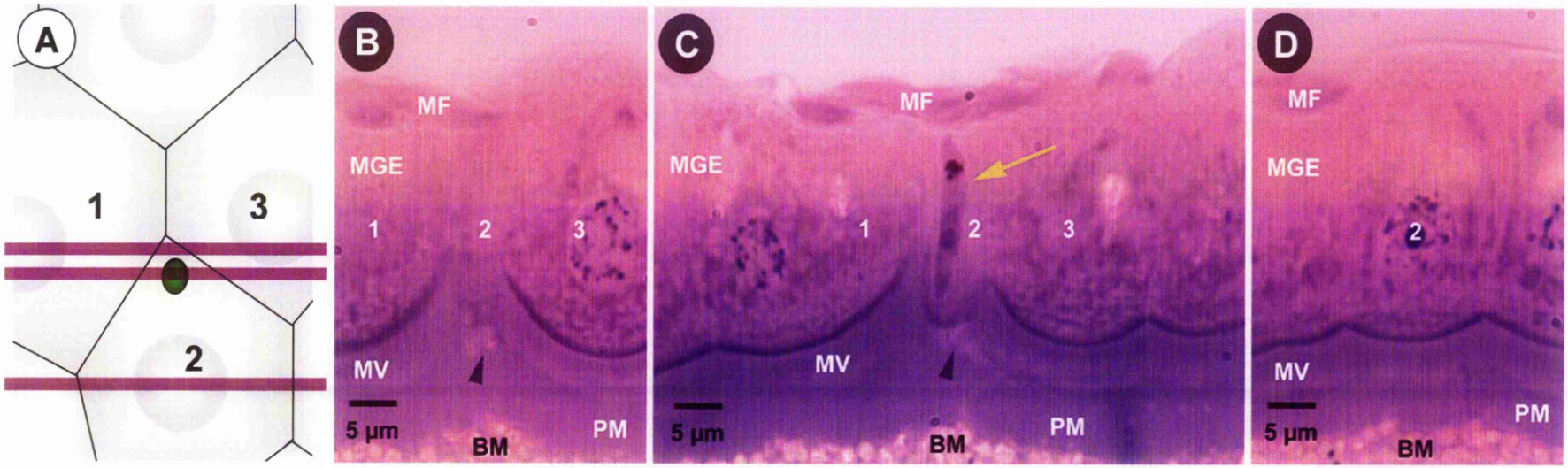


Figure 4.13 ***P. falciparum*** **3D7A ookinete within a morphologically normal** ***An. stephensi*** **midgut epithelial cell.**

(A) Diagram illustrating the relative location of the sections shown in **B to D**. A section taken through the middle of the longitudinal plane of the midgut epithelium, which would be observed if looking down onto the midgut epithelium from above, is illustrated. Purple lines indicate orthogonal planes through which the sections are taken (top to bottom represent **B to D** respectively). The ookinete is shown in green. The sections shown in **B** and **C** are separated from one another by 2 µm while a distance of 14 µm separates the sections shown in **C** and **D**. **(B to D)** Consecutive serial sections illustrating a single intracellular ookinete (yellow arrow in **C**) within a morphologically-normal midgut epithelial cell (2) possessing a centrally-located nucleus and dense microvilli (**D**). The ookinete is located within one corner of midgut epithelial cell 2 (see **A**) parallel to the lateral plasma membranes of the adjacent midgut epithelial cells. Yellowish material is present beneath the apical border of the invaded midgut epithelial cell (dark arrowheads) in **B** and **C**. Note also localised invagination of the midgut epithelium immediately beneath the invading ookinete (**B** and **C**). 24 hrs pbf.

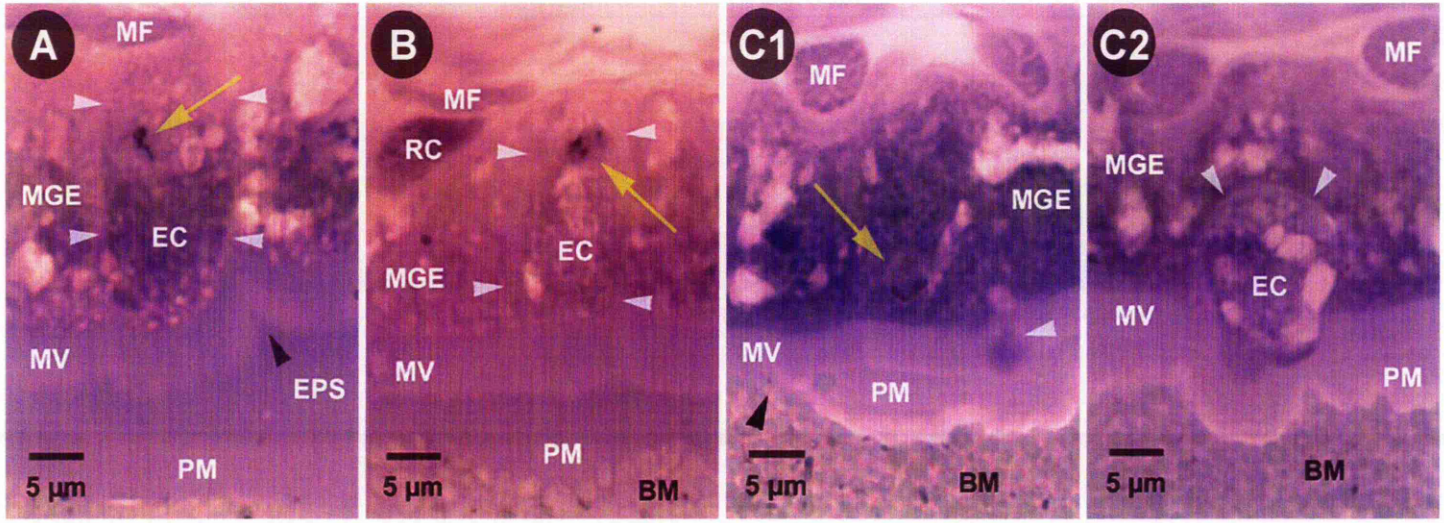


Figure 4.14 Intracellular *P. falciparum* 3D7A ookinetes within morphologically-normal midgut epithelial cells in *An. stephensi*.

(A) Ookinete (yellow arrow) within a morphologically-normal midgut epithelial cell (EC; delineated by white arrowheads) possessing dense microvilli (MV). The apical surface of the midgut epithelium is invaginated (dark arrowhead) on one side of the parasitised midgut epithelial cell. 28 hrs pbf.

(B) Another ookinete within the basal region of a morphologically normal and densely microvillated midgut epithelial cell. A regenerative cell (RC) and muscle fibres (MF) overlying the midgut epithelium are indicated. 28 hrs pbf.

(C1/C2) Consecutive serial sections showing a "rounded" intracellular ookinete (yellow arrow) within the apical region of a morphologically-normal midgut epithelial cell possessing a typical centrally-located nucleus and abundant microvilli. White arrowhead in **C1** indicates part of a protruding morphologically-abnormal midgut epithelial cell seen in fuller view in the section shown in **C2**. Note disruption of the peritrophic matrix and direct contact of the bloodmeal with the microvillar brush border of the midgut epithelium (dark arrowhead in **C1**). 32 hrs pbf.

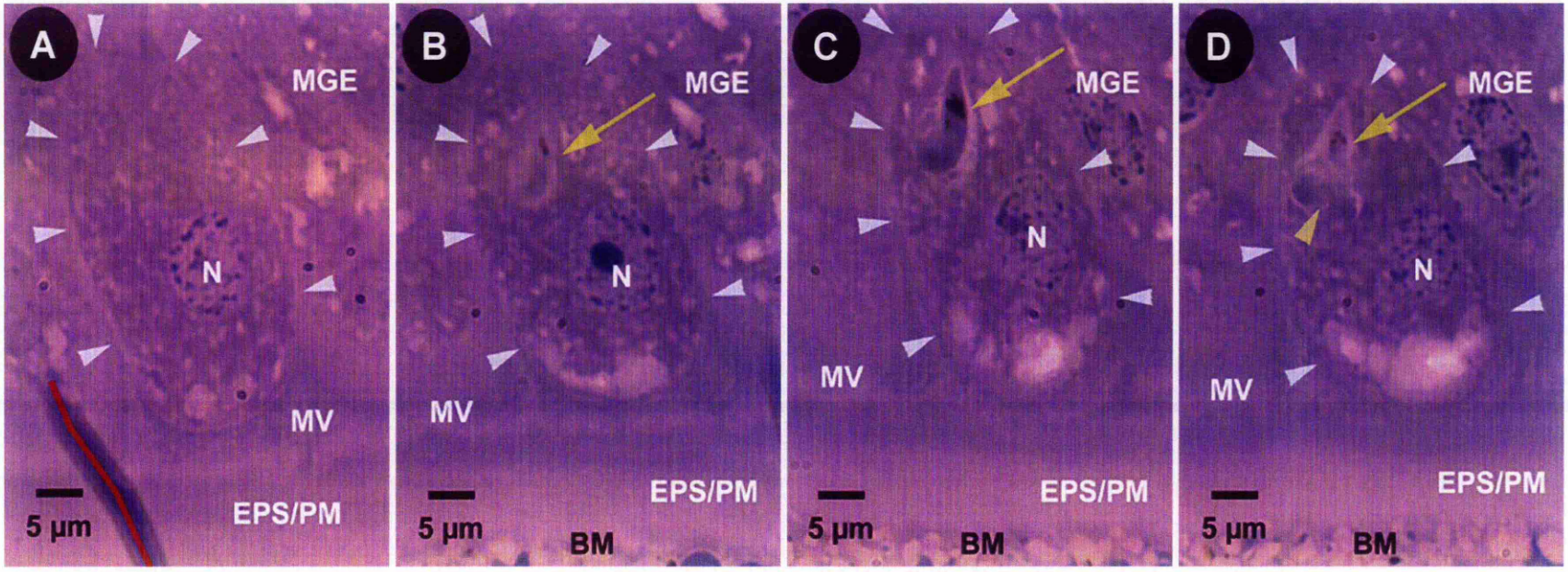


Figure 4.15 Intracellular *P. falciparum* 3D7A ookinete within a microvillated *An. stephensi* midgut epithelial cell.

(A to D) Consecutive serial sections showing a single ookinete (yellow arrows), surrounded by an unstained "halo", within the basal region of a marginally swollen midgut epithelial cell (delineated by white arrowheads). The consecutive serial sections clearly demonstrate the intracellular location of the ookinete. The anterior of the ookinete appears morphologically normal (**B** and **C**) while the posterior region of the malaria parasite is possibly abnormal appearing bloated and diffuse (yellow arrowhead) **(D)**. The infected midgut epithelial cell possesses dense microvilli (MV) similar to those of the surrounding midgut epithelial cells and a morphologically normal nucleus (N) that is, however, situated slightly apically in comparison to the nuclei of the adjacent midgut epithelial cells **(D)**. The bloodmeal (BM) and midgut epithelium (MGE) are also indicated. Red line indicates a crease present in the section shown in **A**. 24 hrs pbf.

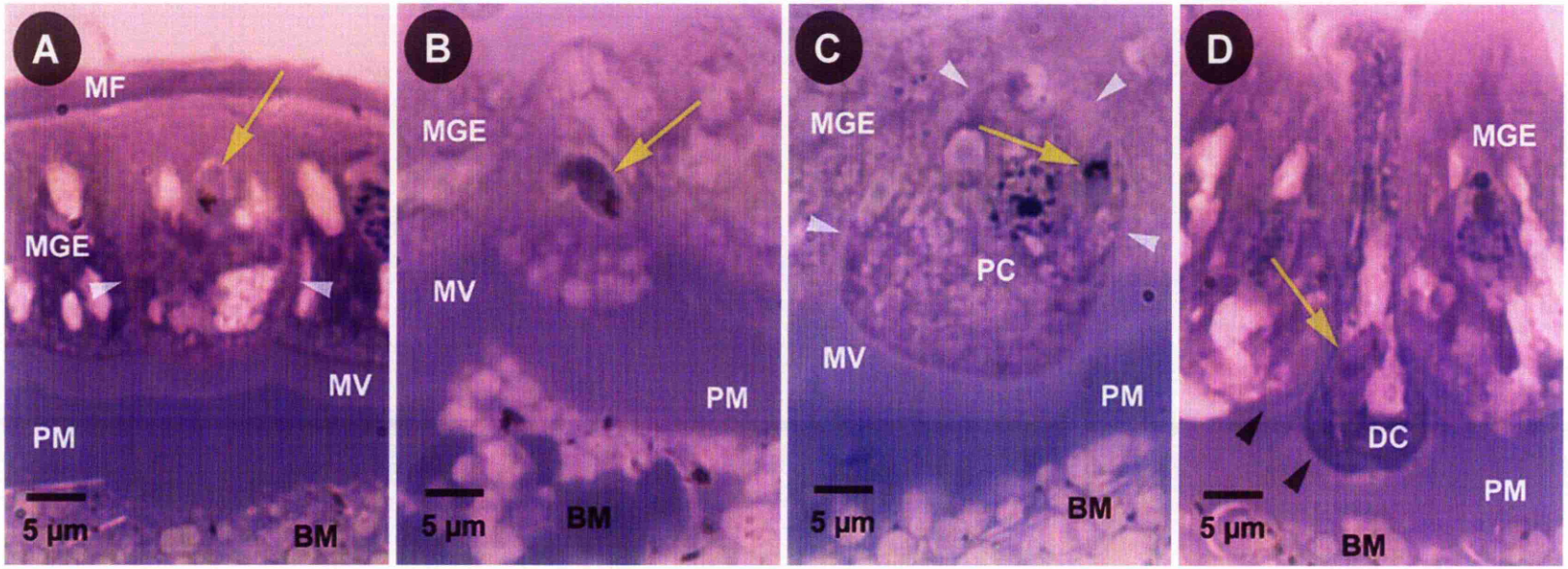


Figure 4.16 ***P. falciparum*** **3D7A ookinetes within midgut epithelial cells from** ***An. stephensi.***

(A) Malaria parasite (yellow arrow) within a midgut epithelial cell, possessing abundant microvilli (MV), which, apart from an apparent minor swelling, and slight protrusion, is morphologically indistinguishable from the surrounding cells of the midgut epithelium (MGE). White arrowheads indicate the lateral plasma membranes of the parasitised midgut epithelial cell. Note unstained "halo" around uppermost surface of the malaria parasite. 28 hrs pbf. **(B)** Intracellular ookinete surrounded by an unstained "halo". 28 hrs pbf.

(C) Another intracellular ookinete apparently in direct contact with the cytoplasm of the invaded midgut epithelial cell (PC), delineated by white arrowheads, which exhibits abnormal protrusion from the midgut epithelium but possesses a normal nucleus and dense microvilli. 36 hrs pbf.

(D) Intracellular ookinete within the apical region of a dark staining, slightly protruding, flaccid midgut epithelial cell (DC), possessing short microvilli (dark arrowheads), which is surrounded by morphologically-normal midgut epithelial cells. 28 hrs pbf.

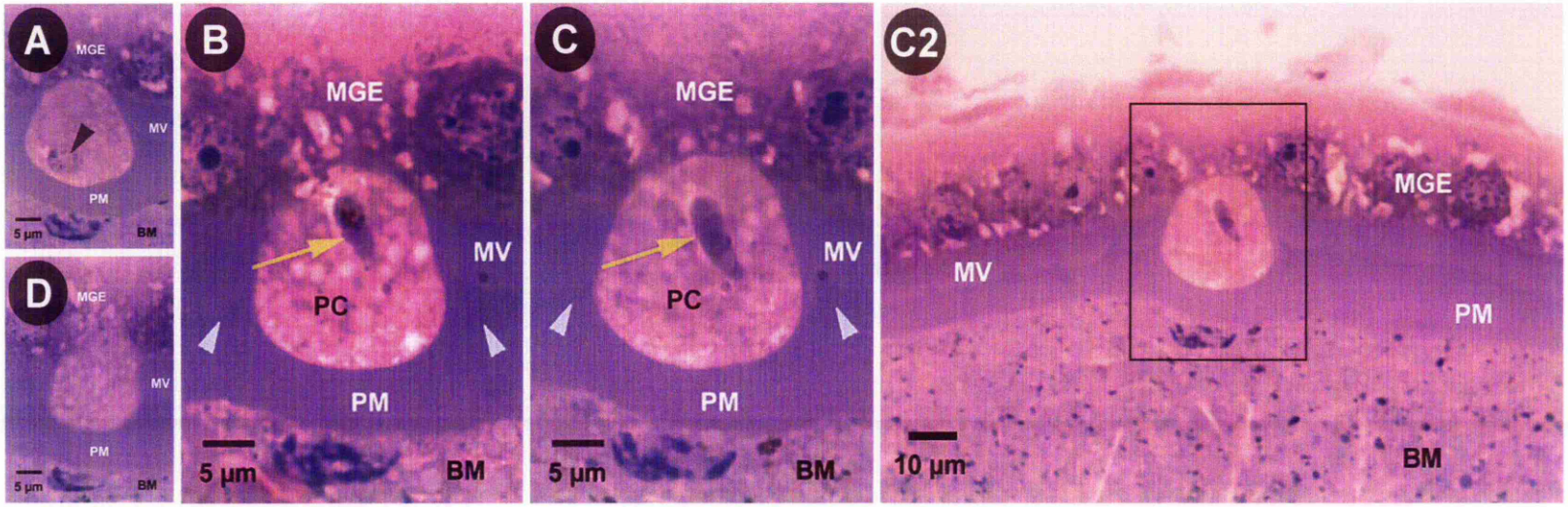


Figure 4.17 Intracellular *P. falciparum* 3D7A ookinete within a lightly staining morphologically-abnormal midgut epithelial cell protruding from the midgut epithelium into the midgut lumen of *An. stephensi*.

(A to D) Consecutive serial sections, labelled in order, showing a single intracellular ookinete (yellow arrows) within the basal region of a single lightly staining midgut epithelial cell (PC) that protrudes from the midgut epithelium into the ectoperitrophic space (white arrowheads) of the midgut lumen. The anterior-most region of the ookinete is presumably shown in **B** and the posterior-most region in **C**. In the previous and subsequent sections (**A** and **D** respectively), the pale protruding midgut epithelial cell alone is present demonstrating the intracellular location of the ookinete present in **B** and **C**. Note the apical location of the nucleus (dark arrowhead in **A**) of the protruding midgut epithelial cell and inward depression of the underlying peritrophic matrix (PM) towards the bloodmeal (BM). **(C2)** Lower magnification image (400X) of the section shown in **C** illustrating degree of protrusion and morphological abnormality of the invaded midgut epithelial cell in comparison to the surrounding normal uninvaded midgut epithelium. The black box outlined in **C2** highlights the region shown at higher magnification (1000X) in **C**. 28 hrs pbf.

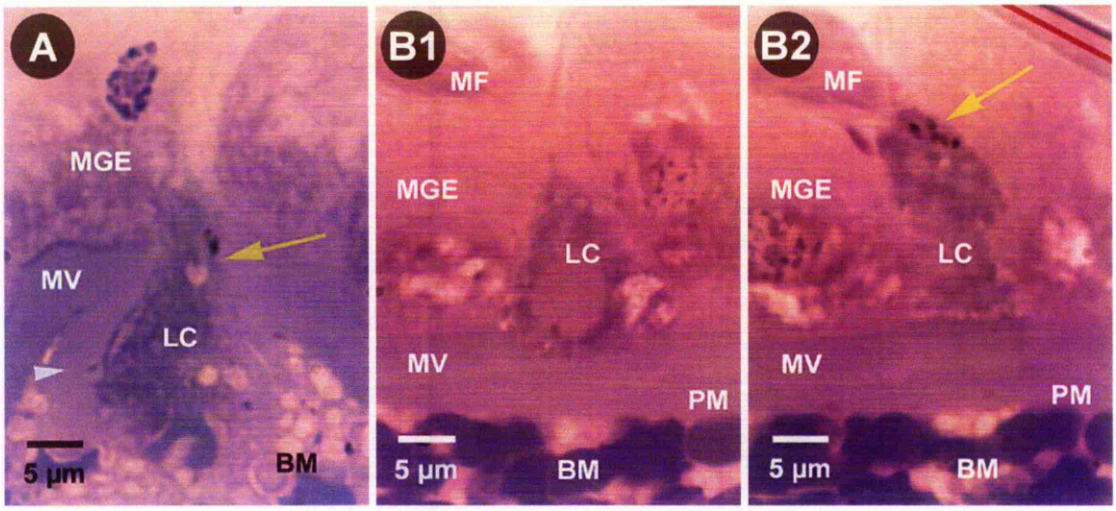


Figure 4.18 ***P. falciparum*** **3D7A ookinetes within "lysing" midgut epithelial cells, exhibiting unusual blue staining, in** ***An. stephensi.***

(A) Ookinete (yellow arrow) within the basal region of a disorganised midgut epithelial cell (LC), which exhibits unusual pale blue staining. The parasitised midgut epithelial cell almost entirely protrudes from the overlying midgut epithelium (MGE) into the bloodmeal (BM) within the midgut lumen. The midgut epithelial cells apparently still possesses dense microvilli (MV)(white arrowhead). 24 hrs pbf.

(B1/B2) Consecutive serial sections showing a single ookinete (yellow arrow in **B2**) within the basal region of another midgut epithelial cell (LC) exhibiting similar unusual blue staining. Although protruding slightly from the apical surface, the parasitised midgut epithelial cell is entirely within the midgut epithelium in this instance (**B1**). A crease is present in the section shown in **B2** (red line). 28 hrs pbf.

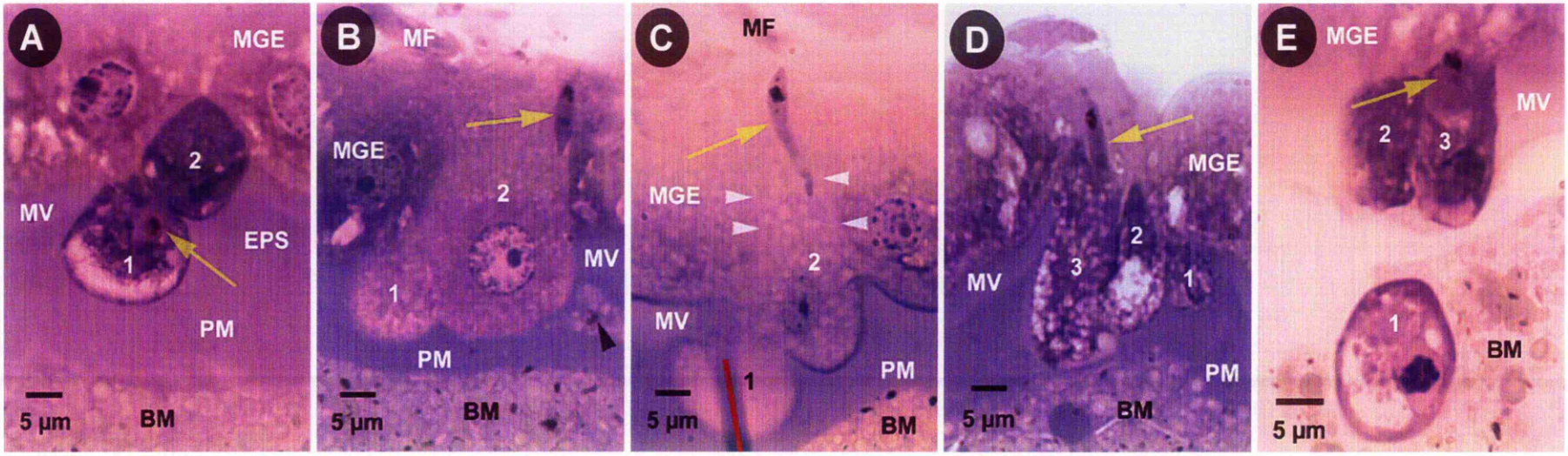


Figure 4.19 ***P. falciparum*** **3D7A ookinetes associated with multiple morphologically-abnormal midgut epithelial cells in** ***An. stephensi.***

(A) Ookinete (yellow arrow) spanning the plasma membranes of two adjacent dark midgut epithelial cells (labelled 1 and 2) protruding from the midgut epithelium (MGE) into the ectoperitrophic space (EPS) of the midgut lumen. 28 hrs pbf. **(B and C)** Different intracellular ookinetes adjacent and parallel to the basal region of the lateral plasma membrane of their respective infected and protruding midgut epithelial cells (2). In both instances, note the second, partially apparent, midgut epithelial cell (1) exhibiting greater protrusion into the midgut lumen. Material from the bloodmeal is present within the ectoperitrophic space (dark arrowhead) in **B**. Red line indicates crease in the section shown in **C**. **B** and **C** are 28 hrs and 24 hrs pbf, respectively. **(D and E)** Different ookinetes emerging from the basal surface of dark protruding midgut epithelial cells (3). In both instances, two further morphologically abnormal dark midgut epithelial cells are present (1 and 2). In **E**, one of these midgut epithelial cells is completely separated from the overlying midgut epithelium and lies entirely within the bloodmeal (BM). Note higher magnification of **E**. Both **D** and **E** are 28 hrs pbf. In all instances, the midgut epithelial cells are numbered according to the presumed order of ookinete invasion.

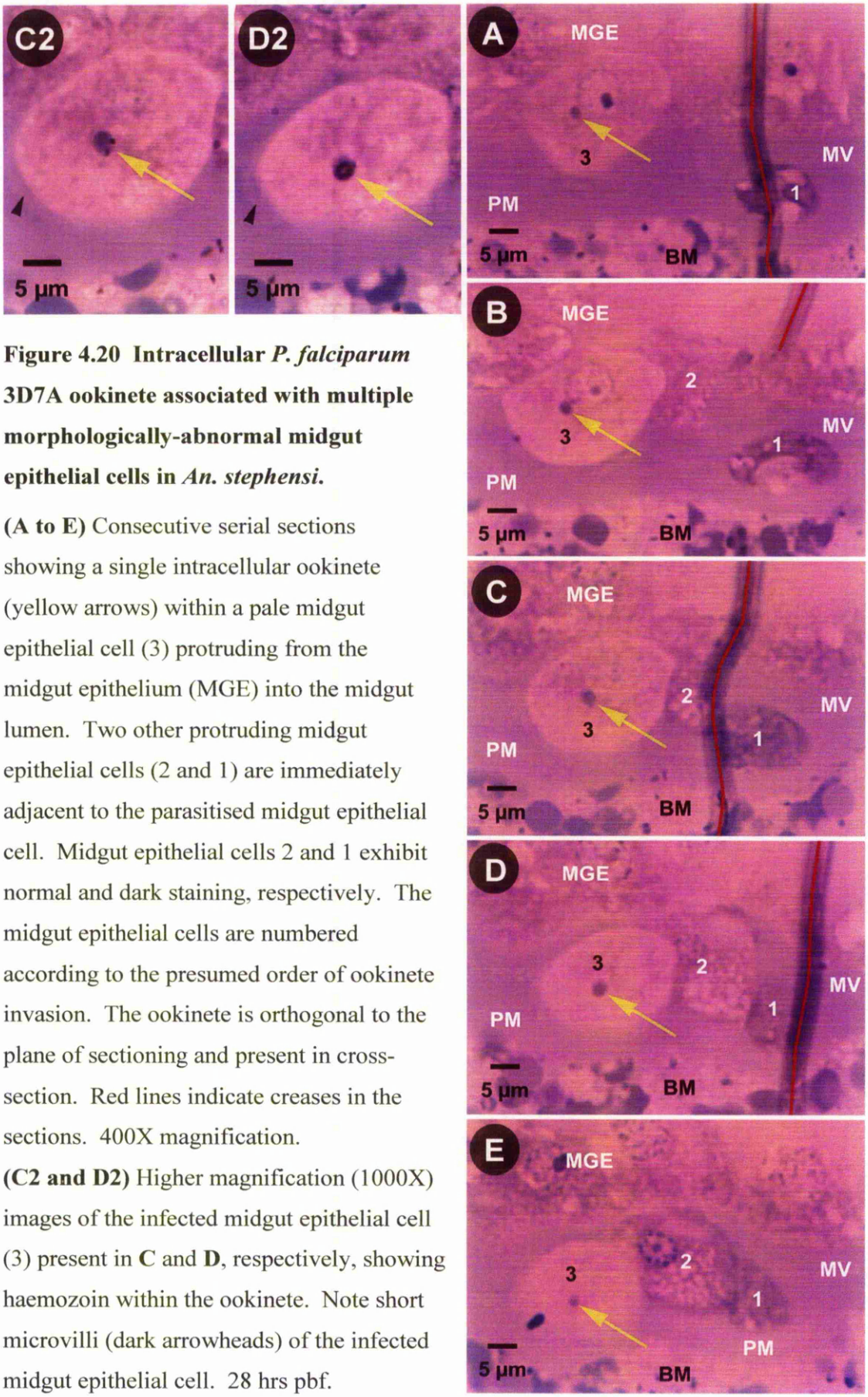


Figure 4.20 Intracellular *P. falciparum* 3D7A ookinete associated with multiple morphologically-abnormal midgut epithelial cells in *An. stephensi*.

(A to E) Consecutive serial sections showing a single intracellular ookinete (yellow arrows) within a pale midgut epithelial cell (3) protruding from the midgut epithelium (MGE) into the midgut lumen. Two other protruding midgut epithelial cells (2 and 1) are immediately adjacent to the parasitised midgut epithelial cell. Midgut epithelial cells 2 and 1 exhibit normal and dark staining, respectively. The midgut epithelial cells are numbered according to the presumed order of ookinete invasion. The ookinete is orthogonal to the plane of sectioning and present in cross-section. Red lines indicate creases in the sections. 400X magnification.

(C2 and D2) Higher magnification (1000X) images of the infected midgut epithelial cell (3) present in **C** and **D**, respectively, showing haemozoin within the ookinete. Note short microvilli (dark arrowheads) of the infected midgut epithelial cell. 28 hrs pbf.

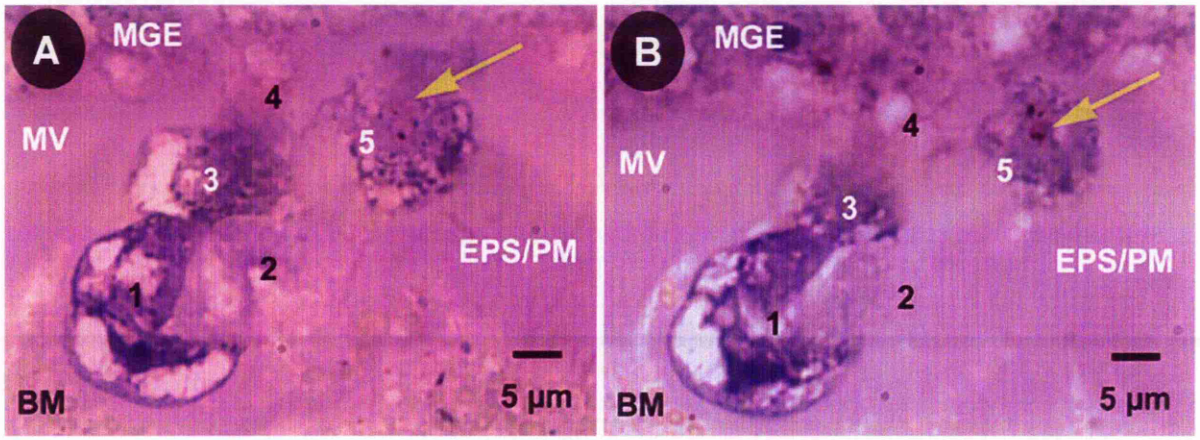


Figure 4.21 Intracellular *P. falciparum* 3D7A ookinete associated with a large cluster of morphologically-abnormal midgut epithelial cells protruding from the midgut epithelium into the midgut lumen of *An. stephensi*.

(A/B) Consecutive serial sections showing a single intracellular ookinete (yellow arrows) within the basal region of a dark midgut epithelial cell (labelled 5) protruding from the midgut epithelium (MGE) into the ectoperitrophic space (EPS) of the midgut lumen. Although the cytoplasm of the dark infected midgut epithelial cell and the ookinete are not easily differentiated, a characteristic cluster of haemozoin is clearly visible within the malaria parasite. A further four morphologically-abnormal midgut epithelial cells (labelled 1, 2, 3, and 4), immediately adjacent to the parasitised midgut epithelial cell, protrude, together with the latter, as a large single mass into the midgut lumen from the overlying midgut epithelium. The midgut epithelial cells are numbered according to the presumed order of ookinete invasion. 1000X magnification. 32 hrs pbf.

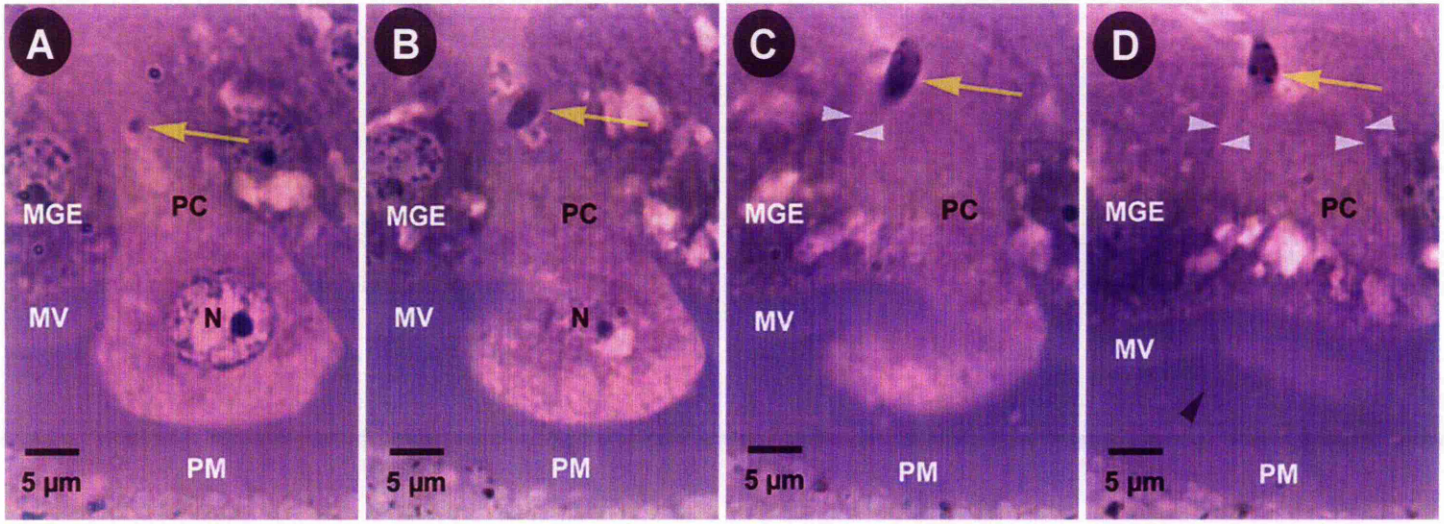


Figure 4.22 ***P. falciparum*** **3D7A ookinete emerging from the basal surface of a pale and protruding** ***An. stephensi*** **midgut epithelial cell.**

(A to D) Four consecutive serial sections showing a single ookinete (yellow arrows) emerging from the basal surface of a single midgut epithelial cell (PC), protruding into the ectoperitrophic space of the midgut lumen, which exhibits abnormal pale staining in comparison to the surrounding morphologically normal uninvaded midgut epithelium (MGE). The posterior-most and anterior-most regions of the ookinete are presumably present in **A** and **D**, respectively. The apical region of the protruding midgut epithelial cell curves underneath the midgut epithelium and progressively disappears from view, while moving from section **A to D**, to reveal the morphologically-normal midgut epithelial cells located behind. The nucleus (N) of the protruding midgut epithelial cell is located apically compared to the central location of the nuclei of the adjacent morphologically-normal midgut epithelial cells (**A** and **B**). Note inward depression of the peritrophic matrix (PM), immediately beneath the pale protruding midgut epithelial cell, towards the bloodmeal. White arrowheads indicate lateral membranes of the pale protruding midgut epithelial cell. Dark arrowhead indicates the ectoperitrophic space. 28 hrs pbf.

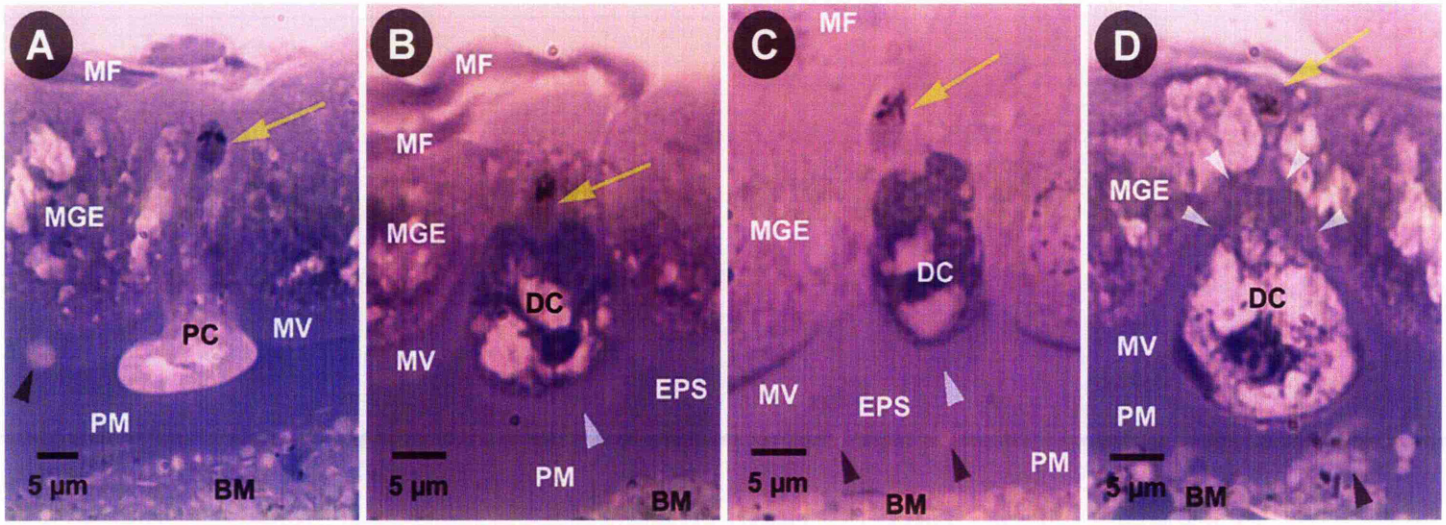


Figure 4.23 ***P. falciparum*** **3D7A ookinetes emerging from the basal region of morphologically-abnormal midgut epithelial cells.**

(A) Ookinete (yellow arrow) emerging from the basal region of a midgut epithelial cell (PC) protruding from the midgut epithelium (MGE) into the ectoperitrophic space (EPS) of the midgut lumen. The apical region of the protruding midgut epithelial cell is lightly stained. "Blebbing" of the apical surface of an adjacent "open" endocrine cell (dark arrowhead) is apparent, although the body of the endocrine cell is not present in the section shown. Note lower magnification of **A** compared to **B**, **C** and **D**. 28 hrs pbf. **(B and C)** Different ookinetes emerging from dark staining and flaccid midgut epithelial cells (DC) possessing condensed and darkly staining nuclei. The dark midgut epithelial cell in **B** possesses short microvilli while the apical surface of dark midgut epithelial cell in **C** apparently lacks microvilli (white arrowheads). Material from the bloodmeal (BM) is apparently present within the ectoperitrophic space in **C** (dark arrowheads). **B** and **C** are 24 and 28 hrs pbf, respectively. **(D)** Ookinete on the basal surface of the midgut epithelium immediately above a dark protruding midgut epithelial cell. 28 hrs pbf. Note: the exact location of ookinetes shown in **A** and **B** (i.e. whether intra- or intercellular) is uncertain while those in **C** and **D** are probably intercellular.

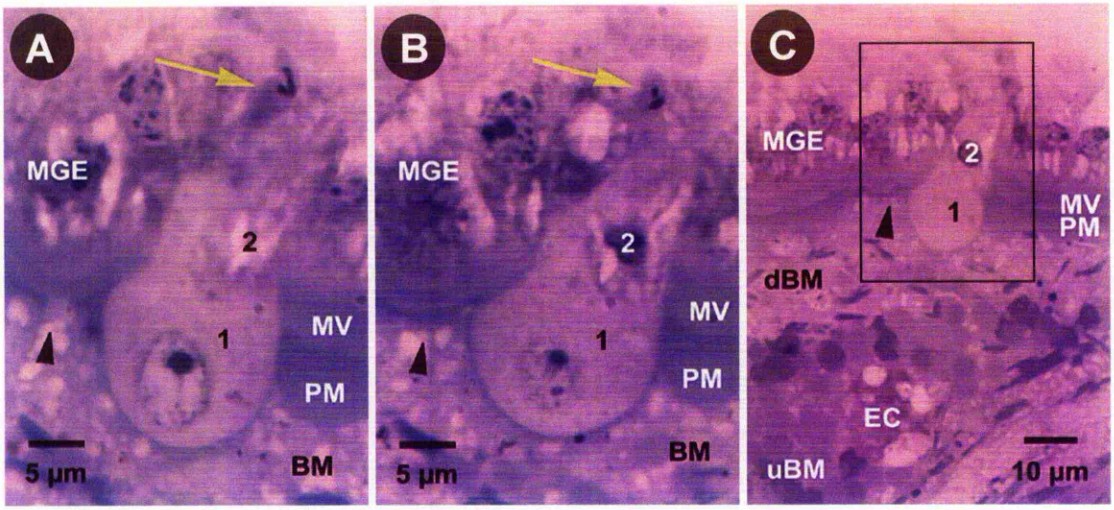


Figure 4.24 *P. falciparum* 3D7A ookinete associated with multiple morphologically-abnormal midgut epithelial cells from *An. stephensi*.

(A to C) Consecutive serial sections showing a single ookinete (yellow arrows) within the basal region of the midgut epithelium. Whether the ookinete is intra- or intercellular is not clear from the sections shown. Immediately beneath the ookinete, on top of one another, are two morphologically-abnormal midgut epithelial cells (labelled 1 and 2), which both stain lightly and protrude into the midgut lumen. The nucleus of the one of these midgut epithelial cells (2) is condensed and dark staining. The two midgut epithelial cells are numbered according to the assumed order of ookinete invasion. **(C)** Lower magnification image (400X) of the section immediately following the section shown in **B**. The black box outlined in **C** indicates the relative location of the images shown at higher magnification (1000X) in the previous two sections (**A** and **B**). At lower magnification, a third midgut epithelial cell (EC), completely separated from the midgut epithelium, is seen, in relatively close proximity, within the central undigested region of the bloodmeal (uBM). The peritrophic matrix (PM) can also be seen to be disrupted immediately beneath, and to the left of, the two protruding midgut epithelial cells (1 and 2) associated with the invading ookinete (dark arrowheads). Consequently, the lower protruding midgut epithelial cell (1) is apparently in direct contact with the peripheral digested region of the bloodmeal (dBM). No other invading malaria parasites were observed within this region of the midgut epithelium. The ookinete shown in **A** and **B** possibly invaded the third midgut epithelial cell located within the bloodmeal prior to invasion of midgut epithelial cells 1 and 2. 36 hrs pbf.

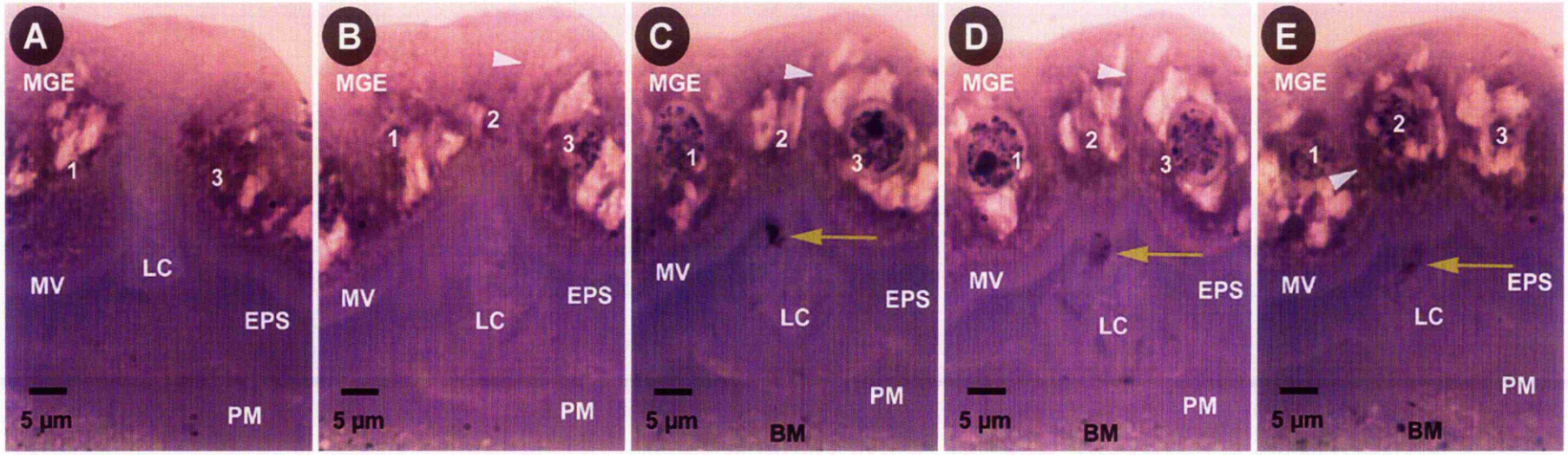


Figure 4.25 ***P. falciparum*** **3D7A ookinete emerging from the basolateral surface of a "lysing" midgut epithelial cell located within the ectoperitrophic space of the midgut lumen of** ***An. stephensi.***

(A to E) Consecutive serial sections showing a single ookinete (yellow arrows) emerging from the basolateral surface of an indistinct and diffuse midgut epithelial cell (LC), exhibiting unusual pale blue staining, which appears to be lysing from the midgut epithelium (MGE) into the ectoperitrophic space (EPS) of the midgut lumen. The apparently lysing midgut epithelial cell, which lacks definite structure, is difficult to discriminate from the ectoperitrophic space *per se* but appears to be spreading throughout this compartment of the midgut lumen (**A**). The lysing midgut epithelial cell is leaving the overlying midgut epithelium, to which it is still attached (**A**), from a site where three adjacent midgut epithelial cells (labelled 1, 2 and 3) converge (**B**). White arrowheads indicate lateral plasma membranes of the adjacent midgut epithelial cells. The ookinete appears to be partly extracellular within the ectoperitrophic space and re-entering the midgut epithelium at the three-cell junction from which the lysing midgut epithelial cell has descended. Note localised invagination of the midgut epithelium at the site of ookinete invasion. 28 hrs pbf.

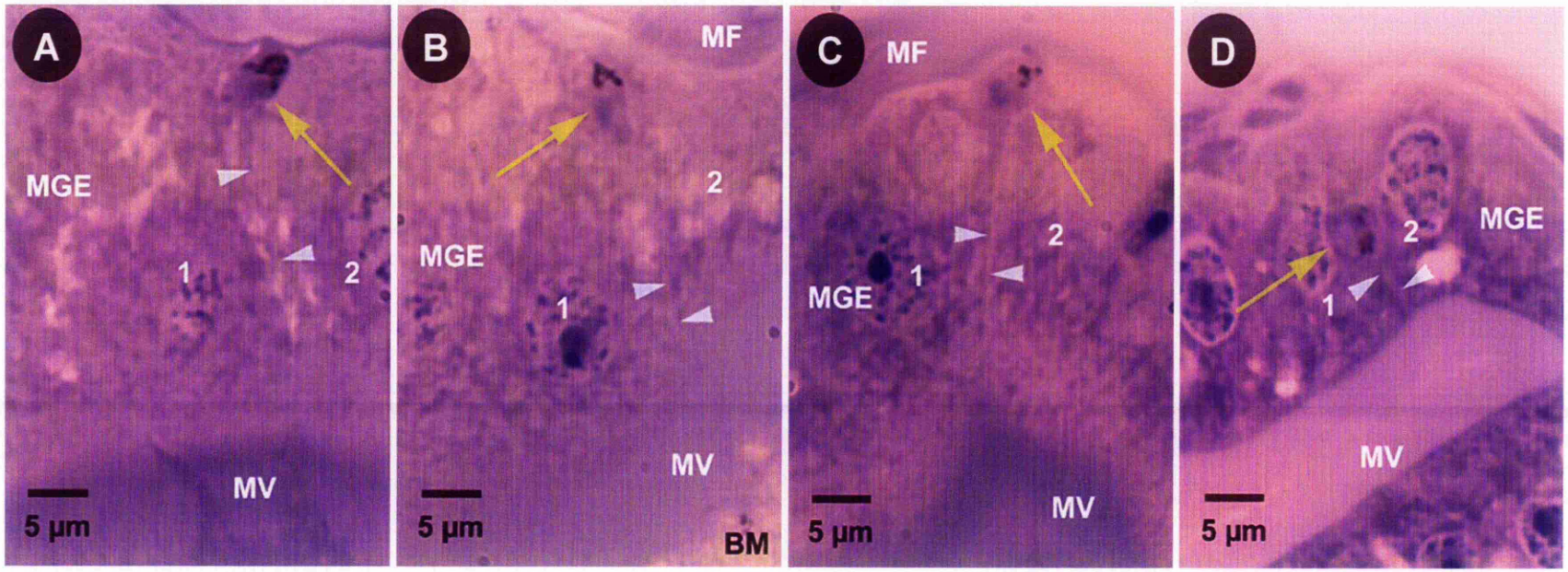


Figure 4.26 ***P. falciparum*** **3D7A ookinetes and oocysts in intercellular locations within the** ***An. stephensi*** **midgut epithelium.**

(A to C) Examples of different ookinetes (yellow arrows) in the basal region of the midgut epithelium (MGE), situated in apparently extracellular locations, immediately within/above the lateral plasma membranes (white arrowheads) of the surrounding/underlying midgut epithelial cells (labelled 1 and 2), which are morphologically normal. **A** and **B** are 28 hrs pbf. **C** is 24 hrs pbf.

(D) Another malaria parasite, either a morphologically abnormal "rounded" ookinete or an ectopic oocyst, situated in an intercellular location, between morphologically-normal midgut epithelial cells, within the central region of the midgut epithelium. D is 44 hrs pbf.

Note: none of the malaria parasites shown exhibited any obvious association with morphologically-abnormal midgut epithelial cells.

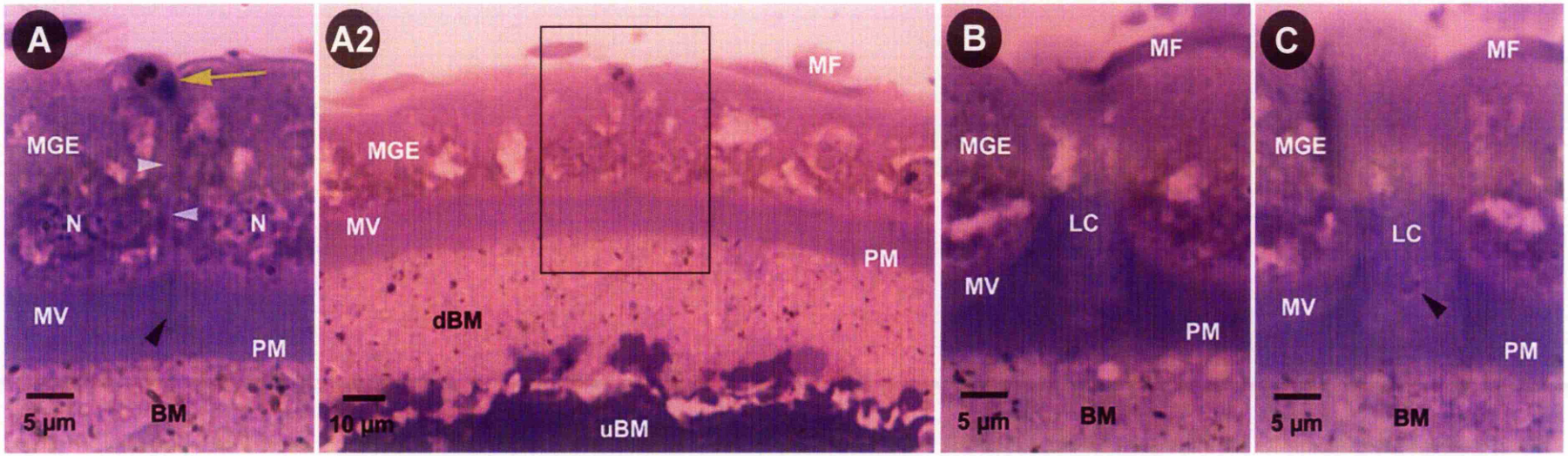


Figure 4.27 Intercellular *P. falciparum* 3D7A ookinete within the basal region of the midgut epithelium of *An. stephensi*.

(A to C) Consecutive serial sections showing a single, apparently extracellular, ookinete (yellow arrow in **A**) in the basal region of the midgut epithelium, situated between two adjacent and morphologically-normal midgut epithelial cells whose nuclei are indicated (N). The ookinete is immediately above the lateral plasma membranes of the two midgut epithelial cells (white arrowheads). A parting within the microvillar brush border of the midgut epithelium (MV) is apparent immediately underneath the lateral membranes of the two midgut epithelial cells (dark arrowhead). **(A2)** Lower magnification image (400X) of the section shown in **A** illustrating that the midgut epithelium (MGE) surrounding the ookinete is morphologically normal in the section shown. The black box outlined in **A2** highlights the region shown at higher magnification (1000X) in **A**. **(B and C)** In subsequent sections, an indistinct pale blue midgut epithelial cell (LC) lysing into the ectoperitrophic space is apparent immediately beneath the ookinete shown in **A**. The nucleus of the lysing midgut epithelial cell is indicated (dark arrowhead). **B** and **C** show two sections immediately adjacent to one another, located approximately 14 µm from the section shown in **A**. 28 hrs pbf.

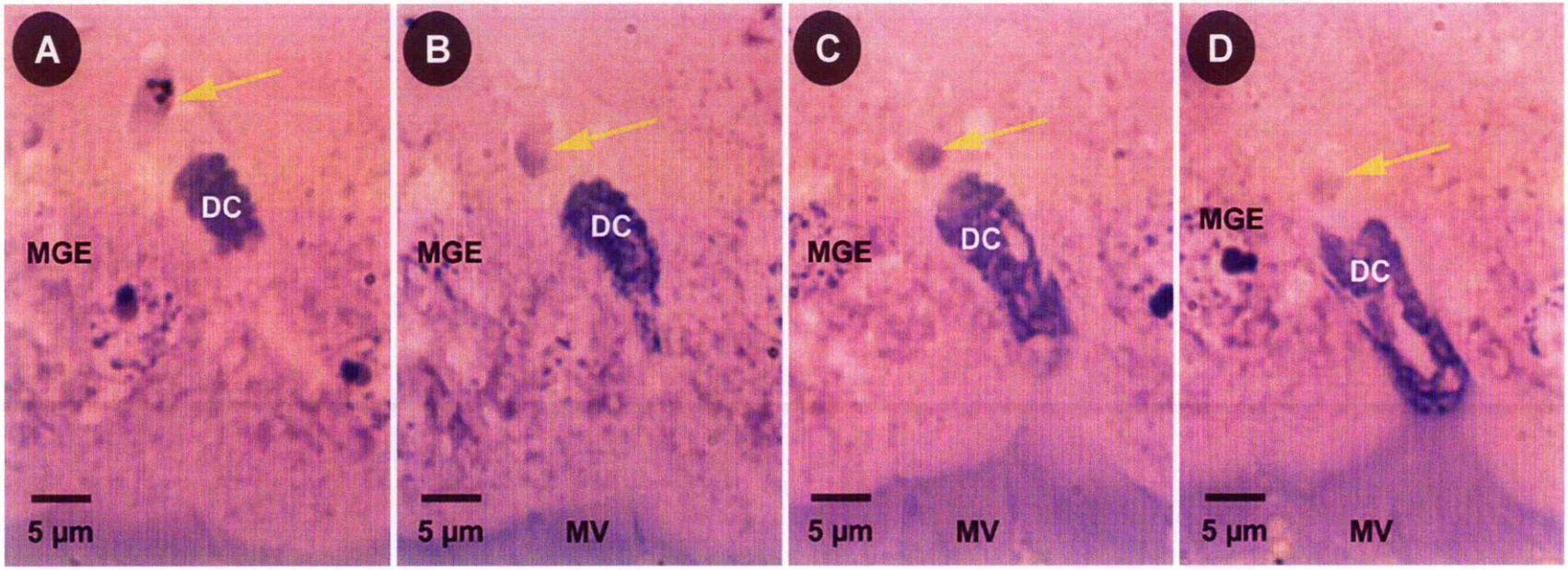


Figure 4.28 Extracellular *P. falciparum* 3D7A ookinete in the basal region of the midgut epithelium of *An. stephensi* associated with a dark staining and flaccid morphologically-abnormal midgut epithelial cell.

(A to D) Consecutive serial sections showing a single ookinete (yellow arrows) in an apparently extracellular location, in the basal region of the midgut epithelium, immediately above a single dark staining and "flaccid" midgut epithelial cell (DC), which is surrounded by morphologically-normal midgut epithelial cells. The anterior- and posterior-most regions of the invading malaria parasite are presumably shown in sections **A** and **D** respectively. The microvillar brush border (MV) of the apical surface of the midgut epithelium is also apparent. 24 hrs pbf.

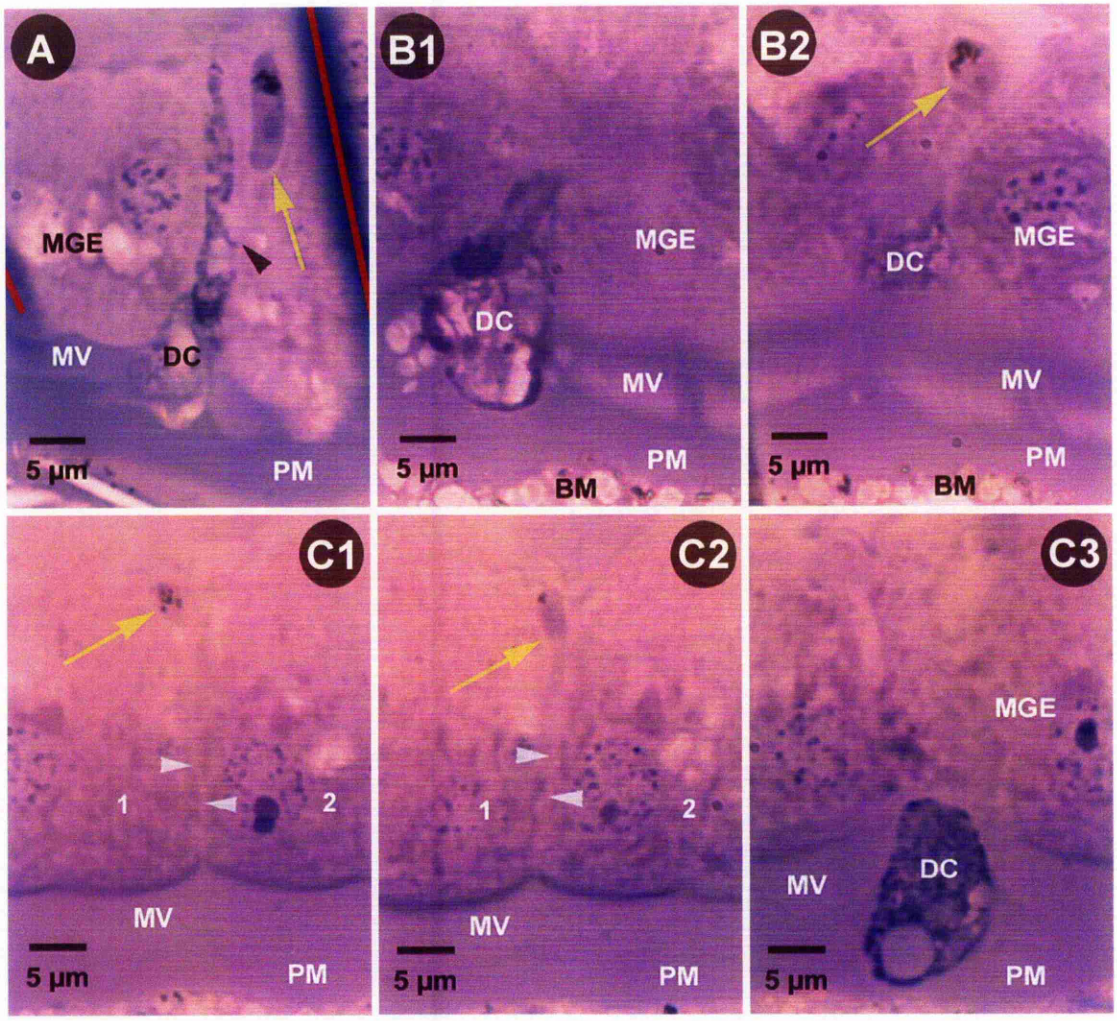


Figure 4.29 Extracellular *P. falciparum* 3D7A ookinetes in the basal region of the *An. stephensi* midgut epithelium associated with abnormal midgut epithelial cells.

(A) Ookinete (yellow arrow) associated with a dark and flaccid midgut epithelial cell (DC). The ookinete is apparently situated where three midgut epithelial cells converge (dark arrowhead). Red lines indicate creases in the section. 24 hrs pbf.

(B1/B2) Consecutive serial sections showing an extracellular ookinete/oocyst located immediately above a dark and flaccid midgut epithelial cell. 28 hrs pbf.

(C1 to C3) Consecutive serial sections showing a single intercellular ookinete (**C1**/**C2**) situated between two adjacent morphologically-normal midgut epithelial cells (1 and 2) and located immediately above an abnormal dark midgut epithelial cell (DC) protruding into the midgut lumen (**C3**). White arrowheads indicate the lateral plasma membranes of the two adjacent morphologically-normal midgut epithelial cells (1 and 2). The sections shown in **C1** and **C2** are immediately adjacent to one another while the section shown in **C3** is located approximately 16 µm from the section in **C2**. 24 hrs pbf.

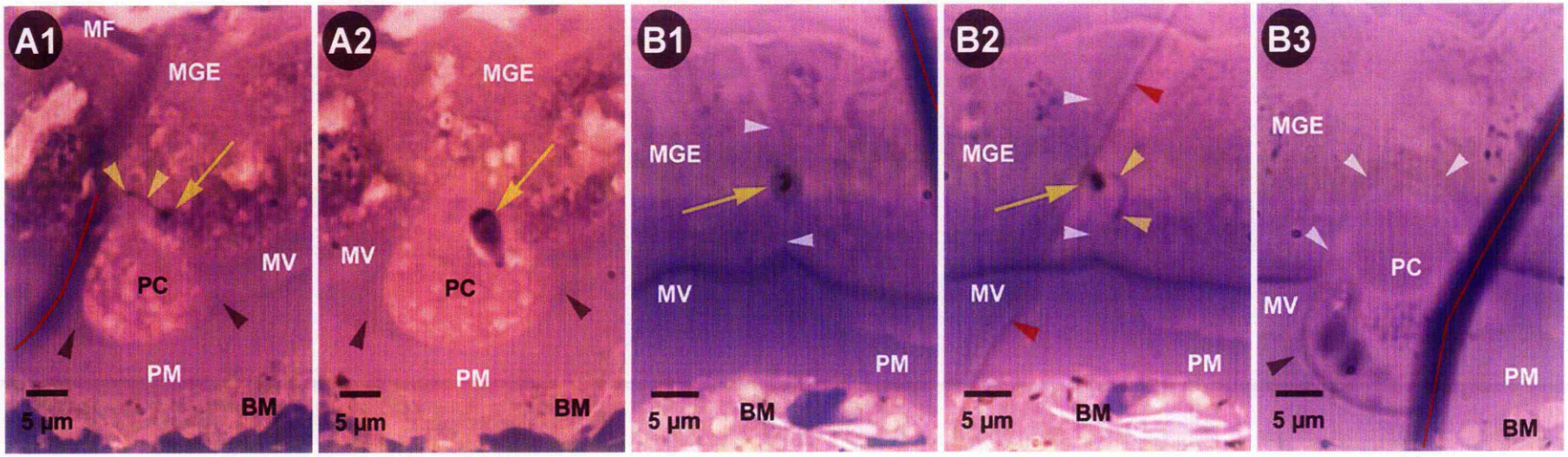


Figure 4.30 "Stalk-form" *P. falciparum* 3D7A ookinetes within the midgut epithelium of *An. stephensi*.

(A1/A2) Consecutive serial sections showing a single "stalk-form" ookinete (yellow arrows and arrowheads) within a pale midgut epithelial cell (PC) protruding from the overlying midgut epithelium (MGE) into the ectoperitrophic space (dark arrowheads). The narrow elongated "stalk" portion of the ookinete (yellow arrowheads) appears to converge with the basal surface of the protruding midgut epithelial cell **(A1)**. Note depression of the peritrophic matrix, towards the bloodmeal (BM), immediately beneath the protruding midgut epithelial cell. 28 hrs pbf. **(B1 to B3)** Consecutive serial sections showing another "stalk-form" ookinete. The anterior "bulb" of this ookinete (yellow arrows) appears to be in an intercellular location within the midgut epithelium, situated between morphologically normal and adjacent midgut epithelial cells (white arrowheads)**(B1/B2)**. Immediately beneath this malaria parasite is a midgut epithelial cell (PC) protruding into the midgut lumen **(B3)**. Sections shown in **B1** and **B2** were immediately adjacent to one another while the section shown in **B3** was located approximately 28 µm from the section shown in **B2**. 28 hrs pbf. Creases of varying magnitude (red lines and arrowheads) are present in some of the sections shown (**A1** and **B2/B3**).

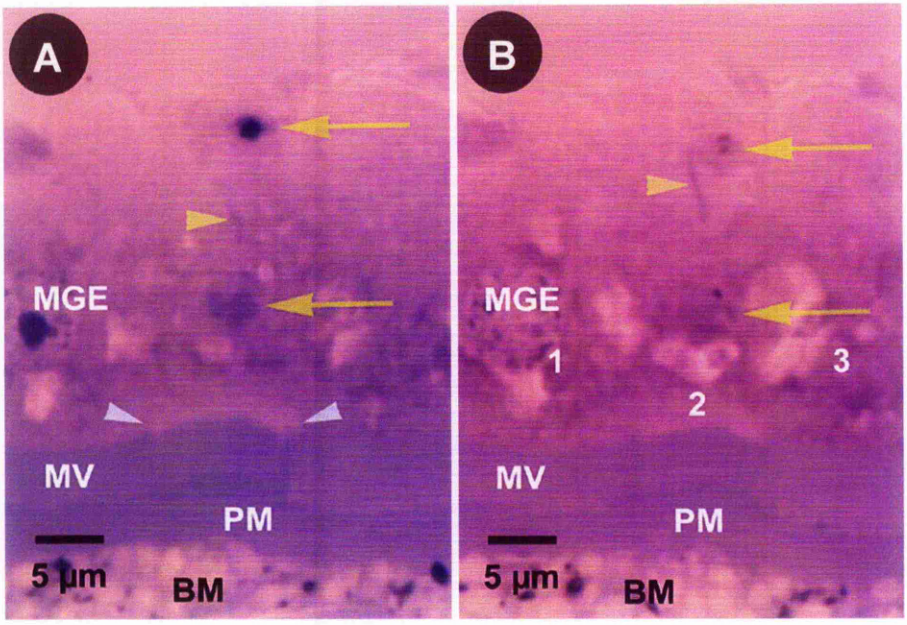


Figure 4.31 A "stalk-form" *P. falciparum* 3D7A ookinete within the midgut epithelium of *An. stephensi*.

(A/B) Consecutive serial sections showing a single "stalk-form" ookinete (yellow arrows and arrowheads) within the midgut epithelium (MGE). The ookinete possesses wide anterior and posterior "bulb" regions (yellow arrows) and a central narrow "stalk" region (yellow arrowheads). Although the cytoplasm of the malaria parasite is faint and difficult to differentiate from the surrounding mosquito tissue, a large clump of haemozoin is apparent in the anterior bulb of the ookinete (in the section shown in **B**) while several small granules of haemozoin are present in both the anterior and posterior bulbs (in the section shown in **A**). Three adjacent midgut epithelial cells (labelled 1, 2 and 3) are apparent around the ookinete, which is seemingly situated within midgut epithelial cell 2. White arrowheads indicate the lateral plasma membranes of these three midgut epithelial cells. The lateral plasma membranes are only distinguishable in the apical region of the midgut epithelium, making it difficult to determine the exact position of the ookinete with respect to the plasma membranes of the midgut epithelial cells. The midgut epithelium surrounding this stalk-form ookinete exhibited no obvious signs of morphological abnormality, except for the slightly concave apical surface of the midgut epithelial cell within which the ookinete is apparently situated. 28 hrs pbf.

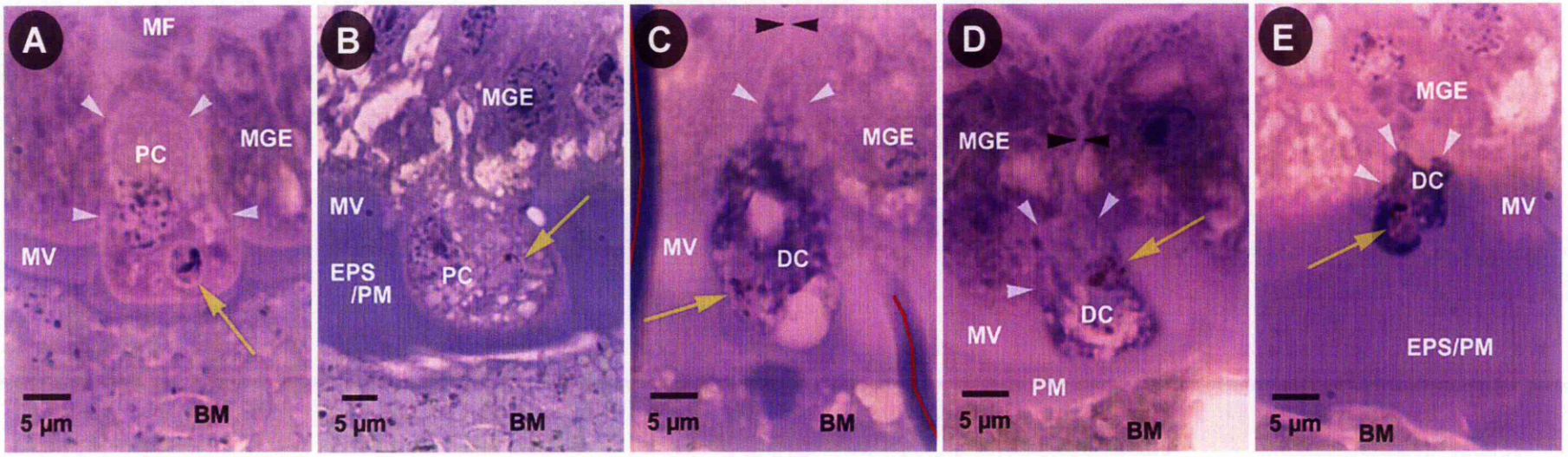


Figure 4.32 Intracellular *P. falciparum* 3D7A ookinetes of dubious morphology within midgut epithelial cells from *An. stephensi*.

(A) "Rounded" intracellular ookinete (yellow arrow) within the apical region of a midgut epithelial cell (PC), possessing shortened microvilli (MV), which is slightly protruding from the midgut epithelium (MGE) towards the bloodmeal (BM). 40 hrs pbf. **(B)** Another "rounded" intracellular ookinete within a midgut epithelial cell exhibiting marked protrusion from the midgut epithelium. Note lower magnification. 28 hrs pbf. **(C to E)** Three different ookinetes all located within morphologically abnormal dark staining midgut epithelial cells (DC) exhibiting various degrees of protrusion from the midgut epithelium into the ectoperitrophic space (EPS) of the midgut lumen. The white arrowheads indicate the lateral and/or basal plasma membranes of the infected midgut epithelial cells. The dark arrowheads indicate the lateral plasma membranes of the surrounding normal and uninfected midgut epithelial cells. Although haemozoin is clearly identifiable, the overall morphology of the ookinetes shown is difficult to discern. However, the ookinetes in **D** and **E** appear abnormally globular and "rounded". Red lines in **C** indicate creases in the section shown. **C** is 28 hrs pbf. **D** and **E** are 32 hrs pbf.

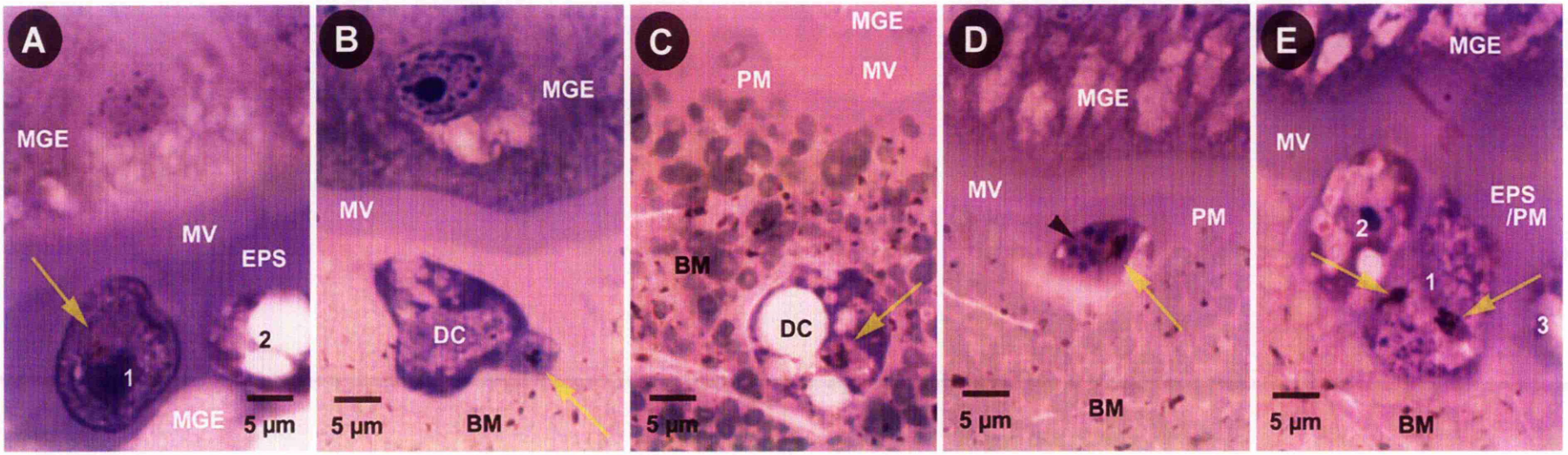


Figure 4.33 ***P. falciparum*** **3D7A ookinetes within morphologically-abnormal midgut epithelial cells located within the midgut lumen and completely separated from the midgut epithelium of** ***An. stephensi.***

(A) Ookinete (yellow arrow) within a dark midgut epithelial cell (1) located within the ectoperitrophic space (EPS) of the midgut lumen and completely separated from the surrounding midgut epithelium (MGE). A second dark midgut epithelial cell (2) is also present within the midgut lumen. 40 hrs pbf. **(B)** A distinct cluster of haemozoin granules (yellow arrow), suggesting the presence of an ookinete, within a dark midgut epithelial cell (DC) located within the bloodmeal (BM) and completely separated from the overlying midgut epithelium. 40 hrs pbf. **(C)** Another dark, and vacuolated, midgut epithelial cell (DC), also located entirely within the bloodmeal, within which is a "rounded" ookinete. 32 hrs pbf. **(D)** A dark midgut epithelial cell, possessing a condensed and fragmented nucleus (arrowhead), completely separated from the midgut epithelium, contains an ookinete. 40 hrs pbf. **(E)** A cluster of three adjacent morphologically-abnormal midgut epithelial cells (1, 2 and 3) completely separated from the midgut epithelium. An apparently fragmented ookinete is present in midgut epithelial cell 1. 40 hrs pbf.

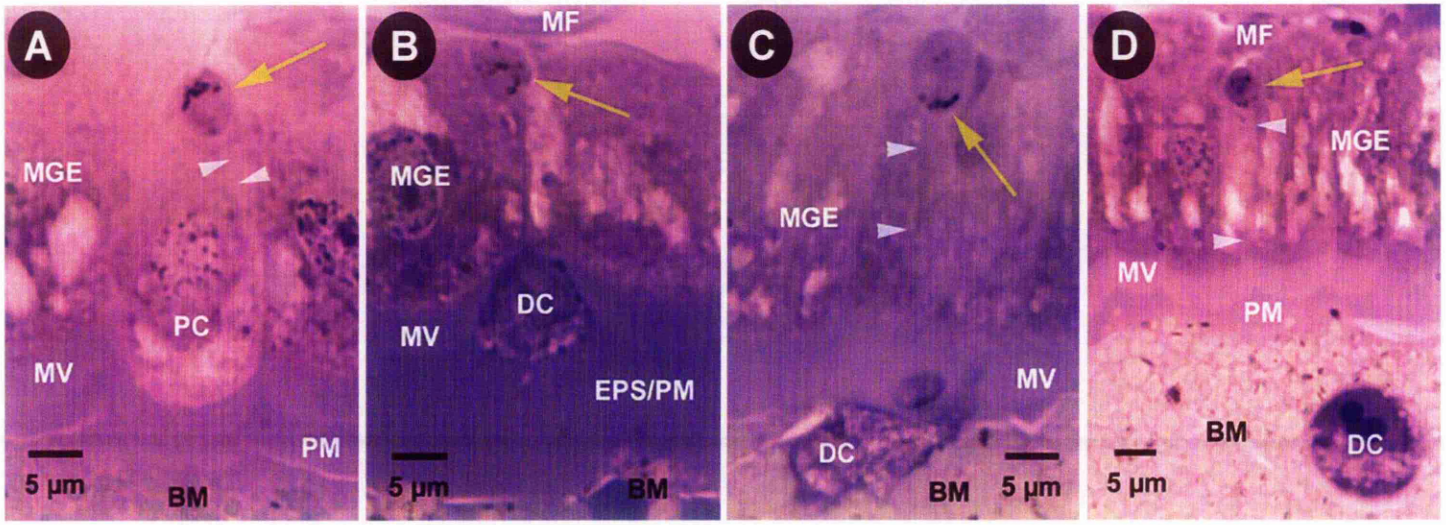


Figure 4.34 ***P. falciparum*** **3D7A oocysts associated with morphologically-abnormal midgut epithelial cells in** ***An. stephensi.***

(A) Oocyst (yellow arrow) on the basal surface of the midgut epithelium (MGE) immediately above a slightly protruding midgut epithelial cell (PC) possessing a slightly apically situated nucleus. The midgut epithelial cell is otherwise morphologically normal possessing abundant microvilli. No other morphologically-abnormal midgut epithelial cells were associated with this malaria parasite. 32 hrs pbf. **(B)** Oocyst on the basal surface of the midgut epithelium immediately above a dark midgut epithelial cell (DC) protruding into the ectoperitrophic space (EPS) of the midgut lumen, which is only partially apparent in the section shown. 28 hrs pbf. **(C)** Oocyst on the basal surface of the midgut epithelium above a dark flaccid midgut epithelial cell (DC) lying within the midgut lumen. 36 hrs pbf. **(D)** Oocyst on the basal surface of morphologically-normal midgut epithelium (MGE). However, a dark spherical midgut epithelial cell (DC), completely separated from the midgut epithelium, is present within the bloodmeal (BM) in close proximity to the malaria parasite. Note lower magnification of image **D**. 36 hrs pbf. All four oocysts shown are located immediately above the lateral plasma membranes of the underlying midgut epithelial cells (white arrowheads).

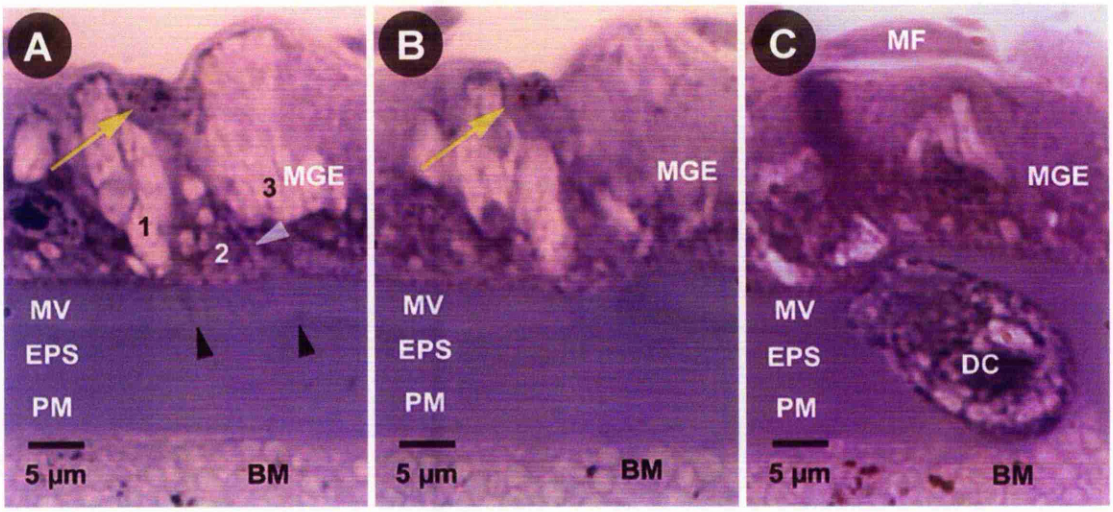


Figure 4.35 ***P. falciparum*** **3D7A ookinete/oocyst associated with a morphologically-abnormal midgut epithelial cell protruding into the midgut lumen in** ***An. stephensi.***

(A to C) Consecutive serial sections showing a single ookinete/oocyst (yellow arrows in **A** and **B**) on the basal surface of the midgut epithelium (MGE). The malaria parasite is apparently situated above where the lateral plasma membranes (white arrowhead) of three adjacent midgut epithelial cells (1, 2 and 3) converge. Dark arrowheads indicate "kinks" in the microvillar brush border (MV) of the midgut epithelium indicative of where the lateral plasma membranes of the adjacent midgut epithelial cells meet. The microvilli of midgut epithelial cell 2 appear slightly disorganised in **A** and **B**. In subsequent sections (**C**), a "turgid" dark staining midgut epithelial cell (DC) is present within the ectoperitrophic space (EPS) of the midgut lumen. Examination of adjacent consecutive serial sections (not shown) suggested that this dark midgut epithelial cell was completely separated from the overlying midgut epithelium. The basal region of the dark midgut epithelial cell abuts the apical surface of midgut epithelial cell 2 (delimited by the dark arrowheads in **A**). The apical surface of the dark midgut epithelial cell appears to pass through the peritrophic matrix (PM) and directly contact the bloodmeal (BM). The sections shown in **A** and **B** are immediately adjacent to one another while the section shown in **C** is approximately 6 µm from the section show in **B**. Note that except for the slightly dishevelled microvilli of midgut epithelial cell 2, the midgut epithelium immediately surrounding the malaria parasite in the sections shown in **A** and **B** appears morphologically normal. 28 hrs pbf.

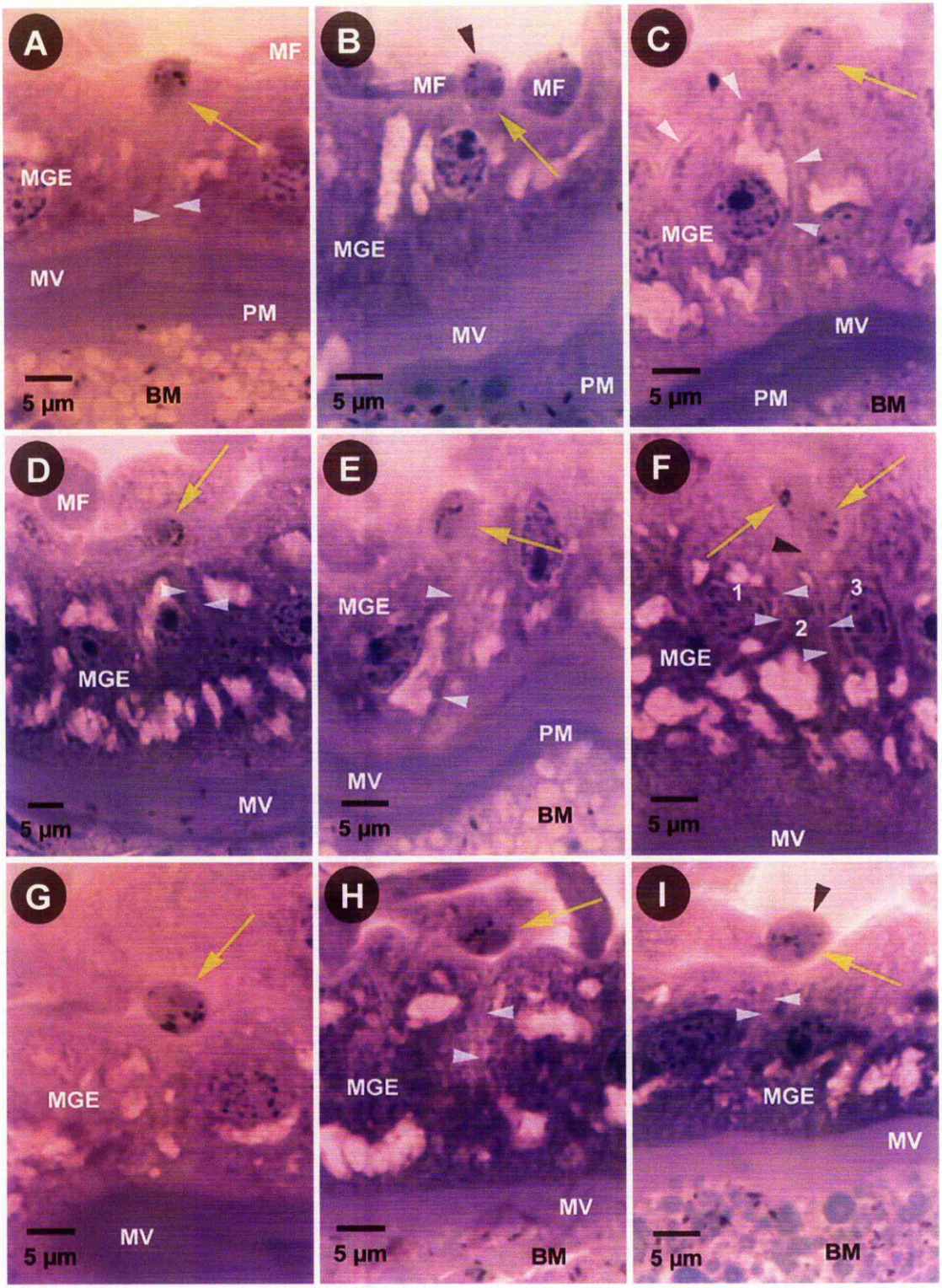


Figure 4.36 ***P. falciparum*** **3D7A oocysts surrounded by morphologically-normal midgut epithelium in** ***An. stephensi.***

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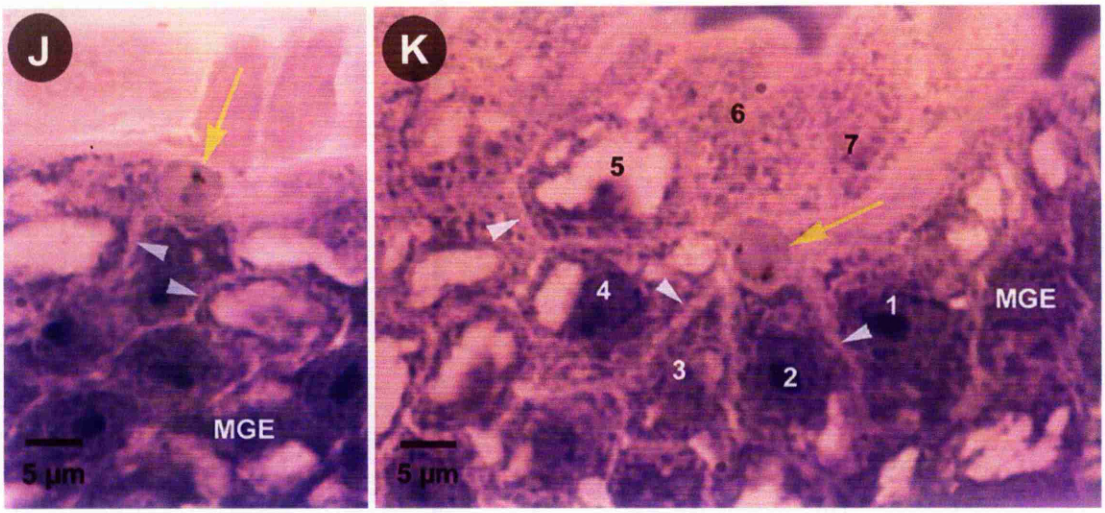


Figure 4.36 ***P. falciparum*** **3D7A oocysts surrounded by morphologically-normal midgut epithelium in** ***An. stephensi.***

Legend to images on this and previous page.

(A to I) Images of sections taken orthogonally to the longitudinal plane of the midgut epithelium showing different early oocysts (yellow arrows) on the basal surface of the midgut epithelium (MGE). The oocysts are located immediately above the lateral plasma membranes (white arrowheads) of the adjacent and underlying midgut epithelial cells. In **F** Two oocysts, located close together and apparently situated above where three adjacent midgut epithelial cells (1, 2 and 3) converge (dark arrowhead), are shown. A faint pink "halo", presumably corresponding to the basal lamina, is apparent around some of the oocysts shown (dark arrowheads in **B** and **I**). Note the apparent absence of the peritrophic matrix (PM) in **H** and **I** despite the continued presence of the digested bloodmeal (BM). **A** is 28 hrs pbf. **B** is 32 hrs pbf. **C to F** are 36 hrs pbf. **G** and **H** are 40 hrs pbf. **I** is 44 hrs pbf.

(J and K) Images of oblique sections through the longitudinal plane of the midgut epithelium showing different early oocysts on the basal surface of the midgut epithelium. The oocyst shown in **K** is surrounded by a "rosette" of adjacent midgut epithelial cells (numbered 1 to 7), which appear to converge underneath the malaria parasite. The midgut epithelium associated with the oocysts was morphologically normal and possessed no signs of the morphologically abnormalities associated with invading ookinetes. **J** and **K** are 32 and 36 hrs pbf, respectively.

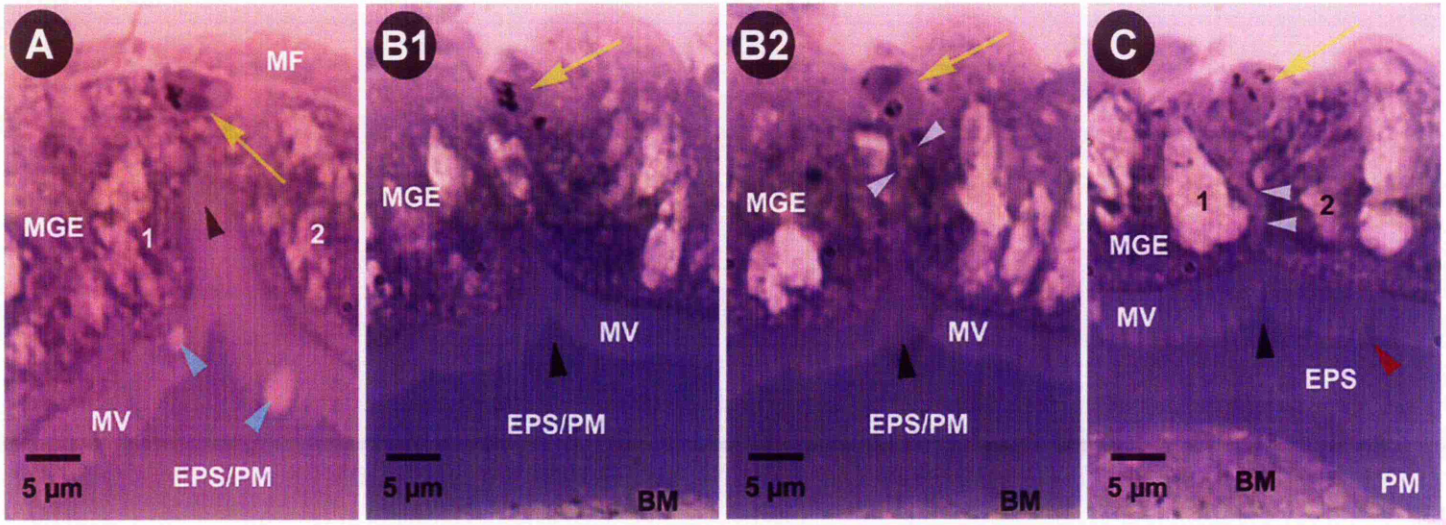


Figure 4.37 ***P. falciparum*** **3D7A ookinetes and oocysts, located on the basal surface of the midgut epithelium, situated above invaginations of the apical surface of the midgut epithelium in** ***An. stephensi.***

Examples of different ookinetes/oocysts (yellow arrows) located on the basal surface of the midgut epithelium (MGE) and situated immediately above invaginations of the apical surface of the midgut epithelium (dark arrowheads) occurring between adjacent midgut epithelial cells (1 and 2). No morphologically-abnormal midgut epithelial cells were associated with these malaria parasites. **(A)** Cyan arrowheads indicate "blebbing" of the apical surface of the midgut epithelial cells. **(B1/B2)** Consecutive serial sections showing a single ookinete/oocyst, apparently situated where three adjacent midgut epithelial cells meet: two distinct lateral plasma membranes converge beneath the malaria parasite (white arrowheads in **B2**). **(C)** The midgut epithelial cells (1 and 2) surrounding the oocyst are morphologically normal, although the microvilli (MV) of the midgut epithelial cells are slightly disorganised and protrude beyond the apical surface of the microvillar brush border of the surrounding midgut epithelium (red arrowhead). White arrowheads indicate lateral plasma membranes of the midgut epithelial cells. **A** is 36hrs pbf. **B** and **C** are 28 hrs pbf.

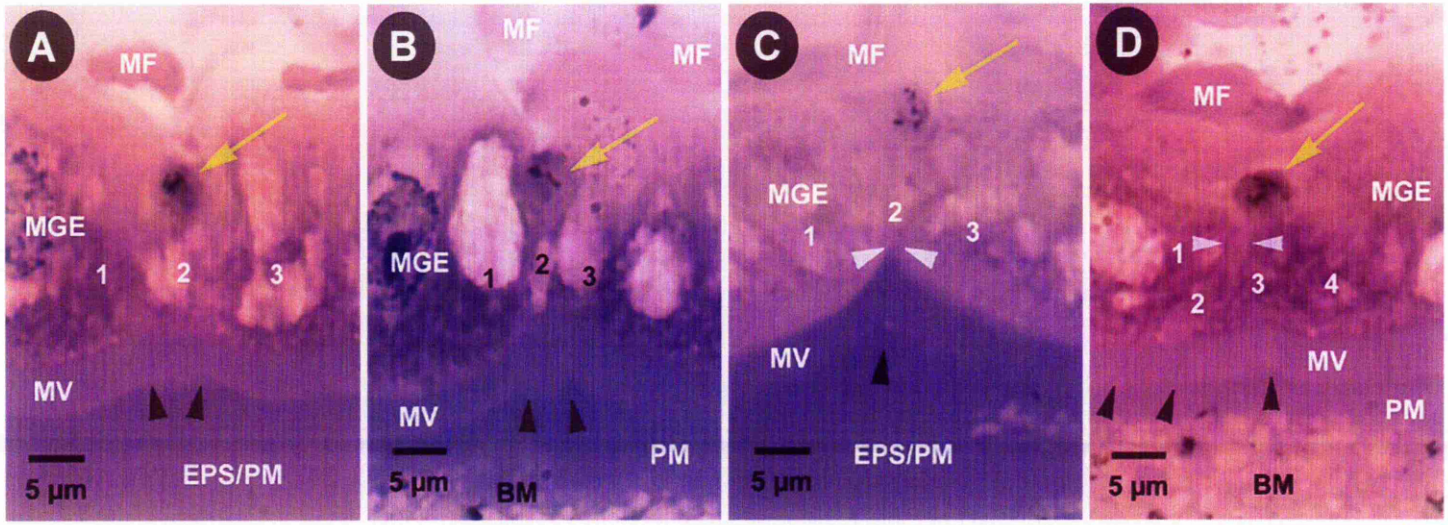


Figure 4.38 ***P. falciparum*** **3D7A ookinetes and oocysts, in the basal region of the midgut epithelium, associated with junctions where multiple adjacent midgut epithelial cells converge in** ***An. stephensi.***

Examples of different ookinetes/oocysts (yellow arrows) located in the basal region of the midgut epithelium (MGE) and associated with junctions where three adjacent midgut epithelial cells (1, 2 and 3) appear to converge. Dark arrowheads indicate "kinks" within the microvillar brush border (MV) of the apical surface of the midgut epithelium indicative of where adjacent midgut epithelial cells meet. White arrowheads indicate the lateral plasma membranes of converging midgut epithelial cells. No morphologically-abnormal midgut epithelial cells were associated with the malaria parasites shown. **(A)** Probably an intracellular ookinete emerging from the basal region of a morphologically-normal midgut epithelial cell. 28 hrs pbf. **(B to D)** Different oocysts on the basal surface of the midgut epithelium. In all three instances, one corner of midgut epithelial cell 2 is only just apparent in the sections shown. Note also the invagination of the apical surface of the midgut epithelium immediately beneath the oocyst shown **B** and **C**. All sections shown are 28 hrs pbf.

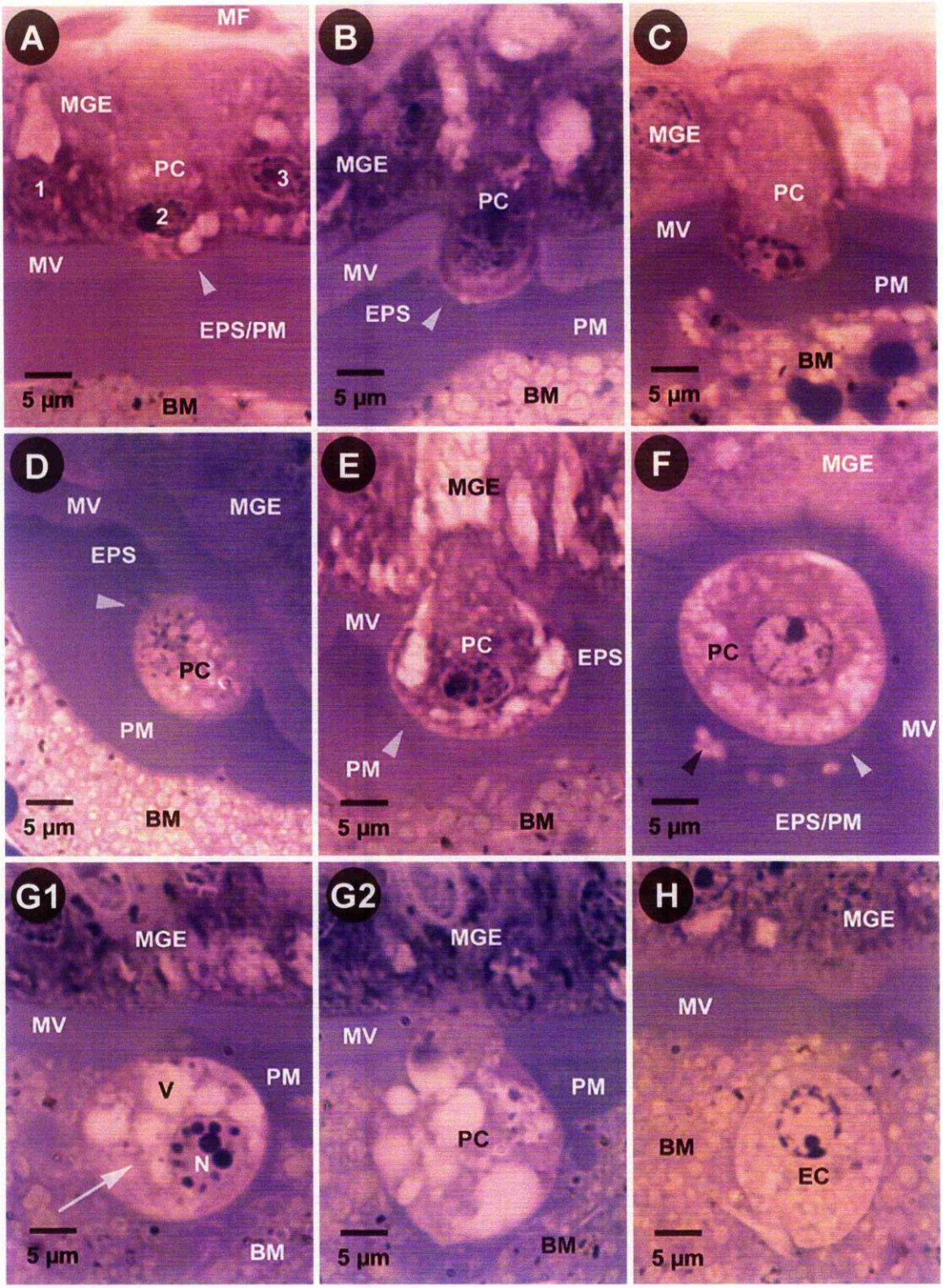


Figure 4.39 Normal and lightly staining "turgid" midgut epithelial cells exhibiting various degrees of extrusion from the midgut epithelium in *An. stephensi*.

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Figure 4.39 Normal and lightly staining "turgid" midgut epithelial cells exhibiting various degrees of extrusion from the midgut epithelium in *An. stephensi.*

Legend to images on previous page.

(A to H) Different examples of morphologically abnormal "turgid" midgut epithelial cells (PC) exhibiting varying degrees of extrusion from the midgut epithelium (MGE) into the ectoperitrophic space (EPS) of the midgut lumen. Although all the sections shown are from mosquitoes challenged with *P. falciparum* 3D7A, none of these extruded midgut epithelial cells exhibited any obvious association with malaria parasites invading the midgut epithelium. The midgut epithelial cells shown either exhibit relatively light staining (**D**, **G** and **H**) or staining of an intensity equivalent to the surrounding morphologically normal midgut epithelium (**A to C** and **F**). In comparison to the morphologically normal midgut epithelial cells, the protruding midgut epithelial cells possess few and, relatively short, microvilli (MV)(white arrowheads)(**A**, **B** and **C to E**). The nuclei of the protruding midgut epithelial cells, although morphologically normal, are also apical situated (**A to E**). For example, in **A**, compare the location of the nuclei of normal midgut epithelial cells (1 and 3) to the nucleus of the protruding midgut epithelial cell (2).

(A to C) Different midgut epithelial cells exhibiting minor protrusion from the midgut epithelium. **A** and **C** are 28 hrs pbf. **B** is 24 hrs pbf.

(D) The apical-most region of a protruding midgut epithelial cell, which appears to be completely separated from the midgut epithelium, is shown. However, in adjacent sections (not shown), the basal region of this midgut epithelial cell is situated within the midgut epithelium. 28 hrs pbf.

(E and F) Different midgut epithelial cells exhibiting marked protrusion from the midgut epithelium. Both **E** and **F** are 28 hrs pbf.

(G1/G2) Consecutive serial sections showing a single turgid midgut epithelial cell (white arrow/PC) situated within the bloodmeal (BM) but attached via a thin isthmus to the overlying midgut epithelium. The midgut epithelial cell apparently contains several large, spherical vacuoles (V) while the nucleus (N) possesses numerous small and intensely staining bodies. No microvilli are apparent around the surface of this midgut epithelial cell. 32 hrs pbf.

(H) A turgid midgut epithelial cell (EC) completely separated from the midgut epithelium and surrounded by the bloodmeal within the midgut lumen. 24 hrs pbf.

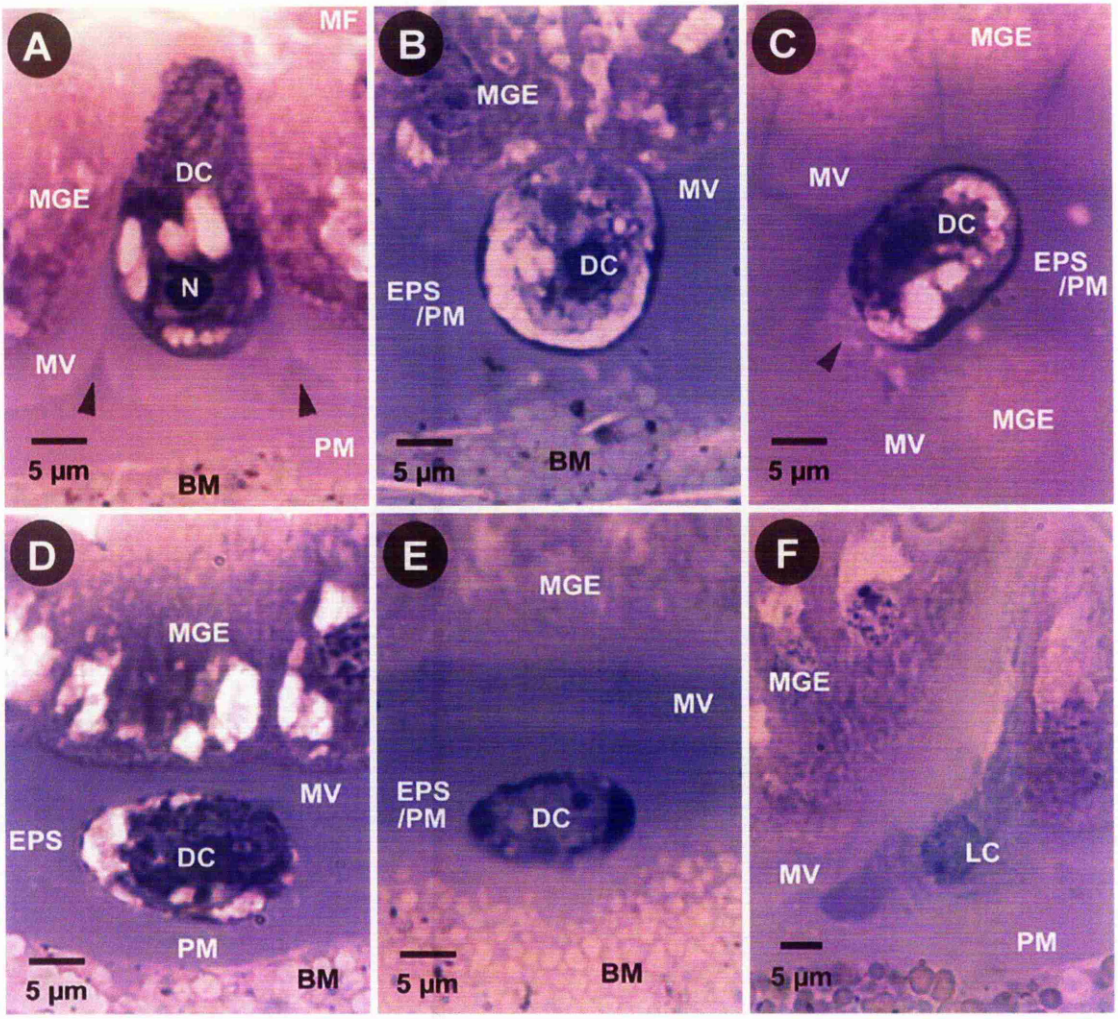


Figure 4.40 Dark and pale blue staining morphologically-abnormal midgut epithelial cells in *An. stephensi*.

(A to E) Different examples of dark staining morphologically-abnormal midgut epithelial cells (DC) exhibiting various degrees of protrusion form the midgut epithelium (MGE) into the ectoperitrophic space (EPS/dark arrowheads) of the midgut lumen. The nuclei of these midgut epithelial cells are single, highly condensed, and intensely staining, masses (**A to C**). **(A)** Dark and slightly "flaccid" midgut epithelial cell possessing dense microvilli (MV) located entirely within the midgut epithelium. **(B to E)** Relatively "turgid" dark staining midgut epithelial cells, some of which are completely separated from the midgut epithelium (**C to E**). **(F)** Oblique section through the longitudinal plane of the midgut epithelium showing a blue staining midgut epithelial cell (LC) "lysing" into the ectoperitrophic space. None of the morphologically-abnormal midgut epithelial cells shown exhibited any obvious association with invading malaria parasites. All sections are 28 hrs pbf.

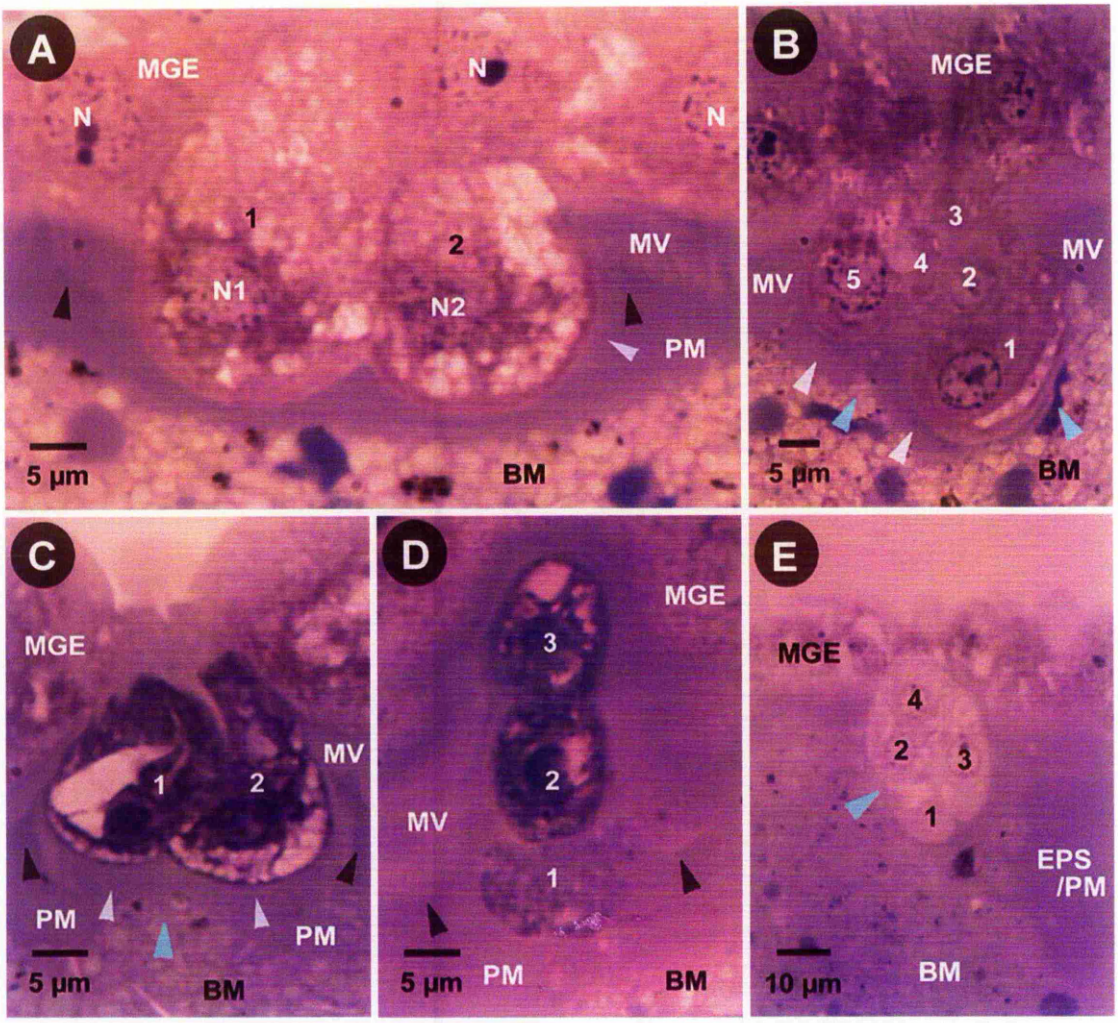


Figure 4.41 Multiple adjacent morphologically-abnormal midgut epithelial cells associated with invading *P. falciparum* 3D7A parasites in *An. stephensi*.

(A) Two adjacent normal staining midgut epithelial cells (1 and 2) protruding from the midgut epithelium (MGE) into the ectoperitrophic space (dark arrowheads). The midgut epithelial cells possess morphologically normal nuclei (N1 and N2) but the microvilli (MV) are sparse and short (white arrowheads). 28 hrs pbf. **(B)** Cluster of five normal staining midgut epithelial cells possessing dense microvilli and protruding into the midgut lumen. 28 hrs pbf. **(C)** Two adjacent dark staining and protruding midgut epithelial cells possessing condensed nuclei but abundant microvilli. 28 hrs pbf. **(D)** A trail of three similar dark staining midgut epithelial cells (1, 2 and 3) protruding into the ectoperitrophic space. 24 hrs pbf. **(E)** Cluster of four lightly staining midgut epithelial cells (1, 2, 3 and 4) protruding into the midgut lumen. 32 hrs pbf. Note absence/disruption of the peritrophic matrix (PM) around the protruding midgut epithelial cells (cyan arrowheads in **B**, **C** and **E**). Associated malaria parasites invading the midgut epithelium are not present in sections shown.

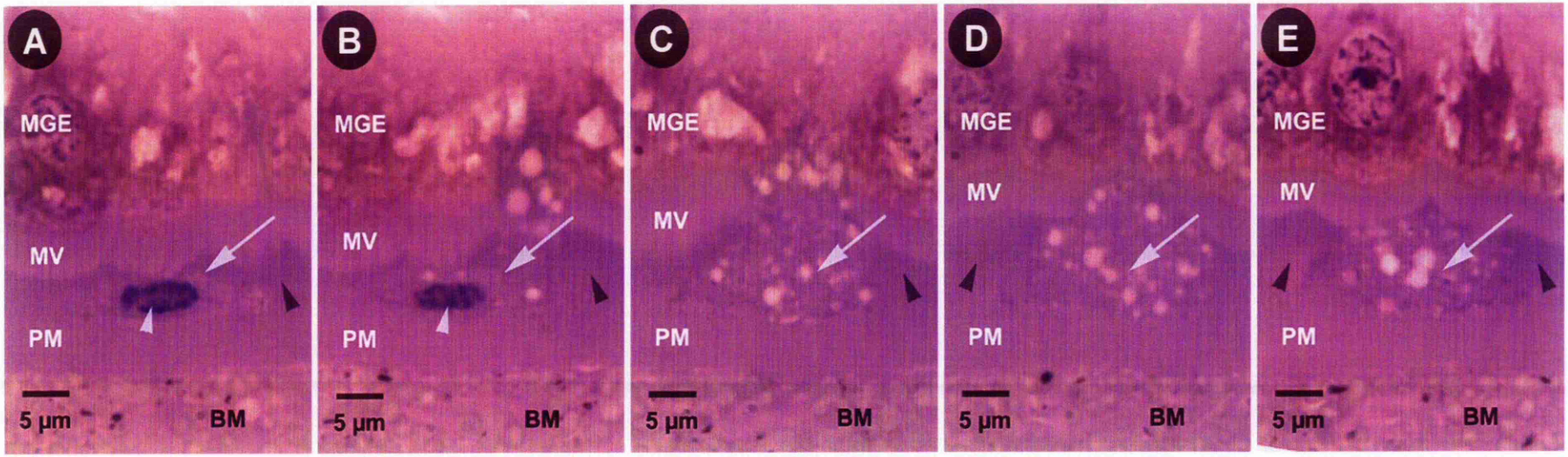


Figure 4.42 Morphologically-abnormal midgut epithelial cell protruding from the midgut epithelium in *An. stephensi*.

(A to E) Consecutive serial sections showing a single "dissolving" morphologically-abnormal midgut epithelial cell (white arrows) which is protruding from the midgut epithelium (MGE) into the ectoperitrophic space (dark arrowheads) of the midgut lumen. The midgut epithelial cell exhibits atypical pale blue staining, compared to the more purple staining characteristic of the midgut epithelium. The pale blue-staining morphologically-abnormal midgut epithelial cell is not easily discriminated from the peritrophic matrix (PM) and the ectoperitrophic space. However, the morphologically-abnormal midgut epithelial cell appears to be spreading, underneath the overlying midgut epithelium, through the ectoperitrophic space (**A** and **E**). Note that the peritrophic matrix does not exhibit the marked inward depression towards the bloodmeal associated with other types of morphologically-abnormal midgut epithelial cell (cf. Figure 4.17 and Figure 4.30A). The nucleus of the midgut epithelial cell (white arrowheads) is indicated in **A** and **B**. 28 hrs pbf.

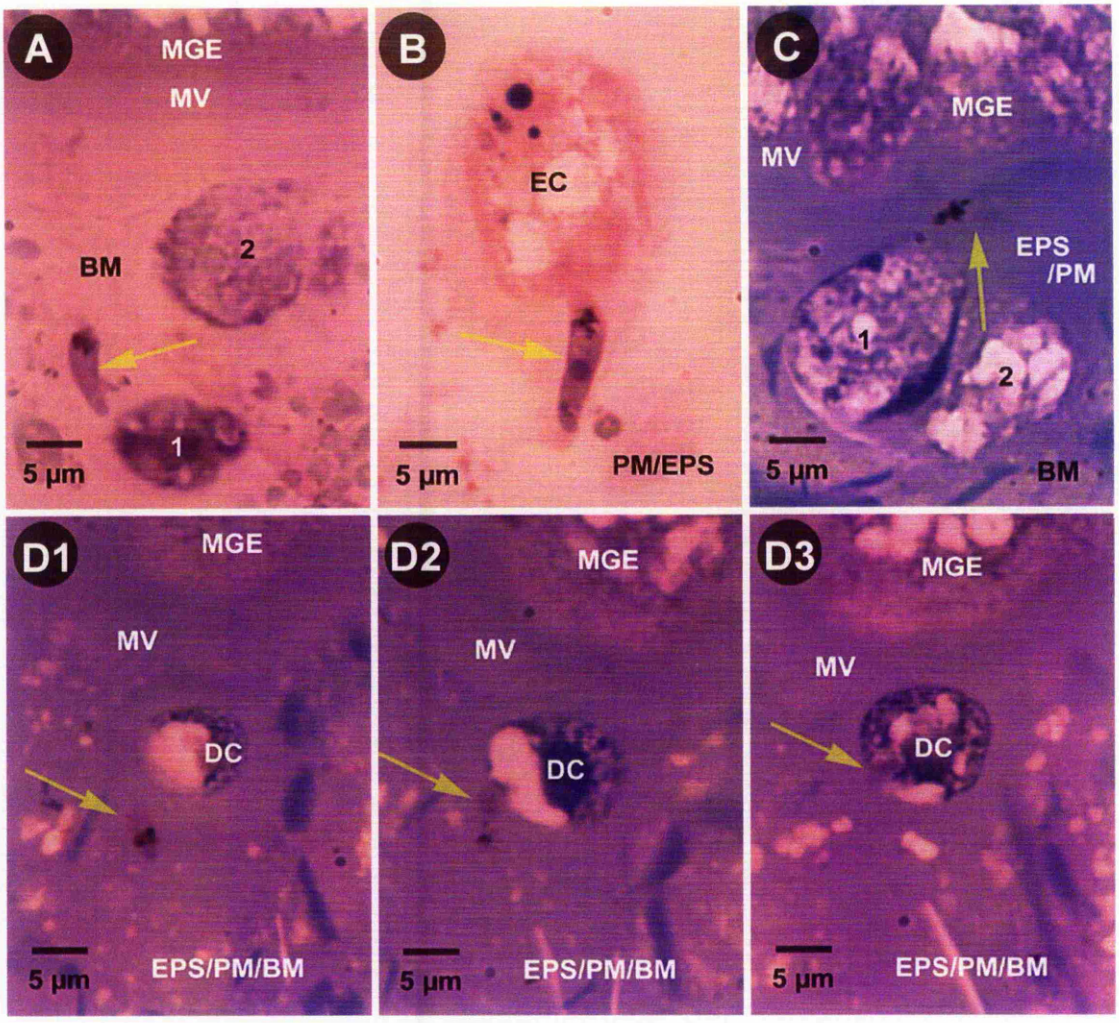


Figure 4.43 Extracellular *P. falciparum* 3D7A ookinetes within the midgut lumen associated with extruded midgut epithelial cells in *An. stephensi*.

(A) Ookinete (yellow arrow) within the bloodmeal (BM) in close proximity to two midgut epithelial cells (1 and 2) completely separated from the midgut epithelium (MGE). 32 hrs pbf.

(B) Extracellular ookinete within the ectoperitrophic space (EPS) in contact with a midgut epithelial cell (EC) separated from the midgut epithelium. 32 hrs pbf.

(C) Another extracellular ookinete within the ectoperitrophic space situated between two dark extruded midgut epithelial cells (1 and 2) and the overlying midgut epithelium. 32 hrs pbf.

(D1 to D3) Consecutive serial sections showing a single ookinete apparently partly within, and partly without, a dark turgid midgut epithelial cell completely separated from the midgut epithelium. The sections shown are oblique to the longitudinal plane of the midgut epithelium. 36 hrs pbf.

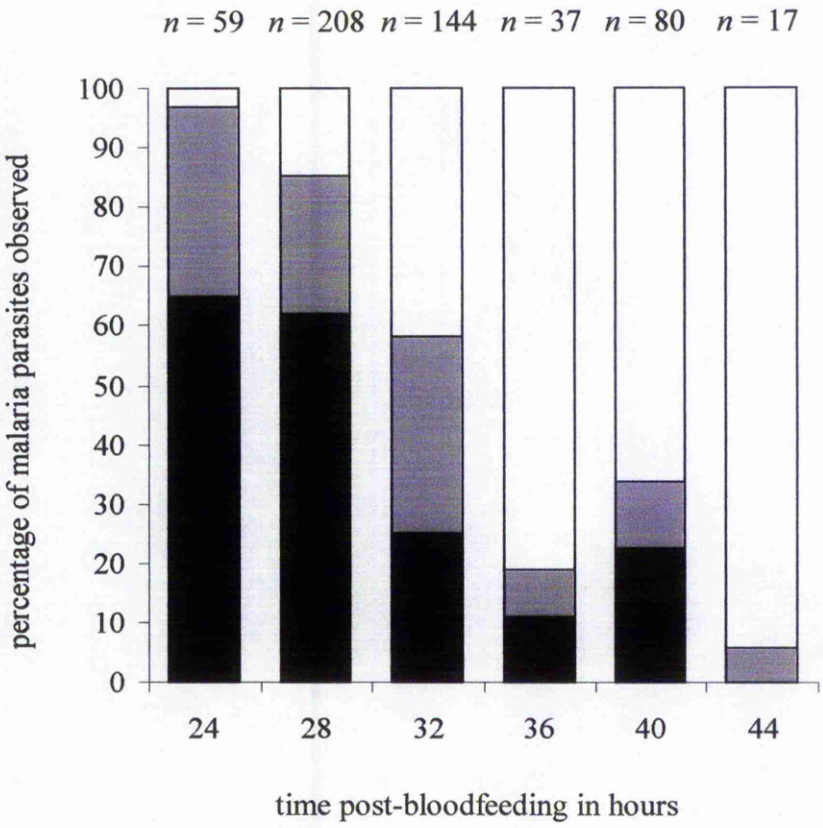


Figure 4.44 Temporal changes in the location within the midgut epithelium of *P. falciparum* 3D7A parasites observed in histological sections from *An. stephensi*.

Bar chart showing the percentage of malaria parasites found within different locations associated with the midgut epithelium at different times after bloodfeeding.

(■) Ookinetes associated with the peritrophic matrix, the ectoperitrophic space and/or the microvillar brush border of the apical surface of the midgut epithelium.

(■) Ookinetes entering or within the midgut epithelium, including intracellular malaria parasites within midgut epithelial cells completely separated from the midgut epithelium and extracellular malaria parasites within the midgut lumen associated with morphologically abnormal midgut epithelial cells.

(□) Oocysts observed on the basal surface of the midgut epithelium.

Numbers (*n*) above each bar indicate the total number of malaria parasites observed within the three different locations at each time point. Data from all time points of both infectious experimental bloodfeeds were pooled and are presented together.

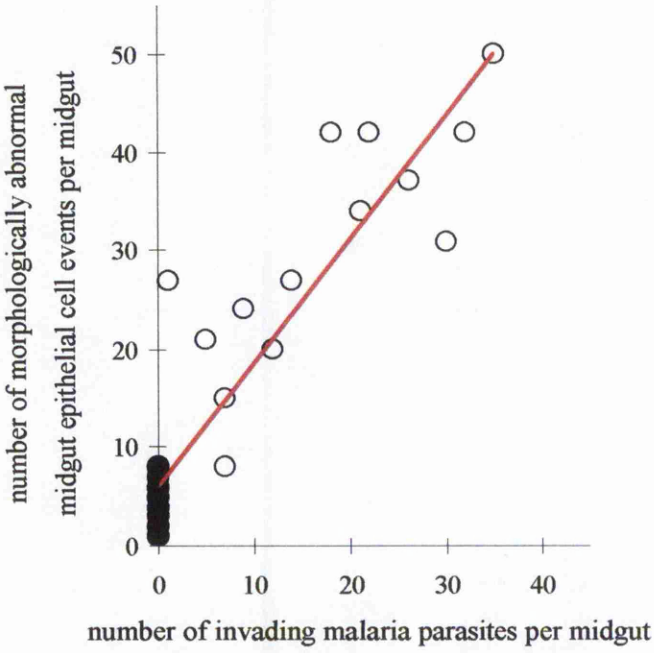


Figure 4.45 Relationship between the number of *P. falciparum* 3D7A parasites invading the midgut epithelium and the occurrence of morphologically abnormal midgut epithelial cells observed in histological sections from *An. stephensi*.

Scatter-plot showing the number of malaria parasites invading the midgut epithelium[1] against the number of morphologically abnormal midgut epithelial cell events observed within each midgut. Each data point represents a single midgut. (●) Mosquitoes fed either uninfected blood or non-infectious infected blood containing asexual erythrocytic stage malaria parasites alone. (○) Mosquitoes fed infectious blood containing both gametocyte and asexual erythrocytic stage malaria parasites. The line of best fit through the observed data determined by simple (bivariate) linear regression analysis is shown (red line). Data from all four experimental feeds were pooled but only midguts sampled between 24 and 36 pbf are plotted and were included in the statistical analysis due to the apparent disappearance of morphologically abnormal midgut epithelial cells with increasing time pbf (see Table 4.8 and Section 4.2.3.2).

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[1] All ookinetes within the midgut epithelium, including intracellular malaria parasites within midgut epithelial cells completely separated from the midgut epithelium and extracellular malaria parasites within the midgut lumen associated with morphologically abnormal midgut epithelial cells, together with oocysts, were classified as "malaria parasites invading the midgut epithelium". Therefore, the single ookinete observed entering a morphologically abnormal midgut epithelial cell was included while the 5 ookinetes observed entering morphologically normal midgut epithelium were excluded from the statistical analysis shown in this figure (see Section 4.3.3.3).

Chapter 5. Proliferative regeneration of the *An. stephensi* midgut epithelium following *P. falciparum* ookinete invasion

5. 1 Introduction

The results presented in Chapter 4 together with previously published work demonstrate that ookinete migration leads to the loss of ookinete-invaded midgut epithelial cells from the midgut epithelium (Becker-Feldman *et al.*, 1985; Han *et al.*, 2000; Zieler & Dvorak, 2000; Vlachou *et al.*, 2004). Whether destruction of ookinete-invaded midgut epithelial cells occurs in all malaria parasite-mosquito vector combinations, or even accompanies all invasion events within a particular malaria parasite-mosquito vector combination is uncertain. Nevertheless, when the level of malaria parasite infection is high, and large numbers of ookinetes simultaneously invade the midgut epithelium, an appreciable proportion of the total number of midgut epithelial cells is apparently destroyed (at least in the parasite-vector combinations that have been investigated). However, there is no direct evidence that ookinete invasion of the midgut epithelium *per se* is detrimental to the mosquito vector. For example, the mortality of very heavily infected mosquitoes is frequently no different from that of uninfected mosquitoes (Meis & Ponnudurai, 1987a; Han *et al.*, 2000; Ferguson & Read, 2002). This observation implies that adult female mosquitoes possess efficient mechanisms to restore the damaged midgut epithelium following malaria parasite infection. Indeed, such mechanisms have already been reported: the actin-based purse-string mechanism mediating extrusion of ookinete-invaded midgut epithelial cells and extension of filopodia/lamellipodia from the surrounding healthy uninvaded midgut epithelium beneath the extruding midgut epithelial cells (Han *et al.*, 2000; Vlachou *et al.*, 2004). Although these mechanisms account for the efficient removal of ookinete-damaged midgut epithelial cells and the maintenance of the integrity of the midgut epithelium during this process, neither mechanism explains how infected mosquitoes survive the loss of an appreciable proportion of the total number of midgut epithelial cells (Shahabuddin, 2002). It is possible that infected mosquitoes do not replace the midgut epithelial cells lost as result of ookinete invasion, and continue their remaining adult life with a midgut epithelium significantly reduced in the number of midgut epithelial cells. When the number of invading ookinetes is low, the mechanisms enabling removal of parasite-invaded midgut epithelial cells from the midgut epithelium

alone may be sufficient to account for the absence of any immediate and obvious negative effects on infected mosquitoes. However, when the number of invading ookinetes is high, the loss of a substantial fraction of the midgut epithelium would be expected to have at least some functional consequences for the mosquito vector. For example, a smaller volume of blood ingested during subsequent bloodfeeding due to the smaller size of the midgut, leading to a reduction in the number of eggs produced by each female mosquito.

An alternative explanation for the apparent absence of any major detrimental effect of ookinete invasion on the mosquito vector is that the midgut epithelium possesses the capacity to replace parasite-invaded midgut epithelial cells. Restoration of the midgut epithelium of infected mosquitoes could be achieved through proliferation and differentiation of midgut epithelial cell precursors generating new midgut epithelial cells that replace the midgut epithelial cells destroyed and lost as a consequence of ookinete invasion. Small pyramidally shaped cells have been found sparsely scattered throughout the basal region of the mosquito midgut epithelium (Hecker *et al.*, 1971a; Hecker *et al.*, 1971b; Hecker, 1977; Han *et al.*, 2000). By analogy to morphologically similar cells found in the midguts of other insects, these small cells are generally presumed to be a population of undifferentiated stem cells, known as regenerative cells, which are capable of dividing and differentiating into functionally mature midgut epithelial cells (Hecker *et al.*, 1971a; Hecker *et al.*, 1971b; Hecker, 1977). However, despite their nominal description, the function of "regenerative" cells in mature adult female mosquitoes is unproven: no evidence has previously been reported that these cells are mitotically active within the midgut epithelium of these mosquitoes (Hecker *et al.*, 1971b; Hecker, 1977; Weaver & Scott, 1990a). Consequently, some investigators have questioned whether regenerative cells are precursor cells with a midgut epithelial cell replacement function in mature adult female mosquitoes (Billingsley, 1990b; Weaver & Scott, 1990b).

In the current Chapter, further observations made from examining the histological sections described in the previous Chapter are reported. Evidence is presented that regenerative cells within the midgut epithelium of adult female *A. stephensi* mosquitoes undergo cell division and subsequently differentiate into normal columnar midgut epithelial cells. The level of regenerative cell division/differentiation in uninfected and *P. falciparum*-infected *A. stephensi* mosquito midguts is also compared. Unsuccessful attempts to confirm the occurrence of cell division within whole mounts of the mosquito

midgut epithelium, using immunofluorescent detection of incorporated 5-Bromo-2'-deoxyuridine, are also described.

5. 2 Materials & Methods

5.2.1 Parasite culture & mosquito infection

Malaria parasite culture and mosquito infections were performed as previously described in Chapter 2, Section 2. 2.

5.2.2 Preparation & examination of histological sections

The observations reported in this Chapter are based upon examination of the same histological sections reported in Chapter 4. The preparation of these Giemsa-stained histological sections from blood-fed mosquito midguts is also described in the previous Chapter, Section 4.2.2. Images were captured and processed as described in Chapter 4. Unless stated otherwise, all images shown were taken at 1000X magnification.

5.2.3 Estimation of the amount of regenerative cell division/differentiation

The relative amount of regenerative cell division/differentiation within each midgut was estimated by counting the total number of dividing/differentiating regenerative cells observed in every tenth histological section from each midgut. As individual midgut epithelial cells persist through multiple adjacent histological sections (typically 4 to 6 adjacent sections), every tenth section was examined to avoid the problem of sampling the same dividing/differentiating regenerative cells multiple times. For similar reasons, it was not possible to determine the absolute number of dividing/differentiating regenerative cells within each midgut from examining a subset of the histological sections alone.

5.2.4 Detection of cell proliferation using 5-Bromo-2'-deoxyuridine incorporation

5-Bromo-2'-deoxyuridine (BrdUrd) is a thymidine analogue that has been widely used in studies of the cell cycle and DNA replication (Pagano, 1995). BrdUrd is incorporated into newly synthesised DNA during the S-phase of the cell cycle and can be detected using specific monoclonal antibodies (Gratzner, 1982). BrdUrd incorporation was used to confirm the presence of mitotically active cells within the midgut epithelium of mature adult female mosquitoes.

One hundred microlitres of fresh BrdUrd (Sigma, Cat. No. B5002) stock solution in ddH_2O were added per 1.0 ml of infectious bloodmeal to give a final concentration of

100 or 325 μM BrdUrd. In some experiments, 5-fluoro-2'-deoxyuridine (FdUrd) (Sigma, Cat. No. F0503) and 2'-deoxycytidine (dCyd) (Sigma, Cat. No. D3897) were also simultaneously added to the bloodmeal to final concentrations of 20 and 40 μM, respectively. One hundred microlitres of ddH_2O were added per 1.0ml of bloodmeal given to a control group of mosquitoes. The bloodmeal mixture was fed to *An. stephensi* mosquitoes using standard membrane-feeding protocols and the mosquitoes maintained as previously described (Chapter 2, Section 2. 2). In one experiment, mosquitoes were also maintained both before and after bloodfeeding on standard glucose solution additionally supplemented with 325 μM BrdUrd.

At appropriate times pbf (1 to 5 days post-bloodfeeding), mosquitoes were dissected, their midguts removed and cut in half longitudinally or kept intact. The resulting midguts were then fixed in 2 to 4% paraformaldehyde in PBS at room temperature for at least 1 hour (or overnight at 4°C). Midguts were subsequently either exposed to 2.0M HCl for 15 to 30 minutes at room temperature or 0.1M HCl containing 0.7% Triton X-100 for 20 minutes at room temperature followed by incubation with *Eco*RI (150 units/ml) (New England Biolabs Ltd., Cat. No. R0101) for 1 hour at 37°C and then exonuclease III (300 units/ml) (New England Biolabs Ltd., Cat. No. M0206) for 1 hour at 37°C. This treatment was used to permeabilise the midgut epithelium and partially denature the DNA to allow access of the α-BrdUrd mAb. After deoxyribonuclease treatment, midguts were washed twice for five minutes each with PBS containing 20 mM EDTA. Midguts were blocked with PBS containing 1.0% BSA / 0.5% Tween-20 / 0.05% NaN_3 (PBT) and then incubated with primary α-BrdUrd (IgG1) mAb antibody (Sigma, Cat. No. B2531; clone BU33) diluted 1:50 to 1:1000 in PBT at room temperature for at least 1 hour or overnight at 4°C. Subsequently, midguts were incubated with secondary FITC-conjugated goat α-mouse IgG antibody (Southern Biotech, c/o Cambridge Bioscience) diluted between 1:20 to 1:50 in PBT for at least 1 hour at room temperature. Midguts were washed twice for five minutes each with PBS or PBT between all incubation steps, which were performed in flat-bottomed 96-well plates. After incubation of the secondary antibody, midguts were mounted under a cover slip with glycerol containing 2.5% DABCO and examined by epifluorescence microscopy using an appropriate FITC filter (Zeiss Filter Set 09).

As BrdUrd is light sensitive, as far as possible, all of the above procedures, including preparation and performance of the blood-feed and maintenance of the mosquitoes thereafter, were undertaken in the dark.

5.2.5 Statistical analysis

In order to maintain comparability between sample groups, and the assumption of a linear relationship between different variables in the regression analysis, only observations made between 24 and 36 hours pbf inclusive were analysed: the observations made at 40 and 44 hours pbf for the second sample group fed infectious gametocyte stage malaria parasites were excluded from the statistical analysis when making comparisons *between* sample groups. For statistical analyses *within* sample groups, all time points were included.

The number of dividing/differentiating regenerative cells within the midguts of mosquitoes given non-infectious and infectious bloodmeals were initially compared using the Kruskal-Wallis test of XLStat© version 7.5.2 (Addinsoft, 1995-2004). Multiple pairwise comparisons were subsequently performed manually using the Dunn test for samples of unequal size (Zar, 1984).

Simple (bivariate) and multiple linear regression analyses were performed using the REG procedure of the statistical software package SAS version 8.2 (SAS Institute Incorporated, Cary, NC, USA 1999-2001) and XLStat©. For the multiple linear regression analysis, four predictor variables were included and the best model chosen from all possible model combinations using Mallow's C_p and the Schwarz Bayesian information criterion (Quinn & Keough, 2002). Hierarchical partitioning was performed to quantify the proportion of variance in the response variable explained by each of the four predictor variables using publicly available software (http://www.R-project.org) (Chevan & Sutherland, 1991; Quinn & Keough, 2002; MacNally & Walsh, 2004). Data from all four experimental (non-infectious and infectious) bloodfeeds were pooled and included in all regression analyses (excluding the observations made at 40 and 44 hours pbf for the second sample group fed infectious gametocyte stage malaria parasites).

5. 3 Results

5.3.1 Histological evidence for division/differentiation of regenerative cells within the midgut epithelium of mature adult female *An. stephensi* mosquitoes

In Giemsa-stained histological sections, regenerative cells appear as small flat

triangular cells, which stain darkly and homogeneously (Figure 5.1A)[1]. Typically, regenerative cells possess a single, relatively large, central nucleus that occupies the majority of the cytosol. In two instances, however, regenerative cells were observed that appeared to contain two conjoined nuclei (Figure 5.1B). In general, regenerative cells occur singly, sparsely scattered throughout the basal region of the posterior midgut epithelium, in which they terminate, and isolated from one another by the surrounding columnar midgut epithelial cells. However, regenerative cells were occasionally observed in adjacent pairs, or, very rarely, triplets (Figure 5.1C and D). In these instances, the adjacent regenerative cells were morphologically similar but typically differed in size, with one of the regenerative cells being larger and extending further into the apical region of the midgut epithelium.

Very rarely single regenerative cells were observed immediately adjacent to small epithelial cells located entirely within the midgut epithelium, between neighbouring normal midgut epithelial cells (Figure 5.1E). These small epithelial cells resembled normal columnar midgut epithelial cells but possessed basally located nuclei and an undefined apical structure.

Regenerative cells were also observed immediately adjacent to another group of morphologically unusual midgut epithelial cells (Figure 5.2 and Figure 5.4). These unusual midgut epithelial cells were morphologically similar to both the rare small epithelial cells with undefined apical structures and normal columnar midgut epithelial cells. However, as well as basally located nuclei, these unusual midgut epithelial cells possessed a distinct apical cavity, regularly shaped ovoids typically lined peripherally by microvilli-like structures and sometimes containing centrally a yellow-staining refractory fibrotic material. These apical cavities were quite clearly distinct from the irregular non-staining regions, devoid of internal structure, frequently observed in all types of midgut epithelial cell (Chapter 4). The size of the apical cavity varied from small to large, but was typically of comparable size to the nuclei of normal columnar midgut epithelial cells. The unusual midgut epithelial cells were found throughout all regions of the midgut epithelium: there was no obvious localisation to any particular

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[1] All images presented in this Chapter are taken from the midguts of *An. stephensi* mosquitoes given an infectious bloodmeal containing cultured *P. falciparum* 3D7A clone gametocytes. However, similar cells were also observed within the midguts of the same mosquito species given uninfected or non-infectious *P. falciparum*-infected bloodmeals and *An. albimanus* given an infectious *P. falciparum* bloodmeal containing gametocytes from the 3D7A clone (images not shown).

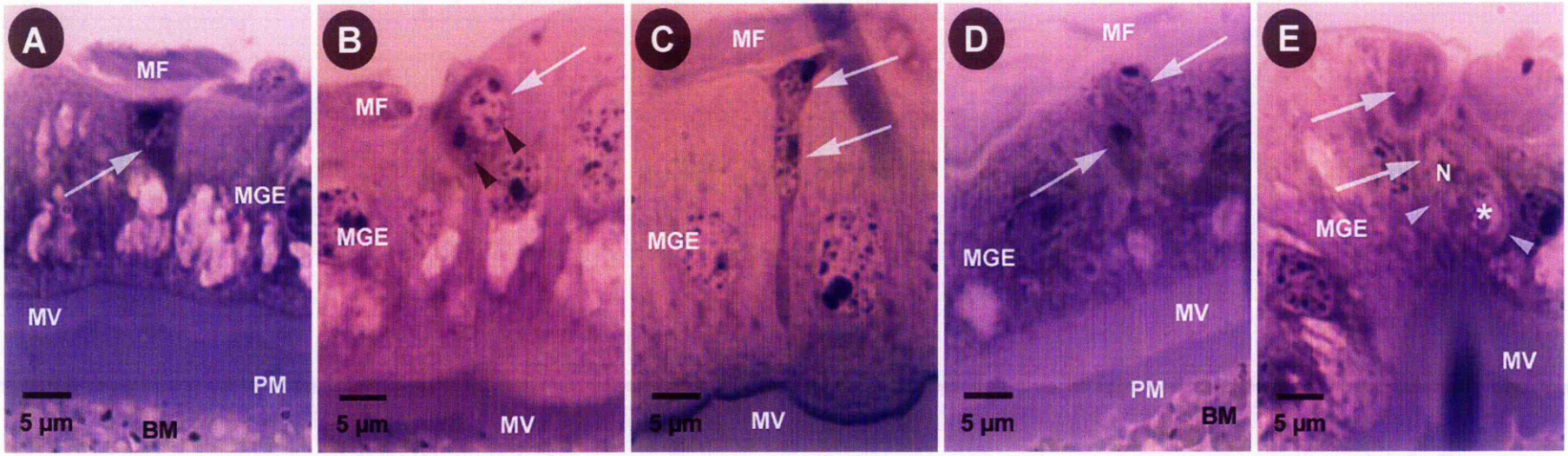


Figure 5.1 Regenerative cells within the midgut epithelium of *An. stephensi*.

(A) Regenerative cell (white arrow) within the basal region of the midgut epithelium (MGE). Note single large nucleus containing a prominent darkly staining nucleolus and muscle fibre (MF) overlying the midgut epithelium immediately above the regenerative cell. 28 hrs pbf.

(B) A single atypical regenerative cell (white arrow) apparently containing two conjoined nuclei (dark arrowheads). 32 hrs pbf.

(C) Two adjacent regenerative cells, one beneath the other and extending further into the apical region of the midgut epithelium. 24 hrs pbf.

(D) Two adjacent regenerative cells in the basal region of the midgut epithelium. Note larger size of the left-hand regenerative cell. 32 hrs pbf.

(E) A "rare small" midgut epithelial cell (lower white arrow) located immediately adjacent to a regenerative cell (upper white arrow). The nucleus (N) and plasma membranes (white arrowheads) of the small epithelial cell are indicated. An indistinct structure, possibly a nascent apical cavity, is present within the anterior region of the small midgut cell (asterisk). 36 hrs pbf.

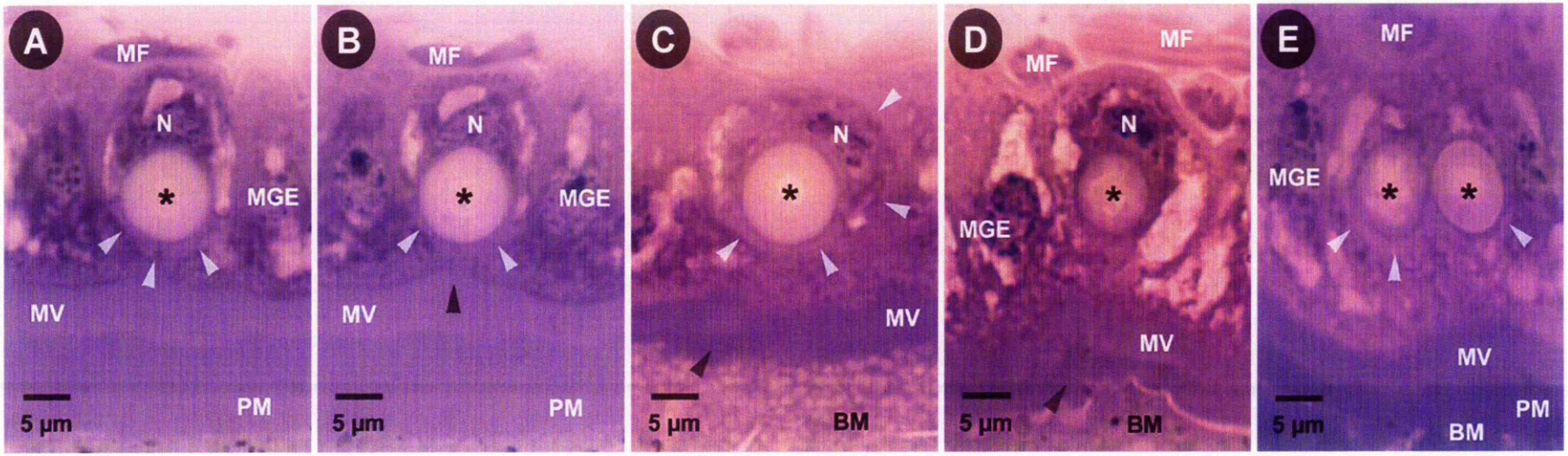


Figure 5.2 Unusual midgut epithelial cells possessing apical cavities lined by microvilli-like structures within *An. stephensi*.

(A and B) Consecutive serial sections showing a single unusual midgut epithelial cell possessing an apical cavity (asterisks) and basally located nucleus (N). The peripheral and central regions of the apical cavity appear distinct. White arrowheads indicate plasma membranes of the unusual midgut epithelial cell, whose anterior-most region terminates within the midgut epithelium. Also note slight invagination of the apical surface of the midgut epithelium immediately beneath the unusual midgut epithelial cell in B (dark arrowhead). 28 hrs pbf.

(C) Another unusual midgut epithelial cell, located entirely within the midgut epithelium, possessing a slightly eccentric nucleus. 28 hrs pbf.

(D) Unusual midgut epithelial cell containing obvious refractory yellow-staining material within the apical cavity. The plasma membranes of this unusual midgut epithelial cell are not readily apparent in the section shown. 32 hrs pbf.

(E) Two immediately adjacent unusual midgut epithelial cells possessing apical cavities lined by structures similar to the luminal microvilli (MV) of the midgut epithelium. The nuclei of the two unusual midgut epithelial cells are not present in the sections shown. 40 hrs pbf.

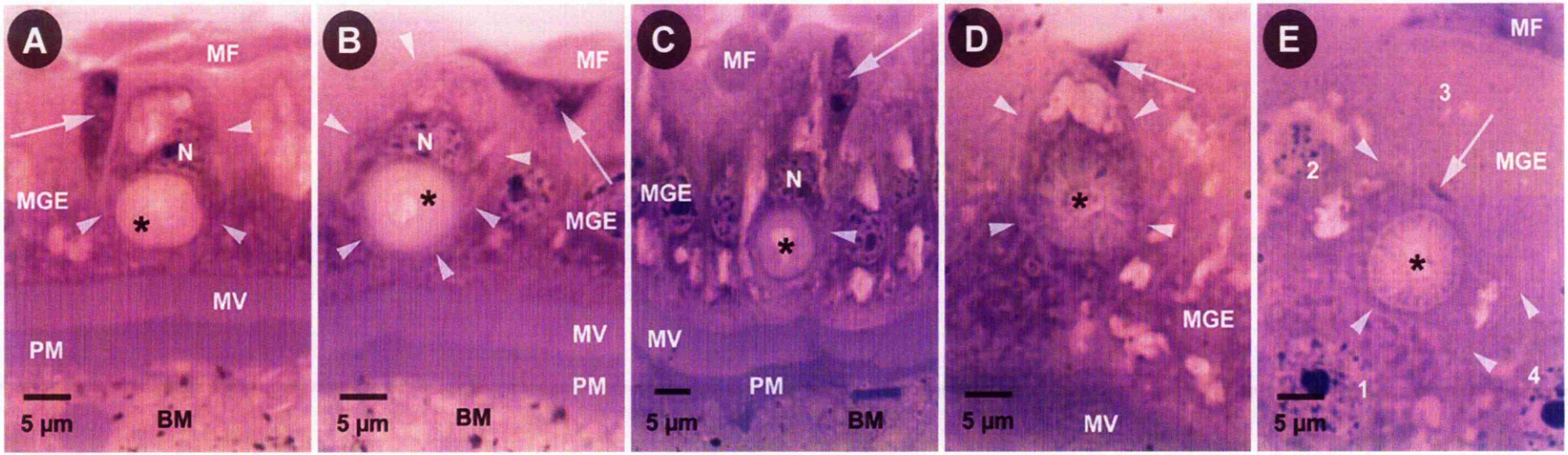


Figure 5.3 Unusual midgut epithelial cells possessing apical cavities found immediately adjacent to regenerative cells in *An. stephensi*.

(A to E) Examples of the unusual midgut epithelial cells found immediately adjacent to regenerative cells (white arrows). The apical cavities (asterisks), basal nuclei (N), and apical and plasma membranes (white arrowheads) of the unusual midgut epithelial cells are indicated.

(D) Oblique section through the plane of the midgut epithelium. Only the apical cavity of the unusual midgut epithelial cell and part of the adjacent regenerative cell are visible in the section shown. Note "striated" structures present around the periphery of the apical cavity, which surround a central mass.

(E) Section through the longitudinal plane of the midgut epithelium (MGE). Again, only the apical cavity of an unusual midgut epithelial cell, surrounded by at least four normal midgut epithelial cells (numbered 1, 2, 3 and 4), is present in the section shown. The narrow apical apex of a regenerative cell is visible immediately adjacent to the unusual midgut epithelial cell (white arrow).

Note lower magnification of C compared to A, B, D and E. (A and B) 28 hours pbf. (C) 36 hrs pbf. (D and E) 24 hrs pbf

area of the midgut. The regenerative cells located immediately adjacent to these unusual midgut epithelial cells were often not observed within the same histological section (Figure 5.2). Indeed, the potential significance of the unusual midgut epithelial cells and their association with regenerative cells was not recognised until both were repeatedly observed adjacent to one another in the same histological sections (Figure 5.3). Subsequent careful examination of consecutive serial sections revealed that the apparently isolated unusual midgut epithelial cells were always located immediately adjacent to a regenerative cell. Occasionally, the unusual midgut epithelial cells were also found in adjacent pairs (Figure 5.2E). These unusual midgut epithelial cells exhibited appreciable variation in size: some of the cells were of similar size to the rare small cells with undefined apical structures while others were of comparable size to normal columnar midgut epithelial cells. Accordingly, the location of the unusual midgut epithelial cells within the midgut epithelium also varied: the smaller cells tended to be positioned basally while the larger cells extended further into the apical region of the midgut epithelium. In most instances, the apical border of the unusual midgut epithelial cells appeared to terminate within the midgut epithelium; the underlying luminal microvilli apparently derived from the surrounding adjacent normal midgut epithelial cells (Figure 5.2 and Figure 5.3). These unusual midgut epithelial cells were clearly located between adjacent normal columnar midgut epithelial cells, which could be observed to meet in the apical region of the midgut epithelium immediately beneath the unusual midgut epithelial cell. Furthermore, the apical surface of the midgut epithelium immediately beneath the unusual midgut epithelial cells was often slightly invaginated where the adjacent underlying normal columnar midgut epithelial cells converged (e.g. Figure 5.2B).

In several instances, unusual midgut epithelial cells possessing apical cavities were observed whose anterior-most region breached the apical surface of the midgut epithelium such that the anterior region of the unusual midgut epithelial cell was directly exposed to midgut lumen (Figure 5.4). No luminal microvilli were apparent immediately beneath these particular unusual midgut epithelial cells, which appeared to possess a "bare" apical surface lacking microvilli.

A single midgut epithelial cell was also observed that possessed an indistinct and unusual "cap-like" apical structure (Figure 5.5). This midgut epithelial cell was immediately adjacent to a regenerative cell but lacked any obvious apical cavity and appeared to possess some luminal microvilli. However, refractory yellow material was

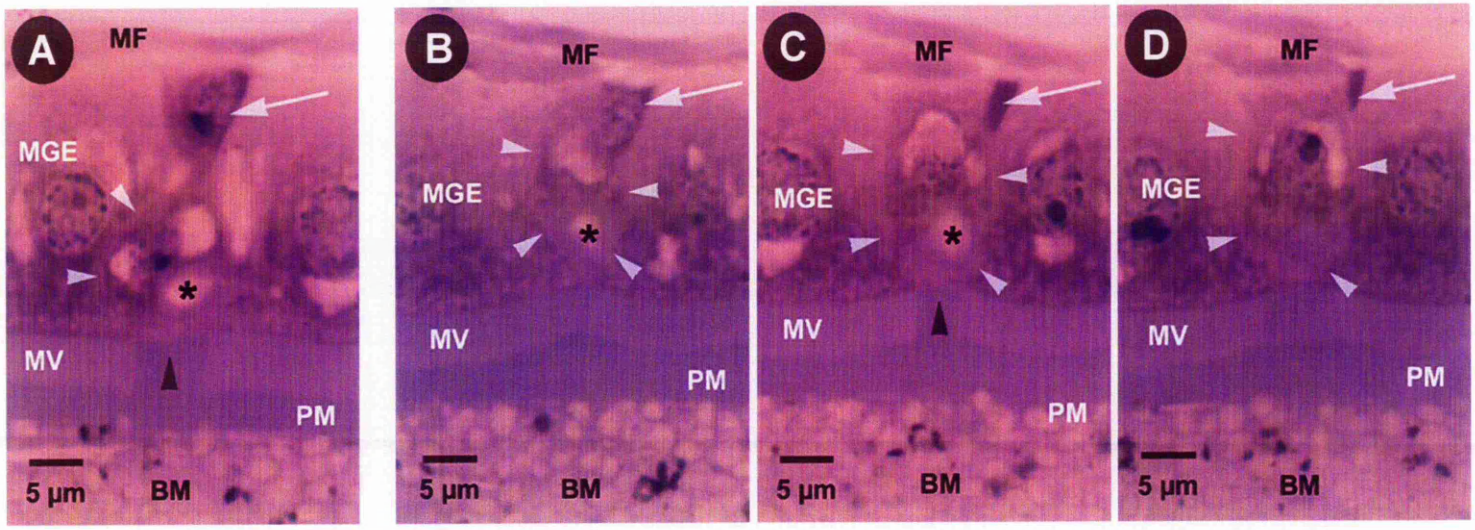


Figure 5.4 Unusual midgut epithelial cells possessing apical cavities extending through the midgut epithelium to the midgut lumen.

(A) An unusual midgut epithelial cell, immediately adjacent to a regenerative cell (white arrow), whose anterior-most region opens through the apical surface of the midgut epithelium to the midgut lumen. Note apparent absence of the luminal microvilli immediately beneath the unusual midgut epithelial cell (dark arrowhead). The apical cavity (asterisk) of the unusual midgut epithelial cell is small and only partly apparent in the section shown. White arrowheads indicate the plasma membranes of the unusual midgut epithelial cell. 28 hrs pbf

(B to D) Consecutive serial sections showing another single unusual midgut epithelial cell possessing an apical cavity, immediately adjacent to a regenerative cell, whose anterior-most region also opens through the midgut epithelium to the midgut lumen. Again, the apical cavity is relatively small and luminal microvilli are absent immediately beneath where the unusual midgut epithelial cell emerges through the apical surface of the midgut epithelium. Also, note slight invagination of the apical surface of the midgut epithelium immediately beneath the unusual midgut epithelial cell in B. 28 hrs pbf.

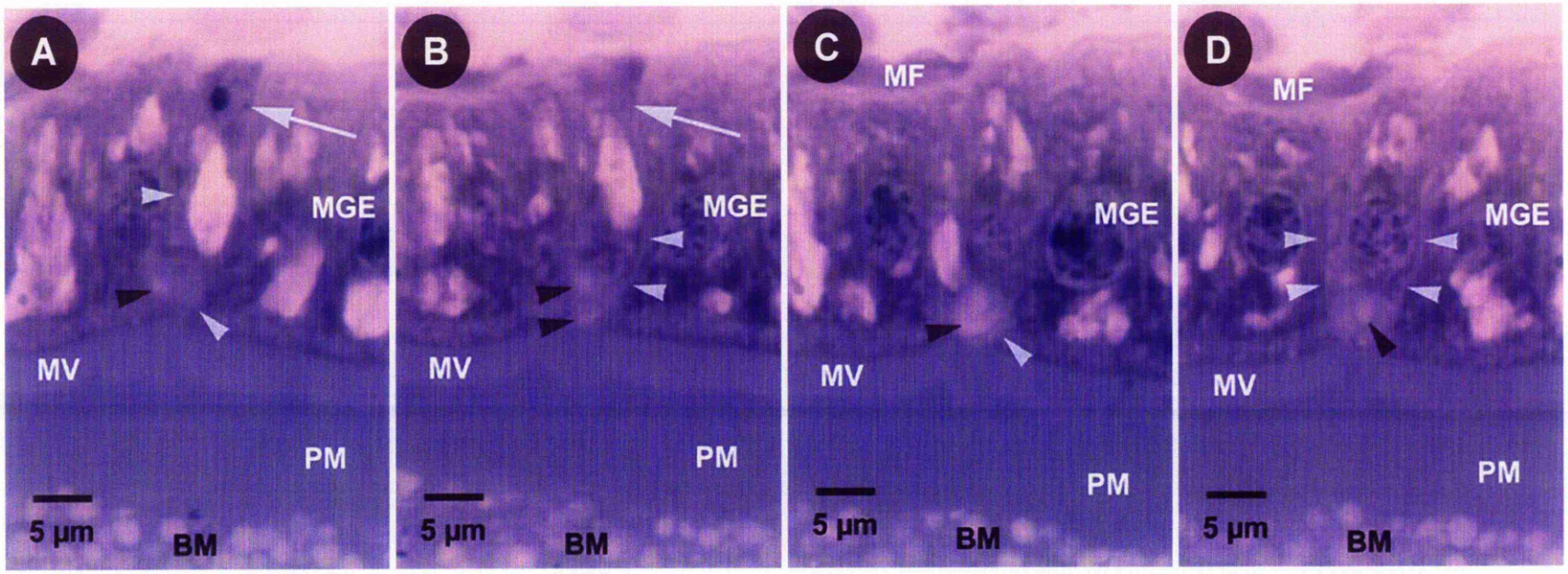


Figure 5.5 Unusual midgut epithelial cell within *An. stephensi* possessing an indistinct apical structure.

(A to D) Consecutive serial sections showing a single midgut epithelial cell, immediately adjacent to a regenerative cell (white arrow), possessing an unusual apical structure. The regenerative cell is only apparent in A and B, while the midgut epithelial cell, although present in all the sections shown, only becomes fully apparent in D. White arrowheads indicate the plasma membranes of the midgut epithelial cell. The apical region of the midgut epithelial cell appears to contain small yellowish clumps (dark arrowheads) reminiscent of the refractory fibrotic material observed within the microvilli-lined apical cavities of unusual midgut epithelial cells. Furthermore, in D, the apical region of the midgut epithelial cell appears to be distinctly delimited. The luminal microvilli (MV) immediately beneath the midgut epithelial cell are possibly less dense in B and C, and appear unusually long, protruding beyond the apical surface of the surrounding microvillar brush border in D. 28 hrs pbf.

present in the anterior-most region of this midgut epithelial cell that was reminiscent of that observed within the apical cavities of the unusual midgut epithelial cells described above.

As well as the relatively commonly encountered morphologies described above, several other "variants" of the unusual midgut epithelial cell possessing apical cavities were also observed extremely rarely (Figure 5.6). Some of these unusual midgut epithelial cells possessed large, darkly staining apical structures reminiscent of typical of apical cavities (Figure 5.6A). In other instances, the apical cavity was extremely large (sometimes bigger than a normal columnar midgut epithelial cell) and contained large amounts of globular, yellow staining material (Figure 5.6B). A single unusual midgut epithelial cell was observed that exhibited the dark intense staining typical of some of the extruding columnar midgut epithelial cells described in the previous Chapter (Figure 5.6C). This unusual midgut epithelial cell was otherwise morphologically "normal" possessing a typical apical cavity lined by microvilli-like structures and an eccentric nucleus.

Regenerative cell doublets and the unusual midgut epithelial cells possessing apical cavities lined by microvilli-like structures were also occasionally observed within close proximity to morphologically abnormal midgut epithelial cells extruding, or entirely separated, from the midgut epithelium (Figure 5.7). In one instance, an unusual midgut epithelial cell possessing an apical cavity was observed immediately above an extruding midgut epithelial cell (Figure 5.7B). However, it was not possible from the histological sections to determine the more general spatial relationships, if any, between these different cell morphologies. As reported in the previous Chapter, malaria parasites invading the midgut epithelium were not observed within regenerative cells or unusual midgut epithelial cells with any of the morphologies described above. Furthermore, there was no apparent spatial relationship between invading malaria parasites and any of these midgut cells.

5.3.2 Quantitative relationships between regenerative cell division / differentiation, *P. falciparum* infection and abnormal midgut epithelial cells

The morphological observations described above are presumed to represent the sequential stages of division and differentiation of regenerative cells into normal columnar midgut epithelial cells (see Section 5.4.1 and Figure 5.9 below). The amount of regenerative cell division/differentiation within each midgut could, therefore, be quantitatively estimated and compared by counting the number of relevant midgut cell

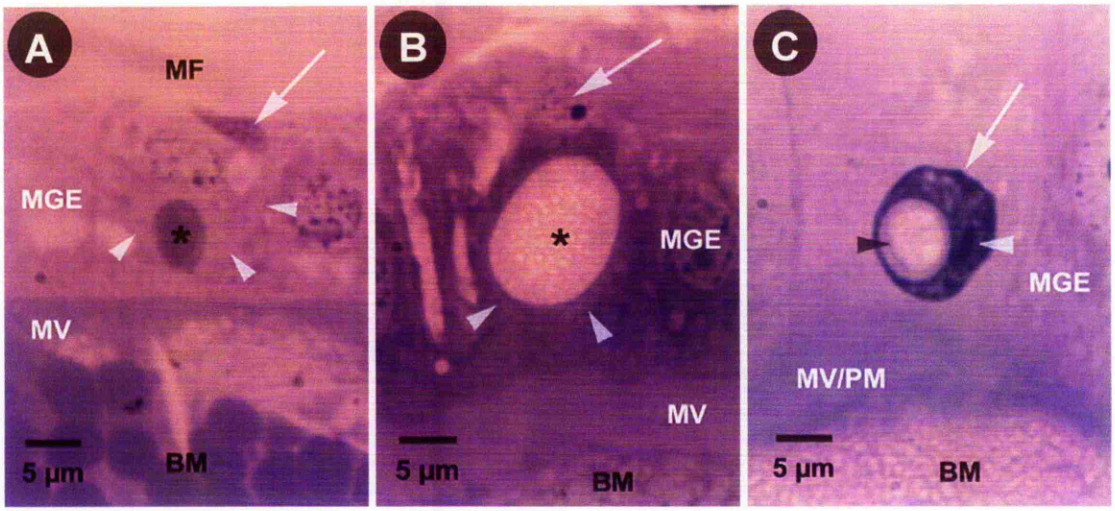


Figure 5.6 Unusual midgut epithelial cells possessing apical cavities from *An. stephensi* with atypical morphology.

(A) An usual midgut epithelial cell, located entirely within the midgut epithelium (MGE) and immediately adjacent to a regenerative cell (white arrow), which possesses a darkly staining apical structure (asterisk) as well as a basally located nucleus. White arrowheads indicate the plasma membranes of the unusual midgut epithelial cell. 24 hrs pbf.

(B) An unusual midgut epithelial cell, which possesses an unusually large apical cavity (asterisk). The apical region of the unusual midgut epithelial cell terminates within the midgut epithelium (white arrowheads). An immediately adjacent regenerative cell is visible while the nucleus of the unusual midgut epithelial cell is not present in the section shown. 36 hrs pbf.

(C) An atypical darkly staining unusual midgut epithelial cell (white arrow) possessing an apical cavity (dark arrowhead) lined by microvilli-like structures and a laterally located nucleus (white arrowhead). The unusual midgut epithelial cell is clearly located entirely within the midgut epithelium (MGE). 24 hrs pbf.

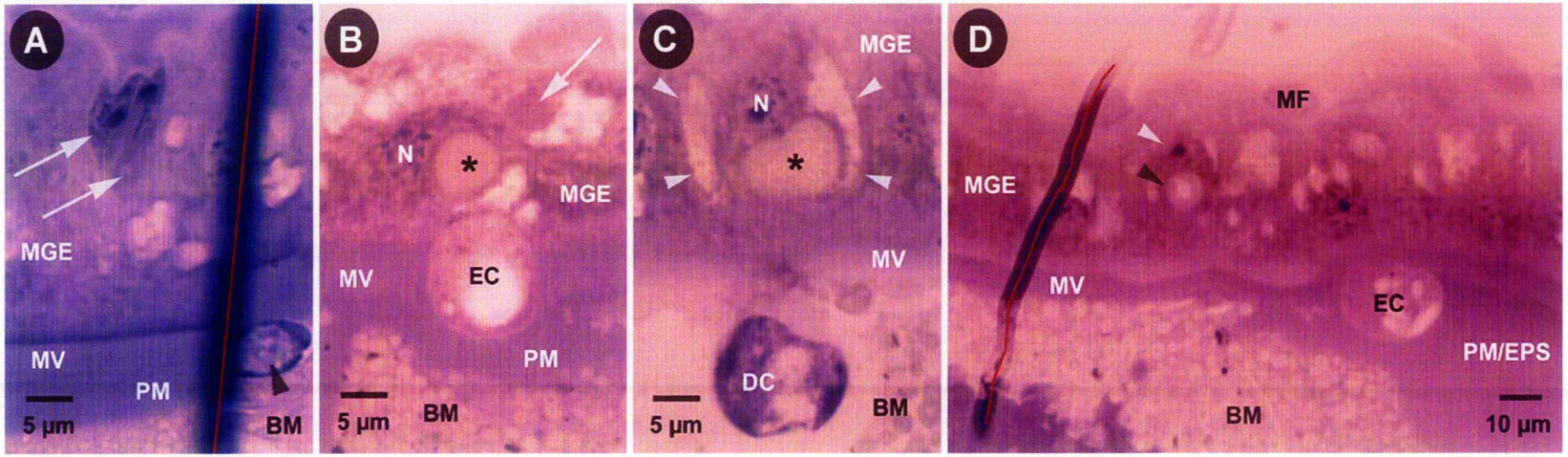


Figure 5.7 Regenerative and unusual midgut epithelial cells possessing apical cavities associated with extruding midgut epithelial cells.

(A) Two adjacent regenerative cells (white arrows) in close proximity to an extruded midgut epithelial cell (dark arrowhead) located within the bloodmeal (BM) beneath the peritrophic matrix (PM). A crease incorporating approximately 10 µm of the width of the section is indicated (red line). 24 hrs pbf.

(B) An unusual midgut epithelial cell possessing an apical cavity (asterisk) and laterally located nucleus (N), adjacent to a partly apparent regenerative cell (white arrow), located immediately above an abnormal midgut epithelial cell (EC) protruding into the midgut lumen. 28 hrs pbf.

(C) An unusual midgut epithelial cell possessing an apical cavity and basally located nucleus, immediately above a dark midgut epithelial cell (DC) present within the bloodmeal and completely separated from the midgut epithelium. 32 hrs pbf.

(D) Lower magnification image (400X) showing an unusual midgut epithelial cell, possessing an apical cavity (dark arrowhead) and basal nucleus (white arrowhead), in close proximity to an extruded midgut epithelial cell (EC) within the ectoperitrophic space (EPS). 28 hrs pbf.

morphologies observed within the histological sections from each midgut (Table 5.1). However, the possible existence of regenerative cell division/differentiation was not appreciated until substantially more than a few thousand histological sections had already been examined! Consequently, as examination of the histological sections was a laborious and tedious task, rather than re-examine *all* of the above mentioned sections again, the *relative* amount of regenerative cell division/differentiation within each midgut was estimated by scoring a fixed proportion of the total number of histological sections from each midgut (Section 5.2.3).

Dividing and differentiating regenerative cells were found in midgut epithelia from mosquitoes fed both uninfected and malaria parasite-infected blood. However, the relative number of dividing and/or differentiating regenerative cells observed within each midgut differed significantly between mosquitoes offered different bloodmeals (Kruskal-Wallis test, $\chi^2_{0.05,\,7.815} = 15.934$, d.f. = 3, $P = 0.0012$) (Table 5.1). The number of dividing and/or differentiating regenerative cells did not differ significantly between either of the two groups of mosquitoes fed non-infectious blood (either uninfected or containing asexual erythrocytic stage parasites alone) or the two groups of mosquitoes fed infectious blood (containing sexual gametocyte stage parasites). In contrast, the number of dividing and/or differentiating regenerative cells was significantly higher in mosquitoes fed gametocytes than asexual erythrocytic stage parasites alone. Although the median number of dividing and/or differentiating regenerative cells was also similarly higher in mosquitoes fed gametocytes than uninfected blood, this difference did not attain statistical significance, possibly because of the small sample sizes involved and low power of the non-parametric multiple comparison test employed (Table 5.1).

The median number of dividing and/or differentiating regenerative cells did not differ significantly over time *within* each of the groups of mosquitoes offered different bloodmeals (Kruskal-Wallis test, uninfected bloodfeed: $\chi^2_{0.05,\,7.815} = 6.557$, d.f. = 3, $P = 0.087$; infected non-infectious bloodfeed: $\chi^2_{0.05,\,7.815} = 4.105$, d.f. = 3, $P = 0.250$; 1st infectious bloodfeed: $\chi^2_{0.05,\,3.841} = 2.193$, d.f. = 1, $P = 0.139$; and 2nd infectious bloodfeed: $\chi^2_{0.05,\,11.070} = 6.376$, d.f. = 3, $P = 0.271$) (Table 5.1). In the second group of mosquitoes fed infectious blood, with the broadest range of time points sampled, the median number of dividing and/or differentiating regenerative cells observed was lower at 24 and 28 hours pbf than 32 and 36 hours pbf, and markedly lower at 40 and 44 hours pbf compared to the other time points sampled (Figure 5.8 and Table 5.1).

Table 5.1 Comparison of the number of dividing and/or differentiating regenerative cells observed in histological sections from *An. stephensi* fed uninfected or *P. falciparum* 3D7A-infected blood.

Experimental feed	Time (hours pbf)	n[a]	Total number of dividing / differentiating regenerative cells	Median number of dividing / differentiating regenerative cells per midgut
Uninfected:	24	2	10	5.0
no malaria	28	2	2	1.0
parasites	32	2	4	2.0
	36	2	18	9.0
	Overall[b]	8	34	3.5^{yz}
Infected:	24	2	4	2.0
asexual stages	28	2	5	2.5
only	32	2	11	5.5
	36	2	1	0.5
	Overall	8	21	2.0^{y}
1st Infectious:	28	2	22	11.0
gametocytes +	32	3	49	16.0
asexual stages	Overall	5	71	14.0^{z}
2nd Infectious:	24	3	24	8.0
gametocytes +	28	2	24	12.0
asexual stages	32	2	36	18.0
	36	2	37	18.5
	(Sub-total	9	121	$13.0)^{z}$
	40	2	7	3.5
	44	2	11	5.5
	Overall	13	149	8.0

[a] The number of midguts examined. Only 10% of the sections from each midgut were examined. [b] Grey boxes highlight summaries of midguts used in statistical analysis. ^{y,z} indicate significant differences, identified in pairwise comparison tests, between experimental feeds, in the overall median number of dividing/differentiating regenerative observed. Different letter indicates significant, while same letter indicates no significant, difference between experimental feeds in the overall number of morphologically abnormal midgut epithelial cell events observed. See main text for details of the statistical analyses.

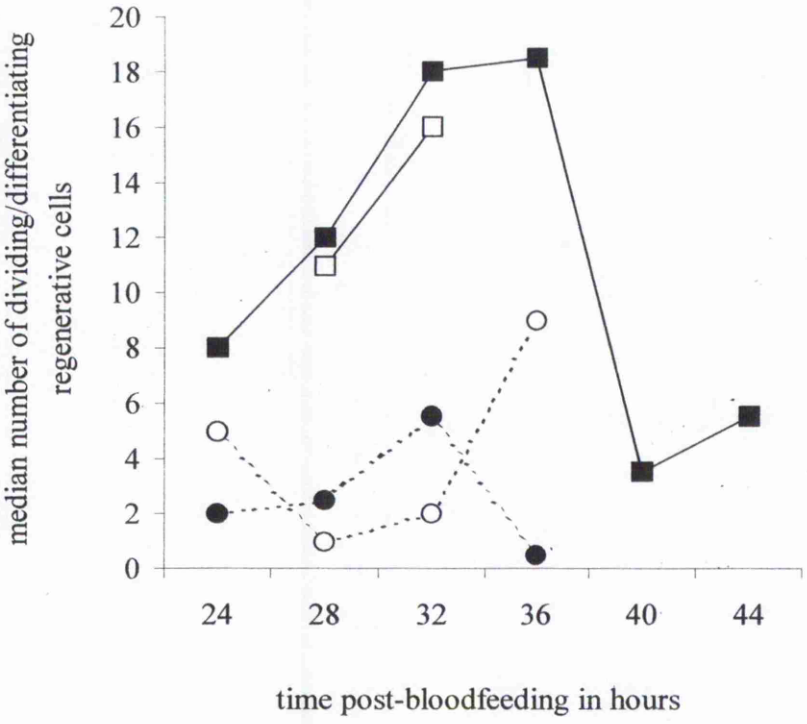


Figure 5.8 Temporal changes in the number of dividing and/or differentiating regenerative cells observed within the midgut epithelium of *An. stephensi*.

Graph showing the change over time in the relative median number of dividing and/or differentiating regenerative cells observed per midgut for four different groups of *An. stephensi* fed either uninfected or malaria parasite-infected blood. Key: (○) mosquitoes fed uninfected blood; (●) mosquitoes fed infected but non-infectious blood containing asexual erythrocytic stage malaria parasites only; (□) 1st group of mosquitoes fed infectious blood containing both asexual and sexual erythrocytic stage malaria parasites; and (■) 2nd group of mosquitoes fed infectious blood containing both asexual and sexual erythrocytic stage malaria parasites. See Table 5.1 for details of the number of mosquitoes examined.

As reported in Chapter 4, *P. falciparum* malaria parasites invading the *An. stephensi* midgut epithelium are significantly associated with morphologically-abnormal midgut epithelial cells extruding into the midgut lumen. Consequently, the relationships between the relative amount of regenerative cell division/proliferation, the absolute numbers of malaria parasites invading the midgut epithelium, and morphologically-abnormal midgut epithelial cells could be investigated using the counts previously reported in Chapter 4.

In bivariate regression analyses, the relative number of dividing and/or differentiating regenerative cells within each midgut exhibited significant positive linear correlations with both the absolute numbers of malaria parasites infecting the midgut epithelium, and of morphologically-abnormal midgut epithelial cells present within each midgut (Table 5.2). The numbers of oocysts and abnormal midgut epithelial cells each accounted for approximately 60% of the variation observed in the number of dividing and/or differentiating regenerative cells ($F_{1,28} = 38.91$, $P < 0.0001$, $r^2 = 0.582$ and $F_{1,28} = 37.71$, $P < 0.0001$, $r^2 = 0.574$ respectively). In contrast, the number of ookinetes invading the midgut epithelium exhibited a relatively weak positive association, explaining approximately 25% of the variation observed in the number of dividing and/or differentiating regenerative cells ($F_{1,28} = 9.15$, $P = 0.005$, $r^2 = 0.246$).

In multiple linear regression analysis, the model with the best fit to the observed data explained over 75% of the variation in number of dividing and/or differentiating regenerative cells within each midgut ($F_{3,26} = 28.47$, $P < 0.0001$, $r^2 = 0.767$) (Table 5.3) (Appendix 2). This model retained three of the four initial predictor variables with the partial regression slopes for each of the retained predictor variables differing significantly from zero (Table 5.3). In contrast to the bivariate analysis, the number of ookinetes invading the midgut epithelium was negatively associated the number of dividing and/or differentiating regenerative cells in the best model identified by multiple linear regression analysis. However, both the numbers of oocysts and abnormal midgut epithelial cells were again positively associated with the occurrence of dividing and/or differentiating regenerative cells.

As previous regression analyses indicated significant covariation between the predictor variables (Table 5.2), hierarchical partitioning was used to estimate the independent and joint correlation of each predictor variable to the response variable (Table 5.4). Hierarchical partitioning confirmed the importance of the three predictor variables retained in the best model identified by multiple linear regression analysis,

Table 5.2 Matrix of Pearson's (product-moment) correlation coefficients (r) describing the linear bivariate relationships between five variables in the midguts of *An. stephensi* mosquitoes 24 to 36 hours post-bloodfeeding (n, the number of midguts examined = 30).

	Time pbf (hours)	Number of ookinetes within the midgut epithelium	Number of oocysts	Number of abnormal midgut epithelial cell events[a]
Number of ookinetes within the midgut epithelium	0.112	-		
Number of oocysts	0.286	0.638*	-	
Number of abnormal midgut epithelial cell events[a]	0.079	0.889*	0.777*	-
Number of dividing/differentiating regenerative cells	0.249	0.496*	0.763*	0.758*

* Indicates correlation coefficient significantly different from zero (i.e. no association between the variables)($F_{1,28} > 9.147$, $P < 0.005$ in all cases). Correlation coefficients without an asterisk were not significantly different from zero ($F_{1,28} < 2.488$, $P > 0.126$ in all cases).

[a] As ookinetes frequently migrate through more than one midgut epithelial cell, clusters of multiple adjacent morphologically abnormal cells were counted as single events.

Table 5.3 Details of the multiple linear regression model with the best fit to the relative number of dividing/differentiating regenerative cells observed within each *An. stephensi* mosquito midgut ($n = 30$).

Model Parameter	Coefficient	t statistic[a]	P value	95% Confidence interval
Intercept	1.747	1.603	0.1209	-0.493 to 3.986
Number of ookinetes within midgut epithelium	-0.991	-3.602	0.0013	-1.557 to -0.426
Number of oocysts	0.367	2.211	0.0361	0.026 to 0.708
Number of abnormal midgut epithelial cell events	0.552	4.540	0.0001	0.302 to 0.802

[a] For a two-tailed test with $\alpha = 0.05$ and 28 d.f., the critical value of $t = \pm 2.05$

Table 5.4 Hierarchical partitioning of the explained variation in the relative number of dividing/differentiating regenerative cells observed within each *An. stephensi* midgut between the four predictor variables included in the multiple linear regression analysis (n = 30).

Predictor variable	Independent effect[a]	Joint effect[a]	z statistic[b]	P value
Time post-bloodfeeding (hours)	0.0276	0.0343	-0.14	0.4443
Number of ookinetes within midgut epithelium	0.1434	0.1028	2.65	< 0.0047
Number of oocysts	0.2619	0.3196	5.43	< 0.0001
Number of abnormal midgut epithelial cell events	0.3345	0.2394	7.14	< 0.0001

[a] Numbers given are partial r^2 values (i.e. proportion of variance in the response variable explained by each predictor variable either independently or jointly with one or more of the other predictor variables). For each predictor variable, the sum of the independent and joint effects equals r^2 when that predictor variable alone is regressed against the response variable (see Table 2 for the relevant r values). The sum of all independent effects equals r^2 for the full regression model containing all four predictor variables.

[b] Statistical significance was determined by performing 1000 randomizations of the observed data matrix (MacNally and Walsh, 2004). For a one-tailed test with $\alpha = 0.05$, the critical value of $z = 1.65$.

indicating the presence of significant associations between dividing/differentiating regenerative cells and invading malaria parasites/abnormal midgut epithelial cells. The numbers of oocysts and abnormal midgut epithelial cells were the most important of the predictor variables, together accounting for over 77% of the explained variation in dividing and/or differentiating regenerative cells in the full regression model, independently of the number of ookinetes invading the midgut epithelium.

5.3.3 Immunofluorescent detection of cell proliferation using BrdUrd incorporation

In five independent experimental feeds, *An. stephensi* mosquitoes were given an infectious *P. falciparum* bloodmeal mixed with BrdUrd. In two of the experiments, the level of oocyst infection at day 10 post-bloodfeeding was assessed and found not to differ significantly between control and BrdUrd-treated mosquitoes (Table 5.5). There was also no evidence that exposure to the levels of BrdUrd used was harmful to the mosquitoes, with greater than 90% of mosquitoes in both control and BrdUrd-treated groups surviving to day 10 post-bloodfeeding in all experimental feeds (data not shown). However, despite examining a total of 42 midguts from batches of heavily infected BrdUrd-treated mosquitoes, using a range of conditions, cell nuclei immunofluorescently labelled with α-BrdUrd mAb were not detected in any of the mosquito midgut epithelia.

5. 4 Discussion

The morphological observations reported in this Chapter from Giemsa-stained histological sections derived from blood-fed *An. stephensi* midguts are interpreted as representing the sequential stages of division and differentiation of regenerative cells into functionally mature midgut epithelial cells. Furthermore, the quantitative associations described above between regenerative cell division/differentiation and abnormal midgut epithelial cells imply that malaria parasite infection triggers proliferative regeneration of the mosquito midgut epithelium following ookinete invasion.

5.4.1 A model for proliferative regeneration of the mosquito midgut epithelium

A model for division and differentiation of regenerative cells within the midgut epithelium of mature adult female mosquitoes is presented in Figure 5.9. Initially, an individual and isolated basally located regenerative cell divides asymmetrically to

Table 5.5 Comparison of the levels of oocyst infection in *An. stephensi* fed gametocytes of *P. falciparum* 3D7A with or without BrdUrd.

Experimental feed	Treatment[a]	*n*[b]	Prevalence[c]	*P* value[d]	Intensity[e]	*z* statistic[f]	*P* value[g]
1	control	16	87.5	1.000	31.0	0.245	0.806
	100 μM BrdUrd	16	81.3	1.000	46.0		
2	control	16	93.8	1.000	43.5	-0.999	0.318
	325μM BrdUrd	16	93.8		20.0		

[a] Mosquitoes were either given a normal infectious bloodmeal containing gametocytes alone (control) or an infectious bloodmeal containing gametocytes supplemented with the stated concentration of BrdUrd.
[b] The number of midguts examined at day 10 pbf.
[c] The percentage of midguts examined harbouring at least one oocyst.
[d] Comparison of the prevalence of oocyst infection, between *An. stephensi* fed gametocytes with or without BrdUrd, using a two-tailed Fisher's Exact test, with $\alpha = 0.05$.
[e] Median number of oocysts observed per midgut (including uninfected midguts).
[f, g] Comparison of the intensity of oocyst infection, between *An. stephensi* fed gametocytes with or without BrdUrd, using a two-tailed Mann-Whitney *U* test, with $\alpha = 0.05$, where the critical value of $z = \pm 1.960$

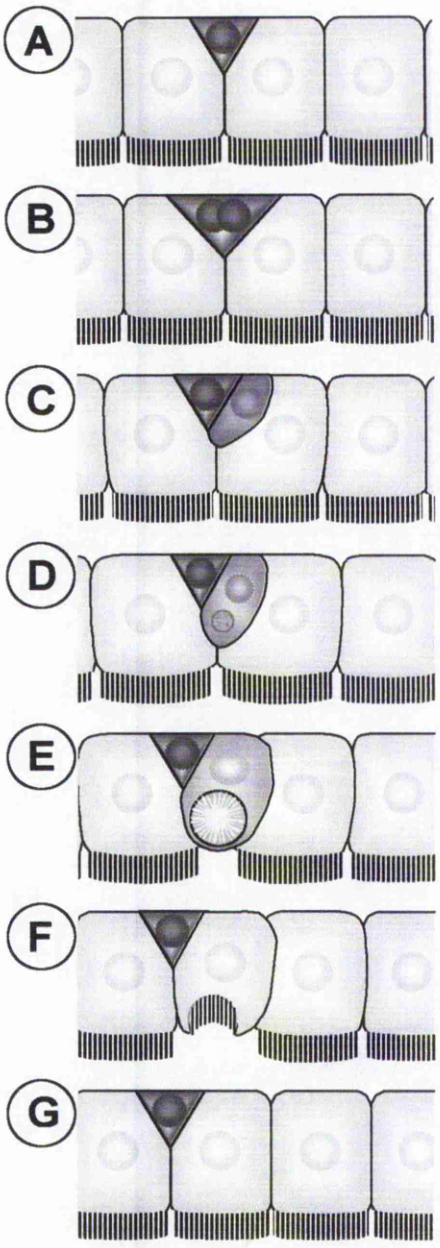


Figure 5.9 A model for division and differentiation of regenerative cells into columnar midgut epithelial cells within the adult mosquito midgut epithelium.

Isolated regenerative cells (A) undergo mitotic replication and division (B) to produce two daughter cells (C). One of the resulting daughter cells remains an undifferentiated regenerative cell while the other daughter cell grows in an apical direction, pushing between the adjacent surrounding midgut epithelial cells (D). The differentiating regenerative cell subsequently develops an apical microvilli-lined cavity (E), which eventually opens through the microvillar brush border of the surrounding midgut epithelium (F) and everts to form the apical microvillated surface of a new columnar midgut epithelial cell (G).

produce two daughter cells: one daughter cell remains an undifferentiated stem cell while the other daughter cell grows in an apical direction, pushing between the adjacent surrounding columnar midgut epithelial cells, towards the midgut lumen. Subsequently, the latter daughter cell begins to differentiate into a mature midgut epithelial cell. During this differentiation, an internal closed microvilli-lined cavity develops in the apical region of the nascent midgut epithelial cell. Eventually, the differentiating regenerative cell emerges through the apical surface of the midgut epithelium enabling the microvilli-lined apical cavity to open and evert into the midgut lumen such that the apical cavity becomes the apical brush border surface of a newly formed columnar midgut epithelial cell. In this manner, new functional midgut epithelial cells can intercalate into the already established monolayer of polarised midgut epithelial cells while maintaining the integrity of the midgut epithelium.

As argued below (Section 5.4.2), damaged midgut epithelial cells undergoing extrusion from the midgut epithelium are believed to trigger division/differentiation of regenerative cells. Therefore, regenerative cell division/differentiation within the midgut epithelium of mature adult female mosquitoes is assumed to be a mechanism of midgut epithelial cell replacement (rather than growth of the size of the midgut epithelium, as in larval insect stages). However, regenerative cell division/differentiation is not expected to play any direct role in extrusion of damaged midgut epithelial cells from the midgut epithelium: midgut epithelial cell extrusion and regenerative cell proliferation are envisioned, to a limited extent, as temporally and spatially separated events. Factors associated with the architectural reorganisation of the midgut epithelium accompanying extrusion of damaged midgut epithelial cells are hypothesized to trigger division/differentiation of nearby regenerative cells. Regenerative cell division/differentiation could be triggered by various stimuli, including mechanotransduction and/or secretion of soluble signalling factors. These stimuli could originate directly from damaged/extruding midgut epithelial cells and/or indirectly from the healthy midgut epithelial cells immediately surrounding the extruding midgut epithelial cells. As regenerative cells are sparsely scattered throughout the midgut epithelium, triggering stimuli are predicted to act over an area sufficiently broad to enable communication with regenerative cells. For similar reasons, differentiating regenerative cells are not necessarily expected to directly replace specific extruded midgut epithelial cells. Although direct replacement may occur if a regenerative cell happens to be located immediately adjacent to an extruding midgut

epithelial cell (e.g. Figure 5.7B), regenerative cell proliferation is, on average, only expected to occur within the approximate region of damage to the midgut epithelium, depending on the location of the regenerative cells relative to the site of the damaged midgut epithelial cells. If midgut epithelial cells are functionally equivalent and, hence, interchangeable as suggested by their morphological similarity (Billingsley, 1990b), the exact location of new replacement midgut epithelial cells is probably less important than that the overall number of midgut epithelial cells within the midgut epithelium is maintained.

5.4.2 *P. falciparum* ookinete invasion indirectly triggers proliferative regeneration of the *An. stephensi* midgut epithelium

Maier, Becker-Feldman & Seitz (1987) previously stated that ookinete-caused midgut epithelial "cell damage can be replaced to a limited extent by neighbouring cells or by cellular regeneration". However, it is unclear if this was a statement of fact or speculation, as no evidence has previously been published of proliferative regeneration of the mosquito midgut epithelium following invasion by ookinetes of the malaria parasite. Although there was no morphological evidence for a spatial association between malaria parasites invading the midgut epithelium and dividing/differentiating regenerative cells from examining histological sections, the results of the quantitative analysis presented here support the interpretation that the presence of malaria parasites is causally related to an increase in regenerative cell division/differentiation. As discussed above (Section 5.4.1), regenerative cell division/differentiation is not necessarily expected to occur at the exact site of damage to the midgut epithelium. Therefore, for similar reasons, invading malaria parasites may also not be located immediately adjacent to dividing/differentiating regenerative cells. Furthermore, according to the model of proliferative regeneration of the mosquito midgut epithelium described in Section 5.4.1, a time lag is expected between the occurrence of damage to midgut epithelial cells and the induction of regenerative cell division/differentiation. Although ookinete invasion rapidly damages invaded midgut epithelial cells, the hypothesized division/differentiation of regenerative cells consequently triggered is unlikely to be morphologically apparent within histological sections until several hours after ookinete invasion (i.e. by the time the infecting malaria parasites have subsequently transformed into oocysts on the basal surface of the midgut epithelium). Therefore, no association should be expected between malaria parasites in the early stages of invasion of the midgut epithelium (i.e. ookinetes) and the occurrence of

regenerative cell division/differentiation. In contrast, within the limits described above, dividing/differentiating regenerative cells are expected to be both temporally and, to a more variable extent, spatially associated with oocysts (i.e. parasites later in the process of infecting the midgut epithelium). Although not statistically significant, the apparent temporal changes in the number of dividing/differentiating regenerative cells observed in mosquitoes given an infectious bloodmeal (Figure 5.8) is consistent with the hypothesis that ookinete invasion triggers proliferative regeneration of the midgut epithelium. This change over time in the number of unusual midgut cells of intermediate morphology is also further evidence that these midgut cell morphologies represent dividing/differentiating regenerative cells (rather than functionally-distinct and terminally-differentiated cell types).

The existence of a time lag between regenerative cell division/differentiation and malaria parasite invasion of the midgut epithelium is suggested by, and explains, the results of the regression analyses described in Section 5.3.2. The weak positive association of invading ookinetes with regenerative cell division/differentiation in bivariate regression analysis is partly explained by covariation of the former with oocysts and abnormal midgut epithelial cells (Table 5.2 and Table 5.4). However, in the presence of oocysts and abnormal midgut epithelial cells, the absence of ookinetes presumably indicates a temporally later stage in the process of malaria parasite infection of the midgut epithelium and this could account for the negative association of ookinetes with regenerative cell division/differentiation in the multivariate analysis. This interpretation is also supported by examination of all the possible models in the multiple linear regression analysis (Appendix 2). In the models where oocysts and/or abnormal midgut epithelial cells are included as predictor variables, the number of ookinetes invading the midgut epithelium either exhibits no or a negative association with dividing/differentiating regenerative cells.

Although malaria parasite infection of the midgut epithelium exhibited a statistically significant positive correlation with division/differentiation of regenerative cells, in biological terms, the presence of malaria parasites is unlikely to directly cause regenerative cell division/differentiation. Rather, malaria parasites are likely to indirectly cause division/differentiation of regenerative cells through the destruction of midgut epithelial cells that accompanies ookinete invasion of the midgut epithelium (Chapter 4). This interpretation is also supported by the results of the regression analyses described in Section 5.3.2. Abnormal midgut epithelial cells were the single

most important predictor variable of regenerative cell division/differentiation, independently accounting for 44% of the explained variation in the latter (Table 5.4) and implying that damage to the midgut epithelium rather than malaria parasites *per se* cause regenerative cell division/differentiation. The presence of a low background level of midgut epithelial cell destruction regardless of the presence of malaria parasites (Chapter 4, Section 4.3.6), would therefore account for the low level of regenerative cell division/differentiation observed in uninfected mosquito midguts. Oocysts were the second most important predictor of regenerative cell division/differentiation, primarily due to covariation with other variables (presumably abnormal midgut epithelial cells) (Table 5.2 and Table 5.4). Surprisingly, however, oocysts independently accounted for 34% of the explained variation in regenerative cell division/differentiation. Although ookinetes were nearly always visibly associated with abnormal midgut epithelial cells in the histological sections, oocysts were frequently found in morphologically normal regions of the midgut epithelium exhibiting no signs of damage (Chapter 4, Section 4.3.3.5). However, these malaria parasites are still believed to enter the midgut epithelium *via* an intracellular route and cause midgut epithelial cell destruction (Chapter 4, Section 4.4.2). Possibly, in these instances, the invaded midgut epithelial cells are no longer visible and have already undergone complete extrusion from the midgut epithelium and subsequent disintegration within midgut lumen. Accordingly, the apparent "independent" effect of oocysts on regenerative cell division/differentiation might still actually be dependent on parasite-induced damage of the midgut epithelium.

Although only morphological evidence for division/differentiation of regenerative cells is provided in the current work, molecular studies support the interpretation that cell proliferation occurs within the mosquito midgut epithelium following ookinete invasion. Vlachou *et al.* (2005) recently identified 650 genes from the mosquito *Anopheles gambiae* differentially expressed in response to *Plasmodium berghei* ookinete invasion of the midgut epithelium using genome-scale transcriptional profiling. At least 30 of the differentially expressed mosquito genes identified in this study have putative/known functions related to various aspects of cell division and/or differentiation (Supplementary material in Vlachou *et al.*, 2005). For example, genes with putative/known roles in nucleotide synthesis, DNA replication, chromosome modelling, cell cycle control and mitotic spindle formation are up-regulated during, or immediately after, ookinete invasion of the midgut epithelium. Although some of these genes are possibly involved in apoptosis of parasite-invaded midgut epithelial cells, the

timing of these molecular events is consistent with the morphological observations of regenerative cell division/differentiation reported here. Further molecular and cell biological studies are currently underway to confirm the occurrence of division and differentiation of regenerative cells, the identity of the unusual midgut epithelial cells with apical microvilli-lined cavities, and the relationship of both to ookinete invasion of the mosquito midgut epithelium.

5.4.3 Failure of BrdUrd incorporation to detect mitotically active cells within the midgut epithelium of mature adult female *An. stephensi* mosquitoes

The results presented in Sections 5.3.1 and 5.3.2 provide strong evidence for proliferative regeneration of the midgut epithelium of mature adult female mosquitoes. However, further evidence is required to formally demonstrate that the unusual midgut epithelial cell morphologies observed in the histological sections are indeed regenerative cells in the process of dividing and then differentiating in to normal columnar midgut epithelial cells. Furthermore, determining general spatial relationships between dividing/differentiating regenerative cells, malaria parasites infecting the midgut epithelium and abnormal midgut epithelial cells from examination of histological sections is both time-consuming and extremely difficult: different aspects of a single event may be present in different and distant histological sections, confounding attempts to discern associations between different observations. Consequently, examination of whole midgut epithelia using specific fluorescent markers was thought desirable to confirm and further investigate the observations made from the histological sections. BrdUrd incorporation (Section 5.2.4) was chosen to investigate whether the mosquito midgut epithelium undergoes proliferative regeneration following ookinete invasion as this is a general technique for detecting actively dividing cells. It was hoped that BrdUrd incorporation would enable the following hypotheses to be tested:

(1) mitotically active cells are present in the midgut epithelium of mature adult female mosquitoes;
(2) the number of these mitotically active cells increases following ookinete invasion of the midgut epithelium;
(3) the number of mitotically active cells is correlated with the level of malaria parasite infection of the midgut epithelium;
(4) the mitotically active cells are spatially associated with both malaria parasites infecting the midgut epithelium and parasite-invaded midgut epithelial cells; and

(5) the mitotically active cells correspond to regenerative cells and the unusual midgut epithelial cells possessing microvilli-lined apical cavities identified in histological sections.

However, no evidence for incorporation of BrdUrd into the nuclei of the cells of the midgut epithelium was found.

The significance of the failure to detect incorporated BrdUrd within midgut epithelium of the *An. stephensi* is uncertain. The absence of a positive control means that it is not possible to conclude that mitotically active midgut cells are absent: proliferating regenerative cells might be present but undetectable by the particular method employed. Alternatively, regenerative cells may not be mitotically active in the midgut epithelium of mature adult female mosquitoes and the various unusual midgut epithelial cell morphologies observed in histological sections are not intermediate forms in the division/differentiation of regenerative cells into normal columnar midgut epithelial cells.

BrdUrd incorporation and immunofluorescent detection has not previously been used to study cell proliferation in mosquitoes, and seems to have only been rarely applied to insects in general. Consequently, little information is available upon which to base an appropriate protocol. In the current work, a variety of protocols, primarily various DNA denaturation methods, were employed in order to address potential problems found with the procedure in other experimental systems. Immunodetection of incorporated BrdUrd requires partial denaturation of labelled DNA, as currently available α-BrdUrd antibodies are unable to recognise BrdUrd within double-stranded DNA (Carduso & Leonhardt, 1995; Giunta & Pucillo, 1995). Three methods for generating single-stranded DNA can be used: (1) treatment with strong acid, (2) thermal denaturation and (3) enzymatic digestion (Dolbeare & Gray, 1988; Takagi *et al.*, 1993). The first two methods are relatively harsh often causing damage to cell morphology, loss of protein antigens, and sample destruction. Acid treatment also sometimes gives insufficient denaturation to detect low levels of BrdUrd incorporation. In contrast, enzymatic treatment preserves cell morphology and protein epitopes, and is at least as sensitive as thermal denaturation in enabling BrdUrd detection. Both acid treatment and enzymatic methods were tried, but no positive fluorescent signal was detected.

It is also possible that insufficient BrdUrd was absorbed into mosquito tissues to enable adequate labelling of dividing cells. BrdUrd is known to be toxic and can inhibit DNA replication at high concentrations. Again, attempts were made to circumvent

potential problems through varying the concentrations of BrdUrd used, the route and frequency of administration, and the addition of ameliorative (dCyd) or enhancing (FdUrd) compounds. dCyd is added to moderate the potentially toxic effects of BrdUrd, which can inhibit DNA replication at high concentrations while FdUrd is a potent inhibitor of thymidylate synthetase used to improve uptake of BrdUrd through reducing production of, and hence competition with, endogenous thymidine (Carduso & Leonhardt, 1995; Giunta & Pucillo, 1995). The presence of BrdUrd and other compounds had no significant effect on the level of oocyst infection implying that ookinete invasion of the midgut epithelium proceeded normally. Consequently, if the midgut epithelial cell destruction resulting from ookinete invasion does trigger regenerative cells to proliferate as outlined in Section 5.4.1, the latter cells should have been present in the samples examined.

5. 5 Summary

Examination of Giemsa-stained histological sections from *An. stephensi* mosquito midguts provided morphological evidence that regenerative cells undergo division and subsequent differentiation into normal columnar midgut epithelial cells *via* unusual intermediate morphological forms possessing apical microvilli-lined cavities. However, attempts to confirm the occurrence of cell division within whole mounts of the mosquito midgut epithelium, using immunofluorescent detection of incorporated 5-Bromo-2'-deoxyuridine, were unsuccessful. The significance of this latter observation for the interpretation of the histological evidence presented is uncertain.

Although evidence for regenerative cell division/differentiation was observed in histological sections from both uninfected and *P. falciparum*-infected *An. stephensi*, the number of apparently dividing/differentiating regenerative cells was significantly higher in midgut epithelia from *P. falciparum*-infected than uninfected mosquitoes. Furthermore, the amount of regenerative cell division/differentiation observed within each midgut was positively correlated with both the levels of malaria parasite infection and midgut epithelial cell destruction. Therefore, the loss of midgut epithelial cells associated with intracellular invasion by ookinetes appears to trigger, and be compensated by, proliferative regeneration of the mosquito midgut epithelium.

Chapter 6. General Discussion

6. 1 Introduction

The results presented in the previous chapters are synthesised and discussed here in greater detail. As the results are to some extent discrete, the discussion is broken into distinct sections on various aspects of malaria parasite infection of the mosquito vector.

6. 2 Comparative susceptibility of *An. albimanus* to *P. falciparum* infection

Overall, *An. albimanus* and *An. stephensi* were simultaneously fed gametocytes of *P. falciparum* 3D7A clone in 13 separate experimental feeds. A total of 361 *An. albimanus* were dissected at day 10 pbf and examined for oocysts. However, oocysts were never observed on the midguts of this mosquito species. In contrast, 330 of the 391 (84.4%) *An .stephensi* mosquitoes examined for oocysts at day 10 pbf were infected with at least one oocyst. For *An . stephensi*, the average prevalence of oocyst infection for the 13 experimental feeds was 86.4% while the average median intensity of oocyst infection was 23.0 oocysts per midgut. The high levels of oocyst infection observed in *An. stephensi* demonstrate that the *P. falciparum* 3D7A gametocytes fed to *An. albimanus* were infective suggesting that *An. albimanus* is refractory to infection with this malaria parasite clone.

As discussed in Chapter 2, Section 2. 4, previous studies using the *P. falciparum* NF54 isolate observed very low levels of mature oocyst infection in *An. albimanus* (Panama) (Vaughan *et al.*, 1994b; Chege *et al.*, 1996). However, the large number of mosquitoes examined here, in repeated experimental feeds, suggest that *An. albimanus* (Panama) is completely refractory to *P. falciparum* 3D7A oocyst infection. Given the large number of midguts examined, the failure to observe oocyst infection with this malaria parasite clone in this mosquito species is unlikely to be due to sampling error.

In contrast to the *P. falciparum* 3D7A clone, the *P. falciparum* HB3B-B2 and 7G8 clones were able to form mature oocysts in both *An. albimanus* and *An. stephensi* albeit at much reduced levels of infection (Table 2.2 and Table 2.3). This demonstrates that:

(1) the *An. albimanus* colony used here is susceptible to *P. falciparum* infection; and
(2) the *P. falciparum* HB3B-B2 and 7G8 clones are not sensitive to the mechanism of refractoriness that prevents the *P. falciparum* 3D7A clone from infecting *An. albimanus*.

Understanding the mechanism of refractoriness of *An. albimanus* to *P. falciparum* 3D7A infection, and why the *P. falciparum* HB3B-B2 and 7G8 clones are insensitive to this mechanism of refractoriness, would be of great interest.

6.2.1 *P. falciparum* 3D7A development in *An. albimanus*

The findings presented in Chapter 3 and Chapter 4 with the *P. falciparum* 3D7A clone are similar to previous studies of malaria parasite development in *An. albimanus*, which have also found the ookinete to oocyst transition to be the key stage determining the outcome of malaria parasite infection in this mosquito species (Chapter 3, Section 3.1.1). Furthermore, the mechanism of refractoriness of *An. albimanus* against *P. falciparum* 3D7A infection is apparently the same as that previously reported for *P. cynomolgi bastianelli* in a Mexican strain of the same mosquito species (Omar, 1968b). For *P. cynomolgi bastianelli* similar numbers of ookinetes formed within the midgut lumen of both mosquito species, but in *An. albimanus* relatively few ookinetes attained the peritrophic matrix and even fewer invaded the midgut epithelium while many ookinetes were observed in both locations in *An. stephensi*. Omar (1968b) also reported that the majority of *P. cynomolgi bastianelli* ookinetes observed in *An. albimanus* rapidly degenerated within the midgut lumen, such that from 30 hours pbf "only remnants of ookinetes could be found" within the endoperitrophic space. In contrast, in *An. stephensi* morphologically normal ookinetes could be found within the digested bloodmeal as late as 51 hours pbf infection implying that these malaria parasite stages were capable of surviving bloodmeal digestion in this mosquito species. Although obviously degenerating ookinetes were seldom observed in the histological sections from *An. albimanus* fed *P. falciparum* 3D7A ookinetes, preliminary vital staining experiments also suggest that the proportion of round form, retort-form and mature ookinetes with disrupted plasma membranes increases significantly in *An. albimanus* relative to *An. stephensi* from 24 hours pbf onwards (data not shown).

Whether the mechanism operating against *P. falciparum* 3D7A and *P. cynomolgi bastianelli* is responsible for the refractoriness of *An. albimanus* to other malaria parasite species/strains is unknown. Several other studies, including those using *P. berghei* and *P. yoelii yoelii*, have also reported formation of mature ookinetes within the bloodmeal of *An. albimanus* but reduced levels of oocyst infection (Eyles & Young, 1950; Vaughan *et al.*, 1991; Vaughan *et al.*, 1994a; Vaughan *et al.*, 1994b; Chege *et al.*, 1996). Although these previously published observations are consistent with the interpretation of malaria parasite destruction within the endoperitrophic space, ookinete

migration from the bloodmeal and invasion of the midgut epithelium were not investigated.

The mechanism of refractoriness operating against *P. falciparum* 3D7A in *An. albimanus* clearly differs from that acting on the human malaria *P. vivax* (VK247 CSP variant) in the same mosquito species, where malaria parasites are killed during or after invasion of the midgut epithelium (Gonzalez-Ceron *et al.*, 2001). In contrast to the studies with *P. vivax* (VK247 CSP variant), the development of *P. falciparum* 3D7A in *An. albimanus* is apparently prevented before ookinete entry into the midgut epithelium. Consequently, separate mechanisms of refractoriness may operate against these two malaria parasite species, and/or *P. falciparum* 3D7A ookinetes have greater susceptibility to factor(s) present throughout all compartments of the midgut which cause destruction of *P. vivax* (VK247 CSP variant). The mechanisms of refractoriness against *P. berghei* and *P. yoelii yoelii* in *An. albimanus* are also unlikely to be (entirely) the same as those acting on *P. vivax* (VK247 CSP variant) as the degenerate oocysts seen with *P. vivax* were not observed for these rodent malaria parasite species (Vaughan *et al.*, 1991; Vaughan *et al.*, 1994a; Gonzalez-Ceron *et al.*, 2001). Possibly, the same mechanism of refractoriness to *P. falciparum* 3D7A oocyst infection in *An. albimanus* is active against a wide range of other malaria parasite species and explains the general resistance of this mosquito species to malaria parasite infection (Chapter 2, Section 2.1.1).

6.2.2 The mechanism of *An. albimanus* refractoriness to *P. falciparum* 3D7A oocyst infection

As reviewed in Chapter 1, Sections 1.5.2.4 and 1.5.2.5, previous studies have identified and characterised mechanisms of mosquito refractoriness to malaria parasite infection which operate during ookinete invasion of the midgut epithelium. However, mosquito immune responses operating during ookinete invasion of the midgut epithelium are apparently not responsible for the loss of *P. falciparum* 3D7A (and *P. cynomolgi bastianelli*) ookinetes within the midgut lumen of *An. albimanus* (Omar, 1968b). Several possible mechanisms could account for the failure of *P. falciparum* 3D7A ookinetes to migrate from the bloodmeal and invade the midgut epithelium in *An. albimanus*.

6.2.2.1 Bloodmeal digestion

The digestive proteases secreted from the mosquito midgut epithelium following

bloodmeal ingestion could create an inimical environment for malaria parasites developing within the midgut lumen (Chapter 1, Section 1.5.2.2). Omar (1968b) believed that the refractoriness and susceptibility of *An. albimanus* and *An. stephensi*, respectively, to *P. cynomolgi bastianellii* oocyst infection was related to differences in the intensity and rate of bloodmeal digestion between these two mosquito species. Omar (1968b) claimed that in *An. albimanus* there was "greater [digestive] enzyme production than in *An. stephensi*". This author also stated that there were "no recognisable differences in the pattern of [bloodmeal digestion] between *A. stephensi* and *A. albimanus*" and he did not mention that the time to completion of bloodmeal digestion was appreciably different between these two mosquito species. Omar's belief of greater digestive enzyme production in *An. albimanus* is apparently based upon the occurrence of "greater secretory activity" of the midgut epithelium, and the greater number of degenerated ookinetes observed within the endoperitrophic space, in this mosquito species. However, the description of "secretory activity" as "manifested as budding vacuoles" and vacuolisation of the midgut epithelium is open to alternative interpretations. "Blebbing" of the apical surface of the midgut epithelium was occasionally observed in the histological sections examined here, and has been reported by others (Weaver & Scott, 1990a; Weaver *et al.*, 1992), but the significance of these cytoplasmic extrusions is contentious. The older literature initially regarded such structures as examples of merocrine secretion of digestive enzymes and later as a form of midgut epithelial cell degeneration (Snodgrass, 1935). However, more recent workers suggest the apical extrusions of midgut epithelial cells observed in sectioned material are fixation artefacts (Brunings & de Priester, 1971).

Analysis of the total protein content of midguts following bloodfeeding (Chapter 3, Section 3.3.1.1), and morphological evidence from histological sections (Chapter 4, Section 4.3.1.2), suggest bloodmeal digestion *is* completed more rapidly in *An. albimanus* than *An. stephensi*. However, the significance of this correlation between duration of bloodmeal digestion and susceptibility to *P. falciparum* 3D7A oocyst infection is uncertain. The differences between *An. albimanus* and *An. stephensi* in time taken to complete bloodmeal digestion could be causally unrelated to the survival of *P. falciparum* 3D7A ookinetes within in the endoperitrophic space. The relatively similar slopes for the decline in average total protein content per midgut over time indicates that the *rate* of bloodmeal digestion is not appreciably different between the two mosquito species (Figure 3.7). Indeed, the earlier time to completion of bloodmeal digestion in

An. albimanus is quite possibly due to the smaller initial volume of blood ingested by this mosquito species compared to *An. stephensi* (rather than higher levels of digestive enzyme activity) (Briegel & Lea, 1975).

Previous studies using *P. falciparum* NF54 compared the processes of bloodmeal acquisition and digestion in *An. albimanus* with two other mosquito species (*An. freeborni* and *An. gambiae*), both highly susceptible to infection with this malaria parasite isolate (Chege *et al.*, 1996; Chege & Beier, 1998). These studies failed to find any differences between the three mosquito species that correlated with their respective susceptibilities to malaria parasite infection.[1] In particular, although the absolute levels of digestive enzyme activity per midgut differed significantly between *An. albimanus* and the other two mosquito species examined, these differences were not associated with differences in susceptibility to malaria infection (Chege *et al.*, 1996). In contrast, although the absolute levels of midgut digestive enzyme activity per midgut were not markedly different between *An. albimanus* and *An. stephensi*, Hörler & Briegel (1995) found appreciable differences in the temporal profiles of trypsin and chymotrypsin activity between these two mosquito species. High levels of constitutive trypsin activity were observed in non-blood-fed *An. albimanus* compared to *An. stephensi* (40 compared to 4% of the maximal activity observed during bloodfeeding, respectively) while peak trypsin activity was attained earlier during bloodmeal digestion in *An. albimanus* (14 compared to 28 to 32 hours pbf) (Hörler & Briegel, 1995). Chymotrypsin activity also peaked earlier in *An. albimanus* compared to *An. stephensi* (20 compared to 36 hours pbf) and exhibited an unusual substrate specificity in the former mosquito species (Hörler & Briegel, 1995). In contrast, the levels and timing of aminopeptidase activity were not markedly different between *An. albimanus* and *An. stephensi* (Hörler & Briegel, 1995). The differences observed by Hörler & Briegel in trypsin and chymotrypsin activities between *An. albimanus* and *An. stephensi* would be consistent with the hypothesis that loss of *P. falciparum* 3D7A ookinetes in the former mosquito species is related to differences in bloodmeal digestion between these mosquito species.

However, these studies did not estimate bloodmeal volume/protein content and,

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[1] Chege & Beier (1998) did report significantly greater agglutination of ingested erythrocytes in *An. albimanus* compared to the other mosquitoes investigated. Possibly, agglutination of ingested erythrocytes could inhibit ookinete migration through the bloodmeal. However, erythrocyte agglutination apparently only persisted up to 12 hours pbf in *An. albimanus* and therefore disappeared before ookinete formation was completed and migration of these malaria parasite stages would begin.

hence, failed to relate digestive enzyme levels to the total amount of blood present within the midguts of each mosquito species (i.e. the *concentration* of the digestive enzymes present within each midgut was not determined) (Hörler & Briegel, 1995; Chege *et al.*, 1996; Chege & Beier, 1998). Consequently, the significance of the previous comparisons of absolute digestive enzyme activity between different mosquito species are difficult to interpret because concentration is likely to be the most important determinant if digestive enzymes are the cause of differences in susceptibility to malaria parasite infection between *An. albimanus* and *An. stephensi*. Furthermore, these studies did not investigate all the digestive enzymes known to be present within the mosquito midgut e.g. carboxypeptidase (Moskalyk, 1998; Edwards *et al.*, 2000; Noriega *et al.*, 2002).

6.2.2.2 Immune responses and co-infecting microorganisms

As discussed in Chapter 1, Sections 1.5.2.4 and 1.5.2.5, malaria parasite infection may be limited by the immune system of the mosquito vector. The reported mosquito immune effector mechanisms acting against malaria parasites operate during and/or after ookinete invasion of the midgut epithelium. There is currently no information about the occurrence of immune effector mechanisms *within* the midgut lumen. Whether the various recognition proteins, antimicrobial peptides and reactive nitrogen/oxygen intermediates identified in mosquitoes are present within the midgut lumen is unknown. Conceivably, some of such immune effector molecules may be present within the midgut lumen and account for the loss of *P. falciparum* 3D7A ookinetes within the endoperitrophic space of *An. albimanus*. Nok, Njoku & Balogun (2002) reported the purification of a sialylated 45kDa protein from *An. albimanus* midguts with potent lytic activity against the protozoan haemoflagellate *Trypanosoma congolense*. Unfortunately, whether this uncharacterised protein is located within the midgut lumen (rather than the midgut epithelium) is unknown.

The most marked difference between *An. albimanus* and *An. stephensi* in histological sections was the abundance of bacteria within the midgut lumen of the former mosquito species and their virtual absence in the latter mosquito species (Chapter 4, Section 4.3.1.2). Previous studies have shown that the presence of antibiotics can significantly increase the level of oocyst infection observed in mosquitoes, an effect attributed to the removal of co-infecting microorganisms suppressing malaria parasite development (Micks & Ferguson, 1961; Pumpuni *et al.*, 1993; Beier *et al.*, 1994; Pumpuni *et al.*, 1996; Luckhart *et al.*, 1998; Lowenberger *et*

al., 1999; Gonzalez-Ceron *et al.*, 2003). Consequently, mosquito immune responses activated by co-infecting microorganisms might also indirectly limit *P. falciparum* 3D7A infection in *An. albimanus*. However, the addition of gentamicin to the glucose solution fed to the mosquitoes had no effect on the level of *P. falciparum* 3D7A oocyst infection in *An. albimanus* (data not shown) suggesting that the presence of bacteria within the midgut lumen is not responsible for the refractoriness of this mosquito species to this malaria parasite clone.

6.2.2.3 The peritrophic matrix and the midgut epithelium

The peritrophic matrix and/or the midgut epithelium may also act as a barrier to malaria parasite development within the mosquito (Chapter 1, Sections 1.5.2.3 and 1.5.2.4). As reported in Chapter 4, in histological sections from *An. stephensi*, ookinetes were most frequently observed penetrating the peritrophic matrix implying that penetration of the peritrophic matrix takes a prolonged period of time in comparison to other stages of malaria parasite migration from the bloodmeal to the basal surface of the midgut epithelium (Omar, 1968b). Consequently, malaria parasites may be exposed to mosquito digestive enzymes for a longer period of time during penetration of the peritrophic matrix than other stages of ookinete migration through the midgut lumen.

Omar (1968b) reported that ookinetes of *P. cynomolgi bastianelli* became aggregated with increasing time pbf at the peritrophic matrix of *An. albimanus* and *An. stephensi*. However, there was no evidence that either the peritrophic matrix and/or the midgut epithelium were barriers to infection with *P. falciparum* 3D7A ookinetes in *An. albimanus*. If the peritrophic matrix was the barrier to *P. falciparum* 3D7A infection in *An. albimanus* ookinetes would be expected to become aggregated at the peritrophic matrix within this mosquito species (Omar, 1968b). Additionally, more ookinetes should be observed at this location within the midgut lumen of *An. albimanus* than in the equivalent location in *An. stephensi* (as the malaria parasites in the latter mosquito species would pass through the peritrophic matrix and onwards to the midgut epithelium). However, significantly fewer ookinetes were associated the peritrophic matrix in *An. albimanus* compared to *An. stephensi*, and no ookinetes were observed within the midgut epithelium of the former mosquito species (Table 4.4 and Table 4.5) suggesting that ookinetes within the bloodmeal of *An. albimanus* are lost *before* the surrounding peritrophic matrix is attained. Furthermore, preliminary work using the addition of *Streptomyces griseus* chitinase to the bloodmeal fed to mosquitoes

(following Shahabuddin *et al.*, 1993; 1995b) suggests that disruption of the peritrophic matrix has no effect on the level of *P. falciparum* 3D7A oocyst infection in *An. albimanus* (data not shown).

6. 3 Population biology of *P. falciparum* development in *An. albimanus* and *An. stephensi*

Numerous previous studies have investigated the population biology of malaria parasite development within the mosquito by comparing the mortality between different parasite life cycle stages (Vaughan *et al.*, 1992; Robert *et al.*, 1994; Vaughan *et al.*, 1994a; Vaughan *et al.*, 1994b; Robert *et al.*, 1995; Awono-Ambene & Robert, 1998; Gouagna *et al.*, 1998; Robert *et al.*, 1998; Gouagna *et al.*, 1999; Alavi *et al.*, 2003; 2004; Okech *et al.*, 2004a; Gouagna *et al.*, 2004a; Gouagna *et al.*, 2004b; Okech *et al.*, 2004b; Zollner *et al.*, 2005).[2] In general, the observations reported here for *P. falciparum* 3D7A in *An. albimanus* and *An. stephensi* concur with previous findings in that marked losses in malaria parasite numbers were observed between successive developmental transitions within these two mosquito species (Figure 6.1). Based upon the studies by Vaughan *et al.* (1992; 1994b), previous workers have stated that laboratory-cultured *P. falciparum* malaria parasites are markedly less efficient than field isolates at infecting mosquitoes (e.g. Sinden, Alavi & Raine, 2004; Okech *et al.*,

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[2] The pioneering studies by Vaughan *et al.* (1992; 1994a; 1994b) introduced the use of the population mortality coefficient, *k*, to study the population dynamics of malaria parasite development within the mosquito. *k*-values are the difference in the logarithms of the malaria parasite density between one life cycle stage and another i.e. the *k*-value for the transition between malaria parasite stage *x* and *y* = $\log_{10}$(average number of stage *x* per mosquito) - $\log_{10}$(average number of stage *y* per mosquito). Consequently, *k*-values allow direct comparison between different malaria parasite stages and are simply manipulated to give the fold-loss, calculated as antilog$_{10}$(*k*), or the yield, the percentage of malaria parasites surviving from one stage to another, calculated as $1 \div$ antilog$_{10}$(*k*). Various refinements of the original "*k*-1" and "*k*-2" categories have been made since the publication of the original studies, adding further complexity and creating some confusion as different authors have used the same epithet (*k*-2 and *k*-3) to refer to different stages of malaria parasite infection of the mosquito vector (Gouagna *et al.*, 1998; Alavi *et al.*, 2003). Consequently, the use of the *k* terminology has generally been avoided for the ease of comprehension of the reader and % survival rates (the yield) between different stages in the malaria parasite life cycle have mostly been used throughout instead as these are intuitively understood. However, previously defined *k* categories have been mentioned in brackets in the main text for those interested and familiar with the terminology who wish to be able to quickly relate the findings presented here with previous work.

2004b). The overall reduction in malaria parasite numbers occurring between macrogametocytes and mature oocysts reported here for *P. falciparum* 3D7A infection of *An. stephensi* (99.5%) is several orders of magnitude less than that reported by Vaughan *et al.* (1994b) for the *P. falciparum* NF54 isolate in this mosquito species (2.3 versus 5.1-fold reduction, respectively) but within the range reported for *An. gambiae* infected with *P. falciparum* from naturally-infected hosts (95.6 to 99.5%) (Gouagna *et al.*, 1998; Lensen *et al.*, 1998; Okech *et al.*, 2004a; Gouagna *et al.*, 2004a; Gouagna *et al.*, 2004b; Okech *et al.*, 2004b).

6.3.1 Macrogametocyte to ookinete transition (*k*-1)

In both *An. albimanus* and *An. stephensi* the greatest absolute reduction in malaria parasite densities during *P. falciparum* 3D7A development within the mosquito vector occurred during the transition from macrogametocytes to mature ookinetes (Table 3.4). Overall, only 5.8 and 4.3%, respectively, of ingested macrogametocytes transformed into mature ookinetes in *An. albimanus* and *An. stephensi*. These losses in malaria parasite numbers are similar to those described in previous laboratory and field studies of *P. falciparum* using a variety of anopheline mosquito species (ranging from 0.01 to 18.2% of macrogametocytes transforming into ookinetes) (Vaughan *et al.*, 1992; Vaughan *et al.*, 1994b; Gouagna *et al.*, 1998; Okech *et al.*, 2004a; Gouagna *et al.*, 2004a; Gouagna *et al.*, 2004b; Okech *et al.*, 2004b). The *overall* efficiency of the macrogametocyte to ookinete transition for *P. falciparum* 3D7A in *An. albimanus* and *An. stephensi* is similar to that reported in field studies. However, the *relative* contributions of the macrogametocyte to macrogamete, and macrogamete to ookinete, transitions are markedly different: the former is much more, and the latter much less, efficient for *P. falciparum* 3D7A (see Sections 6.3.1.1 and 6.3.1.2 below). The reasons for these differences between field and laboratory *P. falciparum* lines are not obvious, but the poor efficiency of the macrogamete to ookinete transition for *P. falciparum* 3D7A presumably accounts for the notorious difficulty of producing *in vitro* cultured ookinetes in the laboratory for this malaria parasite species (Carter *et al.*, 1987; Warburg & Miller, 1992; Warburg & Schneider, 1993). Studies with non-human malaria parasite species found that 19.1% of *P. berghei* macrogametocytes ingested by *An. stephensi* developed into ookinetes whereas only 0.4% of *P. gallinaceum* macrogametocytes developed into ookinetes in *Ae .aegypti* (Alavi *et al.*, 2003; 2004).

The precise reasons for the relatively large losses in malaria parasite numbers that occurs during the macrogametocyte to ookinete transition for a variety of *Plasmodium*

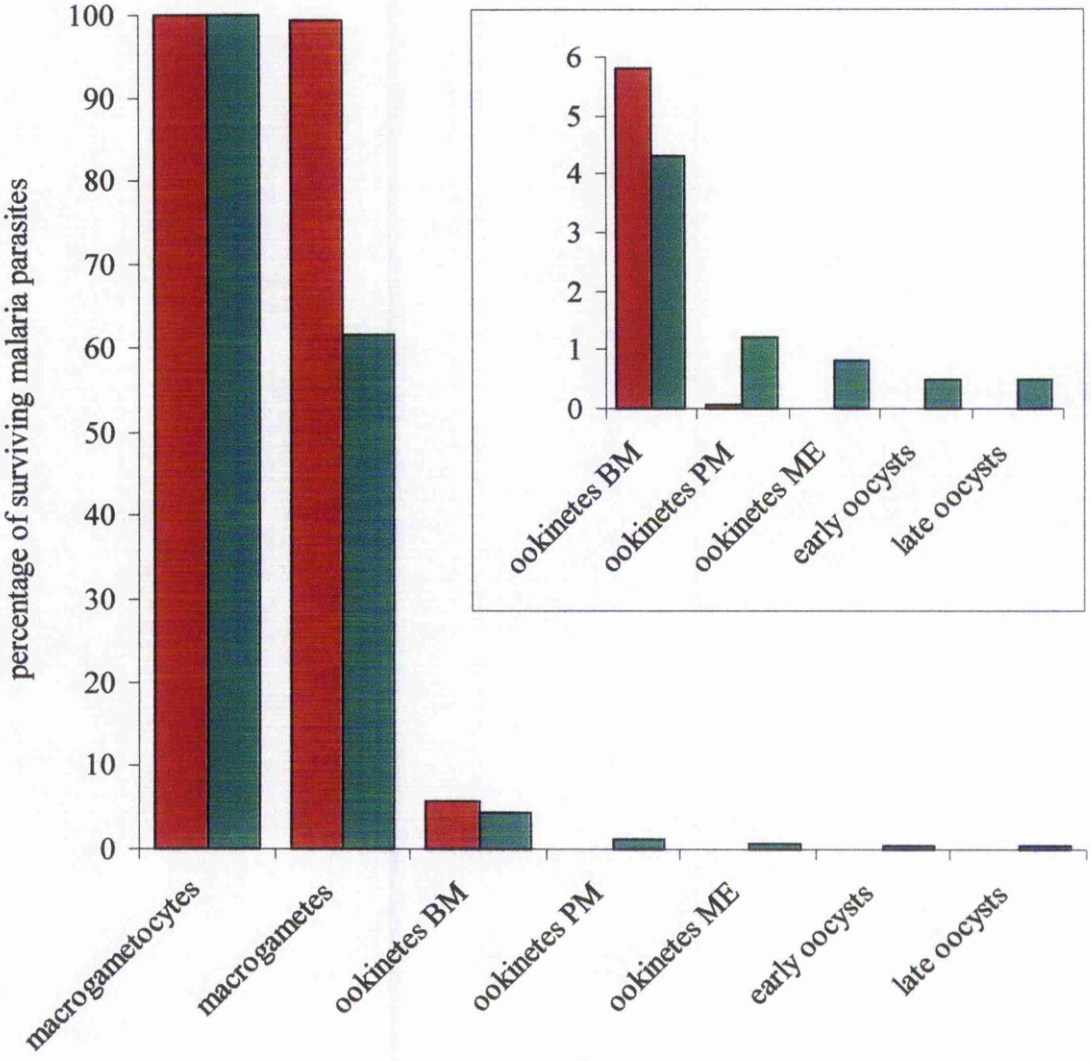


Figure 6.1 Comparison of the survival of different *P. falciparum* 3D7A developmental stages in *An. albimanus* and *An. stephensi*.

Main graph (bottom left): bar chart illustrating the percentage of ingested *P. falciparum* 3D7A macrogametocytes that survive to develop into the different subsequent stages of malaria parasite infection in *An. albimanus* and *An. stephensi*. Inset graph (top right): shows values of the relevant malaria parasite stages from the main graph but on a different scale. Key: (●) *An. albimanus*; (●) *An. stephensi*; ookinetes BM = mature ookinetes forming within the bloodmeal; ookinetes PM = mature ookinetes attaining the peritrophic matrix; ookinetes ME = mature ookinetes entering the midgut epithelium; early oocysts = oocysts formed at day 2/3 pbf; and late oocysts = mature oocysts present at day 10 pbf. The values shown are derived from pooling the data from the different experimental feeds presented in Table 3.4 and Table 4.7.

species in diverse mosquito hosts are unknown (although there are plenty of candidate hypotheses). As reviewed in Chapter 1, Section 1.5.1, a number of *vertebrate* host factors may influence, and negatively affect, malaria parasite development within the bloodmeal. However, many of these vertebrate host factors are irrelevant in the current context e.g. complement, cytokines, specific anti-malaria parasite antibodies, leucocytes/phagocytes, and other inhibitory factors present within the blood associated with malaria "crisis" (acute asexual erythrocytic stage infection of the vertebrate host). This is because heat-inactivated serum and washed human blood depleted of white blood cells, both from (presumed) naïve donors, were used to prepare the experimental feeds given to *An. albimanus* and *An. stephensi* (Chapter 2, Section 2. 2). Consequently, the vertebrate host factors listed above are unlikely to account for the loss of *P. falciparum* 3D7A malaria parasites observed between the macrogametocyte to ookinete transition in *An. albimanus* and *An. stephensi*. This is in agreement with recent field studies which estimated that components present within immune plasma only accounted for between 5 and 20% of the reduction in *P. falciparum* densities during malaria parasite development within the mosquito vector (acting primarily during the macrogamete to ookinete transition) (Gouagna *et al.*, 2004a).

6.3.1.1 Macrogametocyte to macrogamete transition (*k*-1a)

In *An. albimanus* and *An. stephensi*, 99.2 and 61.5%, respectively, of ingested *P. falciparum* 3D7A macrogametocytes underwent gametogenesis and successfully transformed into macrogametes in these two mosquito species (Table 3.4). In comparison to previously published field studies where only approximately 20% of macrogametocytes transformed into macrogametes (Gouagna *et al.*, 2004a), the percentage of *P. falciparum* 3D7A macrogametocytes successfully undergoing gametogenesis was remarkably high in both *An. albimanus* and *An. stephensi*.

The difference in the proportion of macrogametocytes transforming into macrogametes in *An. albimanus* and *An. stephensi* is presumably due to differences between the two mosquito species in factors that either promote and/or inhibit gametogenesis (Chapter 1, Section 1.4.1). Several studies have previously reported that different mosquito species vary in their ability to provide a suitable environment for the occurrence of gametogenesis (Micks *et al.*, 1948; Omar, 1968b; Yoeli, 1973; Alavi *et al.*, 2003). The reasons for the differences between mosquito species in the ability to induce gametogenesis are unknown but could be related to variation in the levels of xanthurenic acid (Arai *et al.*, 2001; Bhattacharyya & Kumar, 2001).

Differences in the efficiency of the macrogametocyte to macrogamete transition between *An. albimanus* and *An. stephensi* would also explain the equivalent peak numbers of round forms (and retort-form and mature ookinetes) observed within the bloodmeals of these two mosquito species despite the volume of blood ingested by *An. albimanus* being approximately 25 to 50% less than *An. stephensi* (Chapter 3, Section 3.3.1.1.4 and Section 3.3.1.1.5). This interpretation could also account for the higher densities of *P. berghei* ookinetes observed within *An. albimanus* compared to *An. stephensi* after correcting for differences in bloodmeal volume (Vaughan *et al.*, 1991).

6.3.1.2 Macrogamete to ookinete transition (*k*-1b)

In *An. albimanus* and *An. stephensi*, 5.9 and 7.0%, respectively, of *P. falciparum* 3D7A macrogametes successfully transformed into mature ookinetes within the bloodmeal of these two mosquito species (Table 3.4). As discussed above (Section 6.3.1.1), the equivalent peak numbers of immature retort-form and mature ookinetes observed in *An. albimanus* and *An. stephensi*, despite the significantly smaller bloodmeal ingested by the former mosquito species, is probably explained by the greater efficiency of gametogenesis in this mosquito species. Previous field studies of *P. falciparum* in *An. gambiae* reported that between 41.6 and 52.5% of macrogametes transform into mature ookinetes (Gouagna *et al.*, 1998; Gouagna *et al.*, 2004a) while laboratory studies estimate that 38.0 of *P. berghei* and 0.5% *P. gallinaceum* macrogametes developed into ookinetes in *An. stephensi* and *Ae. aegypti*, respectively (Alavi *et al.*, 2003; 2004). The efficiency of the macrogamete to ookinete transition observed here for *P. falciparum* 3D7A is, therefore, markedly lower than that reported for *P. falciparum* from naturally infected individuals in *An. gambiae*.

The reasons for the failure of an appreciable proportion of macrogametes to develop into mature ookinetes in a diverse range of malaria parasite-mosquito vector combinations is unknown. Previous studies have not shown when losses in malaria parasite numbers occurs during the macrogamete to ookinete transition. For *P. falciparum* 3D7A in *An. albimanus* and *An. stephensi* the majority of the reduction in malaria parasite numbers observed during this period was due to the failure of round forms to initiate (morphologically apparent) development into ookinetes (rather than retort-forms failing to develop into mature ookinetes) (Table 3.4): approximately 80.1 and 81.6% of macrogametes failed to develop into retort-form ookinetes in these two mosquito species, respectively. The similar levels of *P. falciparum* 3D7A macrogamete to retort-form transformation observed in *An. albimanus* and *An. stephensi* implies that

malaria parasite losses during this transition are either independent of mosquito factors or, if mosquito factors are relevant, these two mosquito species do not differ in this regard.

Malaria parasite losses during the macrogamete to ookinete transition may result from the absence of fertilization or intrinsic aberrations in malaria parasite (independent of the environment of the midgut lumen) (Janse *et al.*, 1985a; Janse *et al.*, 1985b; Al-Olayan, Williams & Hurd, 2002b). If aberrant development is a significant cause of the loss *P. falciparum* 3D7A during the macrogamete to ookinete transition in *An. albimanus* and *An. stephensi*, this must primarily occur at the macrogamete/zygote stage prior to (morphologically-apparent) initiation of differentiation into the ookinete.

Estimating the proportion of zygotes initiating (morphologically-apparent) differentiation into ookinetes that subsequently complete development into the latter malaria parasite stage is complicated by the asynchronous nature of this transformation, and by the loss of mature ookinetes from the midgut lumen as a result of malaria parasite migration from the bloodmeal (and mortality specific to this malaria parasite stage; see Section 6.3.2.1 below). Direct comparison of the cross-sectional peak densities of immature retort-form and mature ookinetes observed at different times (12 and 24 hours pbf, respectively) within the bloodmeal is, therefore, likely to underestimate the efficiency of zygote differentiation into ookinetes. For example, according to such a measure only 29.5 and 37.9% of retort-forms successfully transform into mature ookinetes in *An. albimanus* and *An. stephensi*, respectively (Figure 3.6). However, if the *sum* of retort-form and mature ookinetes observed at 12 and 24 hours pbf are compared there is no significant difference in the combined number of these malaria parasite stages between these two time points (two-tailed *t* test: *An. albimanus* $t_{0.05,\pm 2.228} = -0.838$, $P = 0.421$; and *An. stephensi* $t_{0.05,\pm 2.228} = -0.169$, $P = 0.870$) (Figure 3.6). This implies that over this time period, in both *An. albimanus* and *An. stephensi*, there is no loss in the number of those malaria parasites initiating transformation into ookinetes. There is also no evidence that all retort-forms do not successfully develop into mature ookinetes: the decline in the numbers of retort-forms is commensurate with the increase in the numbers of mature ookinetes. The subsequent decline (from 24 hours pbf onwards) in the number of retort-forms observed in both mosquito species is difficult to interpret because the number of mature ookinetes within the midgut lumen declines simultaneously as invasion of the midgut epithelium begins during this period (Figure 4.44). In *An. albimanus*, after 24 hours pbf, retort-forms may be destroyed

before formation into mature ookinetes is completed (as indicated by the significantly lower number of these malaria parasite stages observed in this mosquito species compared to *An. stephensi*). In contrast, in *An. stephensi*, after 24 hours pbf, the progressive decline and increase, respectively, in the proportion of malaria parasite stages that are retort-forms and matures ookinetes implies completion of differentiation of the former into the latter malaria parasite stages in this mosquito species (Figure 3.6). Loss of some retort-forms cannot be ruled out, especially if these stages are more sensitive to the hostile environment of the mosquito midgut lumen (Gass, 1979), as the changes in the proportion of malaria parasites that are retort-forms and ookinetes could also be explained by differential mortality.

6.3.2 Ookinete to oocyst transition (*k*-2)

Previous studies using a variety of malaria parasite-mosquito vector combinations with widely differing compatibilities have estimated that <1 to >75% of ookinetes within the bloodmeal successfully develop into mature oocysts on the basal surface of the midgut epithelium (Vaughan *et al.*, 1992; Vaughan *et al.*, 1994a; Vaughan *et al.*, 1994b; Gouagna *et al.*, 1998; Kaplan *et al.*, 2001; Alavi *et al.*, 2003; Okech *et al.*, 2004a; Gouagna *et al.*, 2004a; Gouagna *et al.*, 2004b; Okech *et al.*, 2004b; Zollner *et al.*, 2005).

As discussed above (Section 6.2.1), in *An. albimanus* no *P. falciparum* 3D7A ookinetes survive to transform into mature oocysts (i.e. 0% survival during the ookinete to oocyst transition). In contrast, in *An. stephensi* approximately 11% of the *P. falciparum* 3D7A ookinetes forming within the bloodmeal subsequently develop into mature oocysts (Table 3.4). The efficiency of the transformation observed here is an order of magnitude greater than that previously reported for the *P. falciparum* NF54 isolate in the same mosquito species (<0.5% ookinetes surviving to mature oocysts in the latter instance) (Vaughan *et al.*, 1994b). However, the *P. falciparum* 3D7A ookinete to oocyst transition in *An. stephensi* is markedly less efficient than that reported for field isolates of *P. falciparum* in laboratory-reared *An. gambiae* (32.7 to 79.7% survival) (Gouagna *et al.*, 1998; Okech *et al.*, 2004a; Gouagna *et al.*, 2004a; Gouagna *et al.*, 2004b; Okech *et al.*, 2004b).

Although previous studies have estimated the overall efficiency of the ookinete to oocyst transition, few investigations have attempted to estimate the relative contribution of each of the distinct phases in the process of mosquito infection to the overall loss in malaria parasite numbers that occurs during this transition (Awono-Ambene & Robert,

1998; Gouagna *et al.*, 1998; Gouagna *et al.*, 1999; Alavi *et al.*, 2003; Gouagna *et al.*, 2004a). This is partly due to the difficulty of observing, and scoring, malaria parasites located within different compartments of the midgut lumen and the midgut epithelium. Accurately estimating the number of malaria parasites that survive to the complete different phases of the ookinete to oocyst transition is further complicated by the asynchrony of ookinete migration form the bloodmeal and invasion of the midgut epithelium (Figure 4.44). Consequently, the exact reasons for the sometimes appreciable loss of malaria parasites during the ookinete transition into mature oocysts has remained somewhat obscure.

The loss of malaria parasite numbers during the ookinete to oocyst transition has previously been subdivided into several and various categories by different authors. Alavi *et al.* (2003) recognised two subcategories: the loss of malaria parasites between ookinete formation within the bloodmeal and association of these malaria parasite stages with the midgut epithelium (which they denoted *k*-2a); and all subsequent malaria parasite losses occurring between initial association of the ookinetes with the midgut epithelium and the formation of mature oocysts (which they denoted *k*-2b). Gouagna *et al.* (1998) also recognised two, but different, subcategories: loss of malaria parasites between ookinete formation within the bloodmeal and the formation of early oocysts on the basal surface of the midgut epithelium (their *k*-2); and loss of malaria parasites during oocyst development (i.e. between early and late oocyst stages) (their *k*-3). These subcategories can be combined and modified to give four conceptually distinct phases in the ookinete to oocyst transition during which malaria parasite mortality might occur:

(1) ookinete migration within the bloodmeal to the surrounding peritrophic matrix;
(2) ookinete penetration of the peritrophic matrix, migration through the ectoperitrophic space and entry into the midgut epithelium;
(3) ookinete invasion of the midgut epithelium;
(4) the subsequent period of oocyst development.

Although the four phases in the ookinete to oocyst transition defined above were not simultaneously investigated within the same experimental feeds, the data from the separate experimental feeds using direct immunofluorescence microscopy (Chapter 3) and histology (Chapter 4) to investigate *P. falciparum* 3D7A development in *An. stephensi* can be pooled and compared to give estimates of the survival of malaria

parasites during each of these distinct phases in the ookinete to oocyst transition.[1] As these estimates of malaria parasite survival derive from comparing data from separate infection experiments and, in some instances, are based upon very small sample sizes taken at varying time points, care should be taken before inferring too much from these observations. However, with this caveat in mind, combining the data presented in Table 3.4 and Table 4.7 suggests that, on average, the fate of the mature *P. falciparum* 3D7A ookinetes formed within the bloodmeal of *An. stephensi* is as follows: 72.1% are lost within the endoperitrophic space before the peritrophic matrix is reached, 8.6% are lost between penetration of the peritrophic matrix and entry into the midgut epithelium, 8.0% are lost during invasion of the midgut epithelium, while 11.2% successfully transform into oocysts on the basal surface of the midgut epithelium and survive until at least day 10 pbf.

6.3.2.1 Ookinete migration from the bloodmeal to the basal surface of the midgut epithelium

The greatest loss in malaria parasite numbers during the *P. falciparum* 3D7A ookinete to oocyst transition in *An. stephensi* appears to occur *before* ookinete invasion of the mosquito midgut epithelium: approximately 80.7% of mature ookinetes forming within the bloodmeal failed to enter the midgut epithelium. The great majority of ookinetes failing to invade the midgut epithelium (89.3%) are apparently lost within the bloodmeal while a minority of these malaria parasites (11.7%) are lost during penetration of the peritrophic matrix and/or migration through the ectoperitrophic space to the apical surface of the midgut epithelium. Approximately, 19.2% of all mature ookinetes forming within the bloodmeal successfully penetrated the peritrophic matrix, migrated through the ectoperitrophic space and entered the midgut epithelium. However, 35.3% of those ookinetes entering the midgut epithelium did not succeed in transforming into oocysts on the basal surface of the midgut epithelium.

No previous studies have estimated the proportion of mature ookinetes forming within the bloodmeal that attain the peritrophic matrix, reach the midgut epithelium and fail to complete invasion of the midgut epithelium. Alavi *et al.* (2003) estimated *P.*

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[1] Pooling data from separate experimental feeds relies on the assumption that the proportion of malaria parasite losses occurring during different stages in the process of infection of the mosquito is constant across different experimental feeds (even when the overall level of malaria infection varies between experimental feeds).

berghei losses between ookinete formation within the bloodmeal and invasion of midgut epithelium by membrane-feeding *in vitro* cultured ookinetes to *An. stephensi* and *Ae. aegypti*. This experiment enabled comparison of the ability of ookinetes to associate with the midgut epithelia of two mosquito species differing markedly in susceptibility to *P. berghei* infection. However, the relevance of the resulting estimates of malaria parasite survival is somewhat doubtful, as the efficiency of the whole ookinete to oocyst transition for ookinetes developing entirely *in vivo* (17.8%) was greater than that for membrane-fed *in vitro* cultured ookinetes to attain the midgut epithelium alone (7.8%) (Alavi *et al.*, 2003).

The causes of the progressive reduction in *P. falciparum* 3D7A ookinete densities during malaria parasite development in *An. stephensi* are unclear: little evidence was observed in the histological sections of ookinete destruction within *any* compartment of the midgut lumen (Chapter 4). Several authors have reported loss of ookinetes within the endoperitrophic space of mosquito species both susceptible and refractory to malaria parasite infection (Huff, 1934; Omar, 1968b; Sluiters, Visser & van der Kaay, 1986). The largest losses of *P. falciparum* 3D7A ookinetes in *An. stephensi* occurs in the same compartment of the midgut lumen as *An. albimanus* completely refractory to this malaria parasite clone. Does this mean that the mechanism of refractoriness of *An. albimanus* to *P. falciparum* 3D7A infection is also responsible for the loss of ookinetes observed within the endoperitrophic space of *An. stephensi*? The mechanisms previously discussed for the refractoriness of *An. albimanus* to *P. falciparum* 3D7A might also account for the loss of ookinetes within the midgut lumen of *An. stephensi* (Section 6.2.2). If similar mechanisms account for the loss of ookinetes in endoperitrophic spaces of *An. albimanus* and *An. stephensi*, this would suggest that the difference in susceptibility to *P. falciparum* 3D7A oocyst infection between these two mosquito species is quantitative rather than qualitative. Preliminary vital staining experiments suggest that nearly all *P. falciparum* 3D7A retort-form and mature ookinetes within the bloodmeal of *An. stephensi* possess intact plasma membranes until at least 36 hours pbf (data not shown) (cf. *An. albimanus* described in Section 6.2.2.1). This would suggest that different mechanisms are responsible for the loss of *P. falciparum* 3D7A ookinetes within the endoperitrophic spaces of *An. albimanus* and *An. stephensi*. Recent studies have reported that over 60% of *P. berghei* zygotes and ookinetes undergo programmed cell death (apoptosis) within the bloodmeal of *An. stephensi* (Al-Olayan *et al.*, 2002b). Although increased levels of apoptosis might

account for the loss of *P. falciparum* 3D7A ookinetes within the endoperitrophic space of *An. albimanus*, the vital staining experiments suggest apoptosis does not explain the loss of these malaria parasite stages from the same compartment of *An. stephensi*. As discussed in Chapter 1, Section 1.5.2.2, ookinetes within the endoperitrophic space might also be destroyed by mosquito digestive proteases.

In *An. stephensi*, approximately 37.9% of *P. falciparum* 3D7A ookinetes attaining the peritrophic matrix subsequently failed to enter the midgut epithelium. The peritrophic matrix has previously been considered a potentially-significant barrier to malaria parasite infection (Ponnudurai *et al.*, 1988; Billingsley & Rudin, 1992). However, there was little evidence in the histological sections examined here that progressive "hardening" of the peritrophic matrix with increasing time pbf prevented ookinete infection as previously suggested for both *P. falciparum* and *P. cynomolgi bastianelli* in *An. stephensi*, and *P. gallinaceum* in *Ae. aegypti* (Stohler, 1957; Omar, 1968b; Meis & Ponnudurai, 1987b). Meis & Ponnudurai (1987b) reported that "many" apparently "non-viable" ookinetes of the *P. falciparum* NF54 isolate were "stuck" on the peritrophic matrix of *An. stephensi* at 36 hours pbf. However, the *P. falciparum* 3D7A ookinetes observed here in *An. stephensi* did not exhibit marked accumulation at the peritrophic matrix with increasing time pbf. Rather, there was a distinct peak in ookinete association with the peritrophic matrix between 24 and 28 hours pbf and relatively few ookinetes were observed within the peritrophic matrix after this time (Table 4.2) (Figure 4.44). The "wave" of ookinetes associated with the peritrophic matrix is consistent with the majority (at least 62.1%) of these malaria parasite stages successfully negotiating this mosquito structure.

In contrast to the large numbers of *P. falciparum* 3D7A ookinetes associated with the peritrophic matrix, few malaria parasites were observed within the ectoperitrophic space of *An. stephensi* before entry into the midgut epithelium. This is probably because ookinetes rapidly enter the midgut epithelium following penetration of the peritrophic matrix (Omar, 1968b). Such an interpretation suggests that the occasional prolonged gliding of ookinetes over the apical surface of the midgut epithelium prior to invasion observed *in vitro* is artefactual (Zieler & Dvorak, 2000). The paucity of malaria parasites observed within the ectoperitrophic space might result from rapid destruction of ookinetes within this compartment of the midgut epithelium. In particular, the concentration of digestive proteases secreted from the midgut epithelium is likely to be highest within the ectoperitrophic space during the time of ookinete

migration from the bloodmeal and entry into the midgut epithelium (Hörler & Briegel, 1995). There is, however, little direct evidence that ookinetes are destroyed within the ectoperitrophic space (Feldmann *et al.*, 1990), although Danielli *et al.* (2005) reported the presence of lysed transgenic *P. berghei* mutants (lacking P25 and P28) on the luminal surface of the midgut epithelium.

Approximately 64.7% of *P. falciparum* 3D7A ookinetes entering the midgut epithelium of *An. stephensi* successfully completed migration to the basal surface of the midgut epithelium. Several studies have provided estimates of the effect of knocking-out mosquito immune response genes on ookinete losses during migration across the midgut epithelium (Blandin *et al.*, 2004; Osta *et al.*, 2004a; Michel *et al.*, 2005). For example, Blandin *et al.* (2004) estimated that approximately 24.0 and 5.0% of *P. berghei* ookinetes survived invasion of the midgut epithelium of the *An. gambiae* 4arr and L-35 strains selected, respectively, for susceptibility and refractoriness to malaria parasite infection. In contrast, Han *et al.* (2000) stated that at least 95% of *P. berghei* ookinetes successfully completed migration across the midgut epithelium of *An. stephensi*.

The loss of *P. falciparum* 3D7A ookinetes observed during invasion of the midgut epithelium of *An. stephensi* is probably primarily due to mosquito immune responses (e.g. Luckhart *et al.*, 1998), although the exact mechanisms involved are currently unknown and may differ for those previously reported using *P. berghei* (Tahar *et al.*, 2002). Melanisation of *P. falciparum* 3D7A ookinetes/oocysts does not occur in *An. stephensi* and this cannot explain the loss of this malaria parasite clone in this mosquito species during invasion of the midgut epithelium. Some ookinetes might also be destroyed by mechanical processes related to extrusion of invaded midgut epithelial cells from the midgut epithelium into the midgut lumen (rather than mosquito immune responses *per se*). As previously reported, a small number of "stalk-form" ookinetes were observed for *P. falciparum* 3D7A in *An. stephensi* (Chapter 4, Section 4.3.3.4.4) (Vernick *et al.*, 1999; Han *et al.*, 2000). The significance of the stalk-form morphology is currently not understood. Recent *in vivo* observations demonstrate that ookinetes are remarkably plastic and undergo marked deformations during migration across the midgut epithelium (Vlachou *et al.*, 2004). However, some stalk-form ookinetes are apparently severed in two (Han *et al.*, 2000) suggesting that this unusual malaria parasite morphology is pathological rather than an example of the typical transient deformations often accompanying ookinete invasion of the midgut epithelium.

6.3.2.2 Oocyst survival in *An. stephensi*

Several previous studies have investigated oocyst survival for wild *P. falciparum* isolates in *An. arabiensis* and *An. gambiae* estimating that between 11.7 and 83.1 to 97.3% of these malaria parasite stages survive to complete sporogony in these two mosquito species respectively (Awono-Ambene & Robert, 1998; Gouagna *et al.*, 1998; Gouagna *et al.*, 1999; Gouagna *et al.*, 2004a). In the current work, there was no statistically significant reduction in *P. falciparum* 3D7A numbers between the early (day 2 pbf) and late (day 10 pbf) oocyst stages in *An. stephensi* implying that in this particular malaria parasite-mosquito vector combination there was no malaria parasite mortality during the entire period of oocyst development (i.e. approximately 100% survival) (Table 3.2 and Table 3.3).

Given that the process of oocyst development takes at least eight days, the level of *P. falciparum* 3D7A survival observed in *An. stephensi* over this extended period of time is remarkable and implies that these malaria parasite stages possesses extremely efficient mechanisms enabling persistence within the hostile, immune competent, environment of the mosquito vector. Oocyst stage malaria parasites have previously been shown to be susceptible to exogenous antimicrobial peptides (Gwadz *et al.*, 1989; Shahabuddin *et al.*, 1998a), and early oocysts undergo developmental arrest in some natural parasite-vector combinations (Gonzalez-Ceron *et al.*, 2001), providing evidence that oocysts are potentially susceptible to mosquito immune responses. The nature of the mechanisms enabling oocyst survival are currently unknown. Components of the midgut basal lamina are incorporated into the oocyst capsule, which may facilitate evasion of recognition by the mosquito immune system (Adini & Warburg, 1999). Alternatively, oocysts may actively suppress the mosquito immune response, although there is currently no evidence supporting this hypothesis.

Although the levels of early and late *P. falciparum* 3D7A oocyst infection were not significantly different in *An. stephensi*, the intensity of early oocyst infection tended to be lower than the intensity of late oocyst infection (Table 3.6). Gouagna *et al.* (1998; 1999) previously reported that estimates of levels of early oocyst infection might be underestimated by the use of FITC-labelled α-Pfs25 mAb, because ookinetes may not have completed migration across the midgut epithelium at the time of sampling (56 to 72 hours pbf) and/or levels of Pfs25 expressed by oocysts might be reduced due to release of this protein during ookinete invasion of the midgut epithelium. As ookinete

migration across the midgut epithelium was essentially completed by 48 hours pbf (Figure 4.44), only the latter explanation is probably relevant in the current context.

6. 4 Ookinete invasion of the mosquito midgut epithelium

The nature of ookinete invasion of the midgut epithelium has remained controversial, much-debated and unresolved from the end of the nineteenth century until today (Chapter 1, Section 1.4.7 and Chapter 4, Section 4. 1) (Sinden & Billingsley, 2001; Shahabuddin, 2002). The major argument concerns the route of ookinete migration: whether it is intracellular through, or intercellular between, midgut epithelial cells. Recently, a consensus has emerged that both *P. berghei* and *P. gallinaceum* ookinetes enter the midgut epithelium *via* an intracellular route (Meis *et al.*, 1989; Torii *et al.*, 1992; Shahabuddin & Pimenta, 1998; Han *et al.*, 2000; Zieler & Dvorak, 2000). However, two radical and conflicting models of ookinete invasion of the midgut epithelium have been proposed for these malaria parasite species (Shahabuddin & Pimenta, 1998; Han *et al.*, 2000).

The observations of ookinete invasion of the midgut epithelium reported in Chapter 4 used *P. falciparum* 3D7A in a natural vector, *An. stephensi*. The midguts from this mosquito were infected *in vivo* and processed with minimal manipulation to produce histological sections that were examined by light microscopy. Therefore, the observations made are unlikely to be artefacts resulting from either experimental manipulation or using an unnatural malaria parasite-mosquito vector combination. No evidence was found for the existence of a specific subset of morphologically distinct midgut epithelial cells preferentially invaded ookinetes: excluding presumed pathological changes, all normal columnar midgut epithelial cells were morphologically indistinguishable. Instead, the observations made were consistent with *P. falciparum* 3D7A entering morphologically-normal midgut epithelial cells possessing typical centrally-located nuclei and abundant microvilli that subsequently underwent significant pathological changes as a consequence of ookinete invasion. As already discussed, the observations of *P. falciparum* in *An. stephensi* made here differed from those previously made for this malaria parasite-mosquito vector combination (although they are not inconsistent with them) (Chapter 4, Section 4.4.2.1). The observations of *P. falciparum* 3D7A in *An. stephensi* also provided no evidence for the existence of Ross cells or the preferential invasion of a subset of midgut epithelial cells by ookinetes (Shahabuddin & Pimenta, 1998; Cociancich *et al.*, 1999; Shahabuddin, 2002). However, the

observations made with *P. falciparum* 3D7A are consistent with those previously described by the Time Bomb model for the rodent malaria *P. berghei* in *An. stephensi* (Han *et al.*, 2000). Additionally, the observations reported here for *P. falciparum* 3D7A provide further evidence in support of the Time Bomb model, which hypothesised, but did not formally demonstrate (Shahabuddin, 2002), that ookinete-invaded midgut epithelial cells are completely extruded from the midgut epithelium into the midgut lumen.

Although *P. falciparum* 3D7A ookinete invasion of the midgut epithelium of *An. stephensi* is consistent with the Time Bomb model, ookinetes were also observed in intercellular locations between morphologically-normal midgut epithelial cells as previously reported for this malaria parasite-mosquito vector combination (Meis & Ponnudurai, 1987b; Meis *et al.*, 1989). The original studies describing the Time Bomb model only reported *P. berghei* ookinetes in intracellular locations within the midgut epithelium and extracellular locations on the basal surface of (and therefore without) the midgut epithelium (Han *et al.*, 2000). *P. berghei* ookinetes were not reported in intercellular locations in these, or previous, studies (Meis *et al.*, 1989; Han *et al.*, 2000). Previous studies have observed ookinetes in a number of locations associated with the midgut epithelium, consistent with either an intra- or intercellular route of malaria parasite migration across the midgut epithelium (Figure 6.2). Furthermore, ookinetes invading the midgut epithelium have been associated with both morphologically-normal and -abnormal midgut epithelial cells. Significantly, ookinetes invading the midgut epithelium have never previously been observed, within same study of a single malaria parasite-mosquito vector combination, in all the possible locations associated with the midgut epithelium. Consequently, different researchers have concluded that ookinetes take different routes to invade the midgut epithelium, and have different effects on the midgut epithelium, depending on the malaria parasite-mosquito combination (Maier, 1987; Meis & Ponnudurai, 1987a; Meis *et al.*, 1989). In the work presented in Chapter 4, *P. falciparum* 3D7A *was* observed in *all* of the possible locations associated with the midgut epithelium previously described, both in the absence, and in the presence, of morphologically-abnormal midgut epithelial cells. This raises the possibility that ookinetes, regardless of the malaria parasite-mosquito vector combination, invade the midgut epithelium by a common route and have similar effects on the midgut epithelium. Previous studies claiming to observe different routes of ookinete migration across the midgut epithelium were perhaps reporting different aspects of the same

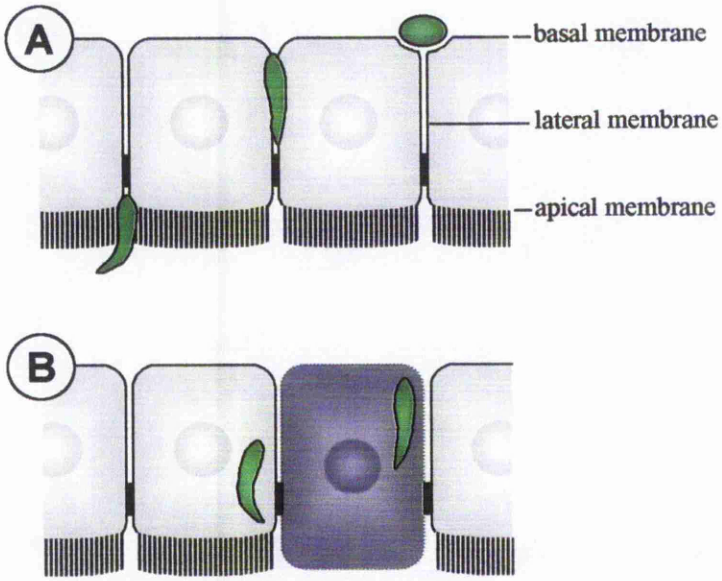


Figure 6.2 The various locations within the midgut epithelium that invading ookinetes have previously been observed in sectioned material examined by light or electron microscopy.

In previous light and electron microscopy investigations of sectioned material, invading malaria parasites have been observed in various locations associated with the midgut epithelium. **(A)** Studies reporting that ookinete migration across the midgut epithelium occurs *via* an intercellular route observed malaria parasites (green) entering the midgut epithelium at sites where adjacent midgut epithelial cells met, in the basal extracellular space between morphologically normal midgut epithelial cells and on the basal surface of the midgut epithelium immediately above the lateral plasma membranes of the underlying, morphologically normal, midgut epithelial cells . **(B)** Studies reporting that ookinete invasion of the midgut epithelium occurred *via* an intracellular route observed malaria parasites inside either morphologically normal or abnormal midgut epithelial cells. The two studies observing ookinetes in both intra- and intercellular locations within the midgut epithelium did not report the occurrence of morphologically abnormal midgut epithelial cells (Torii *et al.*, 1992; Vernick *et al.*, 1995). A single study reported that ookinete-invaded midgut epithelial cells were extruded from the midgut epithelium but did not report intercellular migration by malaria parasites (Becker-Feldman, Maier & Seitz, 1985; Maier, Becker-Feldman & Seitz, 1987).

phenomenon. Careful comparison of the previous literature reveals that *different* authors have observed ookinetes in different locations within the midgut epithelium using the same malaria parasite-mosquito vector combination (Table 1.1 and Chapter 4, Section 4. 1). The major unanswered problems are why many researchers have believed that ookinete invasion could be solely intercellular (or solely intracellular) and have not observed pathological changes in the midgut epithelium following ookinete invasion.

6.4.1 A unified model of ookinete invasion of the midgut epithelium

Based upon the observations reported in Chapter 4, a single "unified" model of the route of ookinete invasion across the mosquito midgut epithelium, synthesising the seemingly contradictory observations of previous studies, is proposed in Figure 6.2 to Figure 6.7. According to this model, ookinete entry into the midgut epithelium is initially intracellular, but occurs through the lateral apical plasma membrane of midgut epithelial cells at sites where three adjacent midgut epithelial cells converge (Figure 6.3 and Figure 6.4). Furthermore, ookinete entry into midgut epithelial cells is accompanied by, at least sometimes, significant invagination of the apical surface of the midgut epithelium localised around the invading malaria parasite (Figure 6.4). Ookinetes subsequently pass intracellularly through one or more midgut epithelial cells, causing pathological changes to the latter which are consequently extruded from the midgut epithelium into the midgut lumen (Figure 6.5 to Figure 6.7). Ookinetes may exit invaded midgut epithelial cells and directly attain the basal extracellular surface of the midgut epithelium (Figure 6.5). Alternatively, ookinetes may exit invaded midgut epithelial cells into the basal intercellular space between adjacent uninvaded morphologically-normal midgut epithelial cells and complete migration to the basal surface of the midgut epithelium *via* an intercellular route (Figure 6.6).

6.4.1.1 Ookinete entry into the midgut epithelium

The initial entry of ookinetes into the midgut epithelium has only rarely been observed in fixed material of *in vivo* infected mosquitoes but was consistently found to occur where the lateral plasma membranes of adjacent midgut epithelial cells converged (Figure 6.3A) (Stohler, 1957; Meis & Ponnudurai, 1987b; Syafruddin *et al.*, 1991). As no obviously-intracellular ookinetes were observed in these studies, this was interpreted as evidence that ookinetes took a solely intercellular route, between adjacent midgut epithelial cells, from the apical to the basal surfaces of the midgut epithelium.

Direct observations of live material *in vitro* and *ex vivo* have demonstrated that

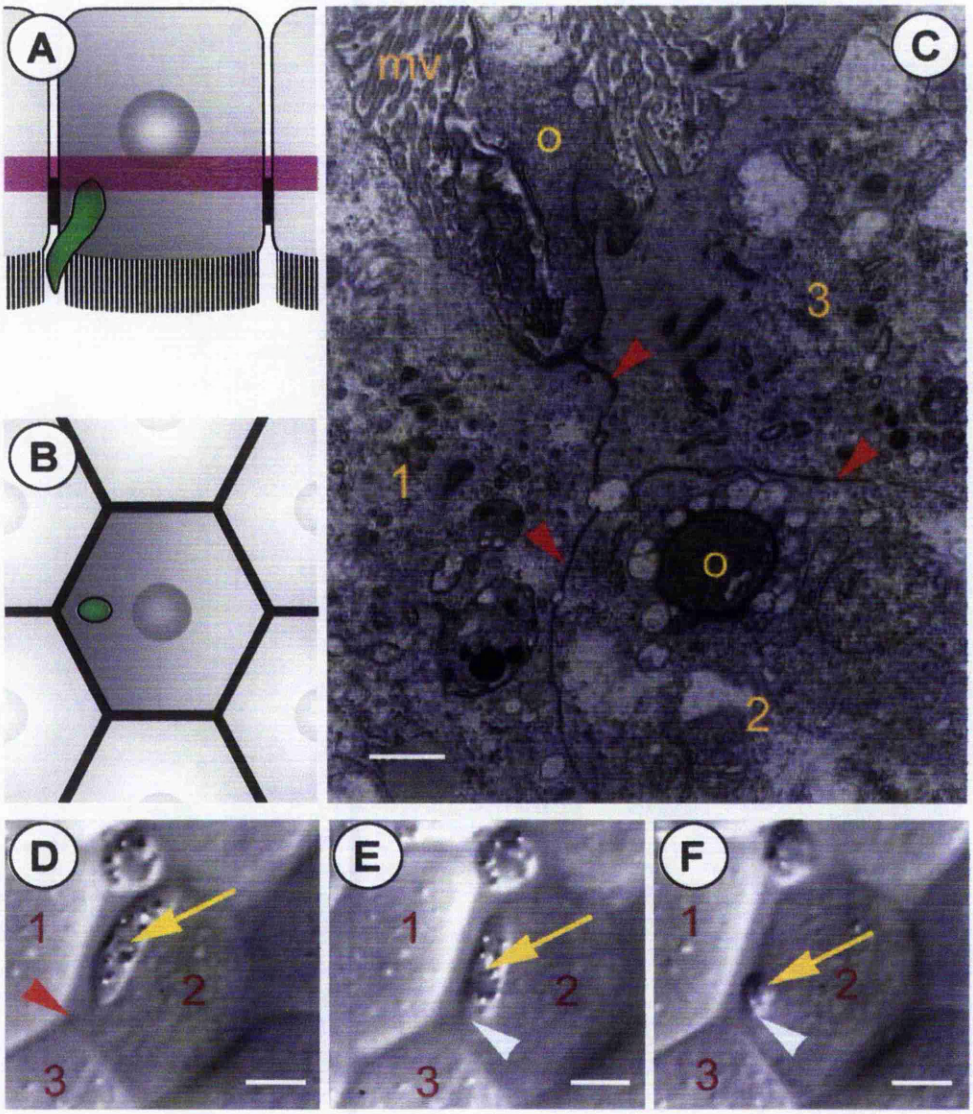


Figure 6.3 Ookinete entry into the midgut epithelium at "three-cell junctions".

(A and B) Ookinetes (shown in green) enter midgut epithelial cells through their lateral apical plasma membrane at sites where three adjacent midgut epithelial cells converge. The purple line in **A** indicates the orthogonal plane through which the section shown in **B** is taken.

(C to F) Re-interpretation of previously published observations also suggests that ookinetes enter the midgut epithelium where three adjacent midgut epithelial cells converge. **(C)** Electron micrograph of a single ookinete of the rodent malaria parasite *Plasmodium yoelii nigeriensis* entering the midgut epithelium of *Anopheles omorii* (Figure 5 in Syafruddin *et al.*, 1991). This ookinete (o) appears to be invading the midgut epithelium intracellularly at a site where *three* adjacent midgut epithelial cells

(numbered 1, 2 and 3) appear to converge. The lateral plasma membranes of the three midgut epithelial cells are indicated by the red arrowheads. Part of the ookinete resides within the middle midgut epithelial cell (2), surrounded by vacuoles, while part of the ookinete is extracellular within the microvilli (mv). Scale bar = 1 μm. **(D to F)** Light micrographs using differential interference contrast, looking down onto the apical surface of the midgut epithelium, showing sequential temporal stages of an ookinete of the avian malaria parasite *Plasmodium gallinaceum* entering the midgut epithelium of *Aedes aegypti* (Figure 1 in Zieler & Dvorak, 2000). Yellow arrow indicates the ookinete. Red arrowhead indicates site where the lateral plasma membranes of three adjacent midgut epithelial cells (1, 2 and 3) converge. White arrowhead indicates the entry site of the ookinete, which is moving downwards, into one of the midgut epithelial cells (2). Scale bars = 5 μm.

Dr Din Syafruddin is gratefully acknowledged for providing an electronic copy of the electron micrograph shown.

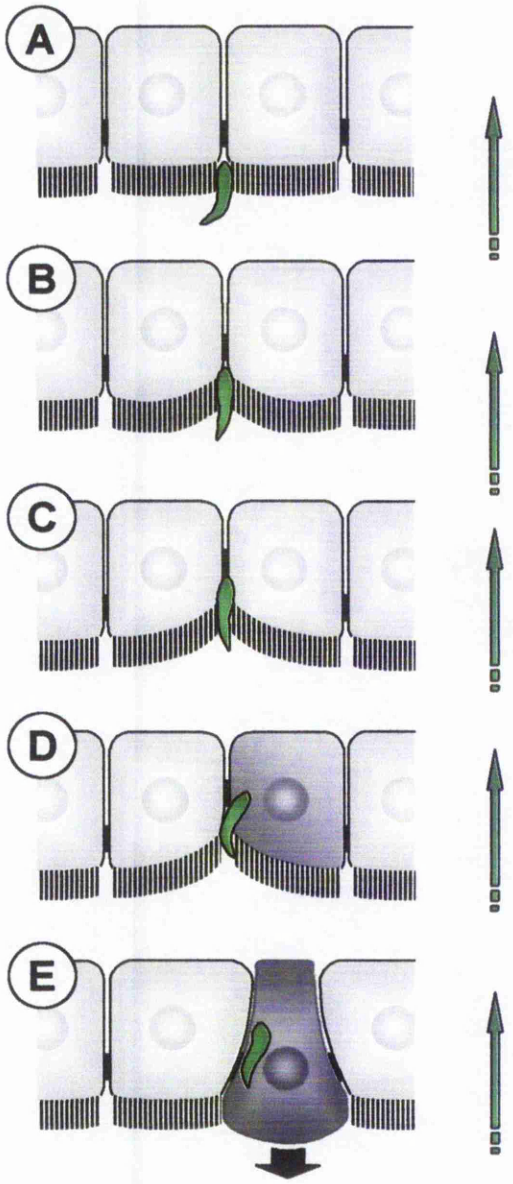


Figure 6.4 Invagination of the midgut epithelium during ookinete invasion.

(A) Ookinetes (green) enter the apical surface of the midgut epithelium at sites where the lateral plasma membranes of adjacent midgut epithelial cells converge. **(B)** ookinete entry causes localised invagination of the apical surface of the midgut epithelium, which **(C)** can be extensive, up to one half to two-thirds the depth of the midgut epithelium. **(D)** Ookinetes subsequently enter into midgut epithelial cells through the lateral apical plasma membrane immediately adjacent to where the intercellular junctions of adjacent midgut epithelial cells begin. **(E)** Ookinetes proceed through the invaded midgut epithelial cell, which exhibits morphological abnormalities including loss of microvilli and protrusion into the midgut lumen (indicate by large black arrow). Green arrows indicate direction of ookinete movement.

ookinete penetration into the midgut epithelium is rapid, taking on average less than two minutes (Freyvogel, 1966; Zieler & Dvorak, 2000), and this probably explains the rarity of observing such events in fixed material. Zieler & Dvorak (2000) observed live malaria parasites *in vitro* noting that ookinete entry into the midgut epithelium was intracellular and invariably occurred through the lateral apical plasma membrane of midgut epithelial cells, immediately adjacent to the site where the intercellular junctions between these midgut epithelial cells began. Consequently, the assumption that ookinetes entering the midgut epithelium where adjacent midgut epithelial cells converge must necessarily be taking an intercellular route is questionable, especially since the malaria parasite species supposedly taking a solely intercellular route across the midgut epithelium have also been observed in intracellular locations (Chapter 4) (Indacochea, 1935; Davies, 1974; Maier *et al.*, 1987). An alternative interpretation is that ookinetes seeming to enter the midgut epithelium *via* an intercellular route could actually be invading the midgut epithelium intracellularly, entering *into* midgut epithelial cells through the lateral apical plasma membrane. Furthermore, the *P. falciparum* 3D7A ookinetes observed here specifically entered the midgut epithelium where the lateral plasma membranes of *three* adjacent midgut epithelial cells converged. Re-interpretation of previous observations also appears to show that ookinetes enter the midgut epithelium at sites where three adjacent midgut epithelial cells converge (Figure 6.3) (Figure 2 in Meis & Ponnudurai, 1987b; Figure 5 in Syafruddin *et al.*, 1991; Figure 1 in Zieler & Dvorak, 2000). Therefore, the site of ookinete entry into the midgut epithelium appears to be conserved across evolutionarily-divergent malaria parasite-mosquito vector combinations.

Although the number of observations is small and more events need to be described before robust inferences can be drawn, ookinetes, either preferentially or perhaps invariably, enter the midgut epithelium where the lateral membranes of three adjacent midgut epithelial cells intersect. Why should ookinetes enter the midgut epithelium where multiple adjacent midgut epithelial cells converge? As the microvillar brush border is often parted where adjacent midgut cells meet, such sites might provide ookinetes with unimpeded access to the plasma membrane of the midgut epithelial cell. Alternatively, the mechanical and structural properties of the midgut epithelium at these sites might be of importance for the invading malaria parasite. In particular, the marked invagination of the apical surface of the midgut epithelium associated with ookinete invasion implies that considerable mechanical force is generated by the malaria parasite

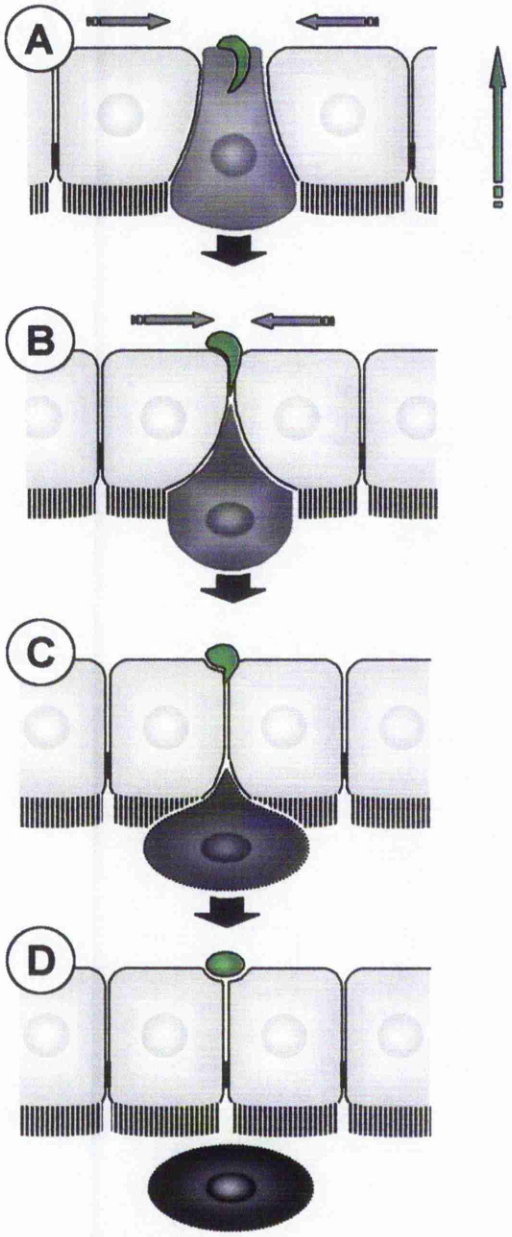


Figure 6.5 Ookinete invasion *via* a solely intracellular route.

(A) If the invaded midgut epithelial cell extrudes relatively slowly from the midgut epithelium, then ookinetes (green) exiting invaded epithelial cells could directly attain the basal surface of the midgut epithelium. **(B to D)** If ookinetes move no further but immediately transform into oocysts, extrusion of invaded cells from beneath the malaria parasites will leave them lying above the lateral plasma membranes of morphologically normal epithelial cells. Large black arrows indicate the direction of movement of the ookinete-invaded midgut epithelial cell from the midgut epithelium into the midgut lumen. Grey arrows indicate movement of the surrounding uninvaded midgut epithelium to fill the void left by the extruding ookinete-invaded midgut epithelial cell.

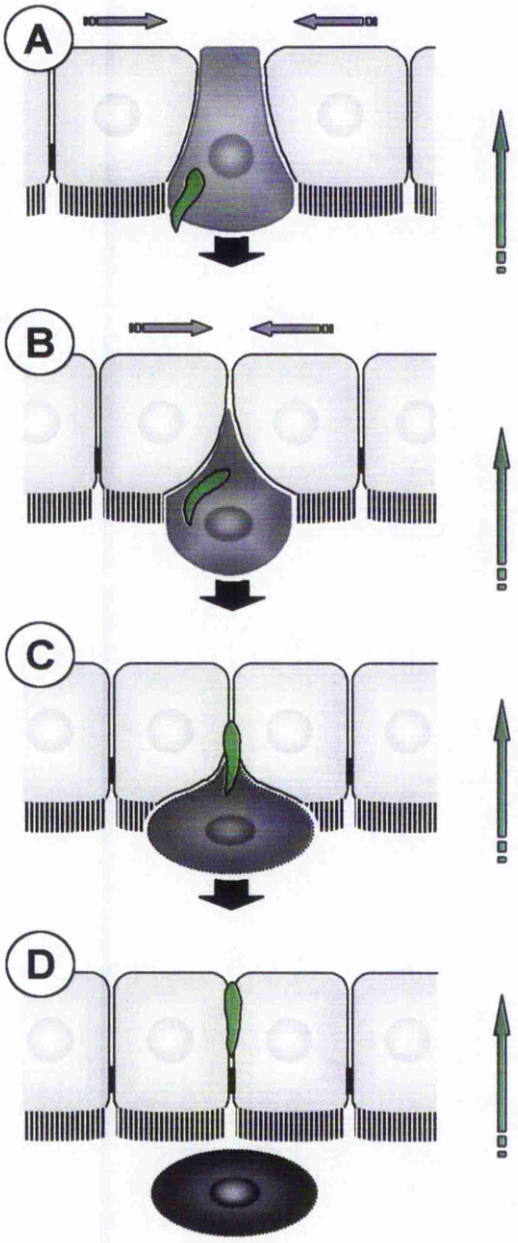


Figure 6.6 Ookinete invasion *via* first intra- and then intercellular routes.

(A to D) If invaded midgut epithelial cells extrude sufficiently rapidly, then ookinetes (green) exiting invaded epithelial cells could find themselves in the basal intercellular space between adjacent healthy epithelial cells despite having entered the midgut epithelium by an intracellular route. Green arrows indicate direction of ookinete movement. Large black arrows indicate the direction of movement of the ookinete-invaded midgut epithelial cell from the midgut epithelium into the midgut lumen. Grey arrows indicate movement of the surrounding uninvaded midgut epithelium to fill the void left by the extruding ookinete-invaded midgut epithelial cell.

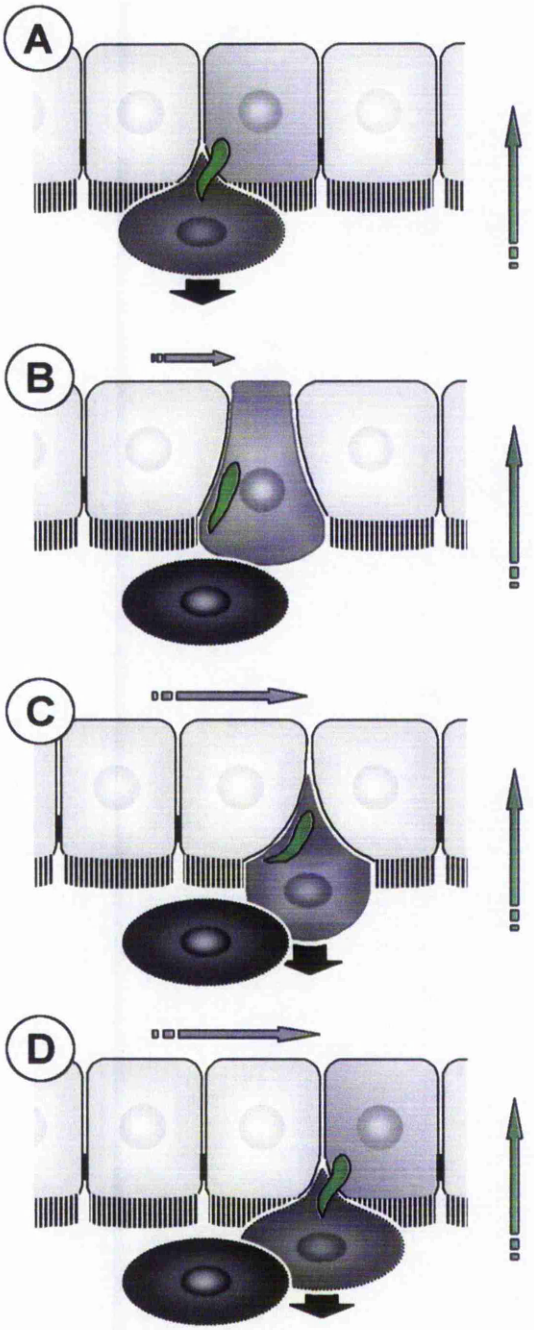


Figure 6.7 Ookinete invasion through multiple adjacent midgut epithelial cells.

(A to D) If extrusion of ookinete-invaded midgut epithelial cells is sufficiently rapid, malaria parasites will be able to pass from the basal surface of the extruding epithelial cell to the apicolateral surface of adjacent uninvaded midgut epithelial cells. Green arrows indicate direction of ookinete movement. Large black arrows indicate the direction of movement of the ookinete-invaded epithelial cells from the midgut epithelium. Grey arrows indicate movement of the surrounding uninvaded midgut epithelium to fill the void left by the extruding ookinete-invaded epithelial cells.

during entry into the midgut epithelium. Consequently, if ookinetes require force to penetrate the apical surface of midgut epithelial cells perhaps this can only be created at sites where the midgut epithelium is sufficiently rigid to provide resistance for the invading malaria parasite to push against. Similarly, entering the midgut epithelium where multiple adjacent midgut epithelial cells converge might also provide the ookinete with greater traction and, hence, enable the generation of greater force. This is because more surfaces of the ookinete will simultaneously contact the midgut epithelium when entry into the midgut epithelium occurs where multiple adjacent midgut epithelial cells converge. Ookinete invasion where multiple adjacent midgut epithelial cells converge might also reduce the occurrence of negative (for the malaria parasite) changes in invaded midgut epithelial cells. For example, ookinete invasion through the central region of the apical plasma membrane might be more likely to cause rapid explosive changes in the invaded midgut epithelial cell which might impede ookinete progress through the midgut epithelium. Ookinetes invading the corners of midgut epithelial cells might also use the groove created by adjacent lateral sides of the midgut epithelial cells as directional guides to reach the basal surface of the midgut epithelium. Consistent with this interpretation, intracellular ookinetes often, but not always, lie adjacent and parallel to the lateral plasma membranes of invaded midgut epithelial cells suggesting movement along the internal surfaces of the plasma membranes of the midgut epithelium. It is tempting to speculate that recognition of specific midgut receptors directs ookinetes to enter the midgut epithelium where multiple adjacent midgut epithelial cells converge. However, topological features of the apical surface of the midgut epithelium alone might be sufficient to lead ookinetes to the requisite sites of entry.

6.4.1.2 Ookinete exit from the midgut epithelium

Ookinetes and oocysts are frequently located within, or immediately above, the lateral plasma membranes of midgut epithelial cells (Huff, 1934; Stohler, 1957; Howard, 1962; Omar, 1968b; Meis *et al.*, 1989; Meis & Ponnudurai, 1989; Syafruddin *et al.*, 1991). This has previously been interpreted as evidence that ookinetes migrate across the midgut epithelium *via* a solely intercellular route. However, the observations reported in Chapter 4 suggest that extrusion of ookinete-invaded midgut epithelial cells accounts for the extracellular location of malaria parasites between, or directly above, the lateral plasma membranes of midgut epithelial cells. An alternative explanation for the occurrence of intercellular ookinetes is that invaded midgut epithelial cells do not

extrude from the midgut epithelium and ookinetes exit invaded midgut epithelial cells through the lateral plasma membrane (Torii *et al.*, 1992). Although this is possible, this has not been observed. If the argument presented here is correct, ookinetes might only exit invaded midgut epithelial cells through the basal membrane and yet still be found in intercellular positions within the midgut epithelium.

6.4.2 Does a "cellular treadmill" explain the route of ookinete migration across the midgut epithelium?

According to the unified model outlined above, extrusion of ookinete-invaded midgut epithelial cells plays a dynamic and decisive role in determining the route of ookinete invasion across the midgut epithelium, requiring malaria parasites to either take an intercellular route to reach the basal surface of the midgut epithelium or invade further midgut epithelial cells until such a route is possible, or the basal surface of the midgut epithelium can be directly attained *via* a solely intracellular route. Consequently, the midgut epithelium can be conceptualised as a "cellular treadmill" of extruding midgut epithelial cells initiated by ookinete invasion. The particular route taken by any individual ookinete will depend on the specific dynamic interactions between the malaria parasite and the midgut epithelium. In particular, the relative rates of ookinete movement through midgut epithelial cells and the extrusion of invaded midgut epithelial cells from the midgut epithelium into the midgut lumen are predicted to determine the route of ookinete migration across the midgut epithelium. If the ookinete migrates through the midgut epithelial cell at sufficient speed, the basal surface of the midgut epithelium may be directly attained before appreciable extrusion of the invaded midgut epithelial cell (Figure 6.5). Alternatively, if the speed of ookinete translocation through the invaded midgut epithelial cell is slower, the midgut epithelial cell may have undergone appreciable extrusion from the midgut epithelium by the time of ookinete exit forcing the malaria parasite to take an intercellular route (Figure 6.6). Finally, if the rate of ookinete invasion is relatively slow, the invaded midgut epithelial cell may have undergone almost complete extrusion from the midgut epithelium confronting the exiting malaria parasite with further midgut epithelial cells (Figure 6.7).

This "Cellular Treadmill" model corresponds well with the current and previous observations of ookinete invasion of the midgut epithelium, and accounts for the considerable diversity observed in the exact route of ookinete invasion taken (Chapter 4) (Han *et al.*, 2000; Zieler & Dvorak, 2000). However, it is uncertain whether this is the entire explanation for the different routes ookinetes use to cross the midgut

epithelium. The Cellular Treadmill model implies that in the absence of extrusion of invaded midgut epithelial cells ookinetes should neither take intercellular routes nor invade multiple adjacent midgut epithelial cells. Although the existence of both of the latter phenomena would not falsify the Cellular Treadmill model, if they occur, other factors would be required to explain them. In this regard, the Time Bomb model and the Cellular Treadmill model make subtly distinct predictions. According to the Cellular Treadmill model, lateral intracellular movement within the midgut epithelium is apparent rather than real: ookinetes need only travel in a single, basally-orientated direction to move into adjacent midgut epithelial cells (Figure 6.7). Consequently, intracellular migration through multiple adjacent midgut epithelial cells could be considered as an incidental, non-adaptive, consequence of the mosquito midgut tissue-repair mechanism of extruding damaged midgut epithelial cells into the midgut lumen. In contrast, the Time Bomb model proposes that ookinetes exhibit 'true' lateral movement within the midgut epithelium (passage across both lateral plasma membranes of adjacent midgut epithelial cells, rather than passage across the basal and apical plasma membranes of adjacent midgut epithelial cells that have become apposed through extrusion of the invaded midgut epithelial cell; Figure 6.7). Malaria parasites are proposed to alter the orientation of their movement within midgut epithelial cells in an adaptively purposeful manner in order to avoid the "time bomb" of deleterious mosquito responses triggered by ookinete invasion (Han *et al.*, 2000; Han & Barillas-Mury, 2002; Kumar *et al.*, 2004; Kumar & Barillas-Mury, 2005). These two differing models of lateral intracellular movement through the midgut epithelium are not necessarily mutually exclusive and both may be relevant to varying extents in different situations. In this respect, current evidence supporting the Cellular Treadmill model is equivocal. Trails of partially and then completely extruded midgut epithelial cells are sometimes present behind invading ookinetes suggesting a treadmill effect (e.g. Figure 4.20 and Figure 4.21) and malaria parasites can be observed apparently moving from the basal surface of extruded midgut epithelial cells into the apicolateral surface of the adjacent midgut epithelial cells (Figure 4.19E and Figure 4.25) (Becker-Feldman *et al.*, 1985). However, ookinetes have been observed *in vitro* moving between adjacent midgut epithelial cells apparently exhibiting little or no extrusion from the midgut epithelium (Zieler & Dvorak, 2000), and *in vivo* clusters of ookinete-invaded midgut epithelial cells exhibit similar degrees of extrusion (Figure 4.19D) (Han *et al.*, 2000). Unambiguous identification of the midgut epithelial cell plasma membranes crossed

during ookinete lateral intracellular migration through the midgut epithelium, and observation of the orientation and direction of ookinete movement within midgut epithelial cells, should help to clarify these issues and can now be done using *in vivo* imaging techniques (Vlachou *et al.*, 2004; Amino *et al.*, 2005).

Another difference between the Time Bomb and the Cellular Treadmill models relates to the route of ookinete movement after malaria parasite exit from invaded midgut epithelial cells. Han *et al.* (2000) claimed that after exiting invaded midgut epithelial cells, *P. berghei* ookinetes migrated extracellularly *over* the basal surface of the midgut epithelium, away from their initial site of intracellular invasion of the midgut epithelium, as a means of escaping the deleterious responses of the invaded midgut epithelial cell. This interpretation was based upon the observation that ookinetes on the basal surface of the midgut epithelium were located several midgut epithelial cell diameters from morphologically-abnormal midgut epithelial cells through which the malaria parasites were presumed to have invaded. This finding was surprising because ookinete movement is generally assumed to immediately cease when malaria parasites reach the basal lamina surrounding the outer surface of the midgut epithelium (Chapter 1, Section 1.4.8). However, there was no evidence that *P. falciparum* 3D7A ookinetes migrated extracellularly *over* the basal surface of the midgut epithelium in *An. stephensi*: all malaria parasites entirely without the midgut epithelium appeared to possess the rounded form of oocyst stages. This may reflect species-specific differences between *P. berghei* and *P. falciparum*, although malaria parasites of the latter species were also sometimes found on the basal surface of the midgut epithelium located some distance from morphologically-abnormal midgut epithelial cells through which they were presumed to have previously migrated. An alternative interpretation is that ookinete movement might always cease upon reaching the basal surface of the midgut epithelium and malaria parasites distant from their initial intracellular invasion site might have undergone extensive intercellular migrations *within*, rather than extracellularly over, the midgut epithelium as previously proposed (Torii *et al.*, 1992).

The Cellular Treadmill model can also provide explanations for certain aspects of the 'stalk-form' ookinete morphology, which are unaccounted for by the Time Bomb model (Vernick *et al.*, 1999; Han *et al.*, 2000). Stalk-form ookinetes are dumb-bell shaped possessing a narrow central stalk connecting bulbous anterior and posterior parts. The central region varies in length from a highly localised constriction (no apparent stalk) to a thin string of homogenous breadth as long as a normal sausage-

shaped ookinete. Localised (relatively minor) constrictions have been observed around other apicomplexan parasites during migration across biological barriers, but the elongated central stalk has been reported only for ookinetes invading the midgut epithelium; this suggests that it results from a specific interaction between the malaria parasite and the midgut epithelium (Vernick *et al.*, 1999). Only a small proportion of ookinetes invading the midgut epithelium are stalk-form, implying that it is either a rare or transient condition (Chapter 4, Section 4.3.3.4.4) (Han *et al.*, 2000). Han *et al.* (2000) reported that the central constrictions/stalks were surrounded by rings/aggregates of contracting actin formed in the basal region of extruding midgut epithelial cells following ookinete invasion. Although the contracting actin rings/aggregates explain the occurrence of localised constrictions within ookinetes, it is not obvious why *elongated* stalks should be found within these malaria parasite stages. Stalk-form ookinetes have only previously been observed exiting invaded midgut epithelial cells (i.e. traversing the plasma membrane of the invaded midgut epithelial cell) (Vernick *et al.*, 1999; Han *et al.*, 2000). Therefore, one possibility is that the elongated stalks are created through ookinetes being "stretched" by invaded midgut epithelial cells as the latter are extruded from the midgut epithelium into midgut lumen. An unexpected observation made here with *P. falciparum* is that an apparently stalk-form ookinete was observed in the process of *entering* the midgut epithelium (Figure 4.11B). A similarly constricted *P. berghei* ookinete has also been observed entering the midgut epithelium of *An. stephensi*, although no "stalk" was reported for this particular malaria parasite (Limviroj *et al.*, 2002). This implies that midgut epithelial cell extrusion *per se* does not have a role in producing elongated stalks. Actin aggregates have been observed around ookinetes on the luminal side of the midgut epithelium, possibly in the process of entering midgut epithelial cells (Danielli *et al.*, 2005; Baton & Ranford-Cartwright, 2005a), and around an ookinete constricted by the apical plasma membrane of an extruding midgut epithelial cell (Gupta *et al.*, 2005).

6.4.3 Is the unified model of ookinete invasion of the midgut epithelium generally applicable?

Many aspects of ookinete migration across the midgut epithelium are conserved across evolutionarily-distant malaria parasite and mosquito species implying a universal route of ookinete migration (Torii *et al.*, 1992; Zieler & Dvorak, 2000). In the malaria parasite-mosquito vector combinations that have been studied, the following aspects of ookinete migration across the midgut epithelium appear to be the same:

(1) the site of entry into the midgut epithelium (through the apicolateral plasma membrane where three adjacent midgut epithelial cells converge);
(2) intracellular migration through the midgut epithelium;
(3) pathological changes to invaded midgut epithelial cells;
(4) the occurrence of ookinetes in intercellular locations within the midgut epithelium;
(5) the presence of basal extracellular malaria parasites immediately above the lateral plasma membranes of the underlying midgut epithelial cells;
(6) the lateral intracellular migration of ookinetes through multiple adjacent midgut epithelial cells; and
(7) the occurrence of altered ookinete morphology (stalk-forms) associated with the passage across the plasma membranes of midgut epithelial cells.

Additionally, extrusion of ookinete-invaded midgut epithelial cells from the midgut epithelium has been observed in all of the mammalian malaria parasite-anopheline mosquito vector species combinations that have been investigated (Chapter 4) (Omar, 1968b; Becker-Feldman *et al.*, 1985; Maier *et al.*, 1987; Paskewitz *et al.*, 1988; Meis *et al.*, 1989; Han *et al.*, 2000; Vlachou *et al.*, 2004; Kadota *et al.*, 2004; Kumar *et al.*, 2004; Danielli *et al.*, 2005).[1] In contrast, at the time of formulating the unified model of the route of ookinete invasion across the midgut epithelium, extrusion of ookinete-invaded midgut epithelial cells had not been reported in any of the avian malaria parasite-culicine mosquito vector species combinations that had been investigated (Reichenow, 1932; Huff, 1934; Stohler, 1957; Maier, 1973; Mehlhorn *et al.*, 1980; Torii *et al.*, 1992; Shahabuddin & Pimenta, 1998; Zieler & Dvorak, 2000). However, recent studies using the *P. gallinaceum-Ae. aegypti* combination have reported extrusion of ookinete-invaded midgut epithelial cells within this culicine mosquito species (Gupta *et al.*, 2005). Although the mechanism of midgut epithelial cell extrusion from the midgut epithelium apparently differs for culicine compared to anopheline mosquitoes, the observations made in this latter study are consistent with the unified model of the route

[1] Although Omar (1968b) did not state that *An. stephensi* midgut epithelial cells invaded by *P. cynomolgi bastianelli* ookinetes exhibited either pathology or extrusion from the midgut epithelium, his Figure 13 clearly shows a midgut epithelial cell within the midgut lumen, completely separated from the overlying midgut epithelium, in close proximity to oocysts on the basal surface of the midgut epithelium, while his Figures 6 and 10 apparently show midgut epithelial cells protruding from the midgut epithelium.

of ookinete invasion across the midgut epithelium proposed here (Gupta *et al.*, 2005).

Re-interpretation of a previous study also suggests that extrusion of midgut epithelial cells occurs in *Cu. pipiens* infected with the avian malaria parasites *P. cathemerium* and *P. relictum* (Huff, 1934). Huff (1934) proposed that ookinetes (referred to as "zygotes" by this author) did not "bore" through the midgut epithelium but rounded up *within the midgut lumen* immediately adjacent to apical surface of the midgut epithelium. Subsequently, the malaria parasites grew in size forcing themselves intercellularly between adjacent midgut epithelial cells until the basal surface of the midgut epithelium was attained. Maier (1973) observed conventional intracellular ookinete invasion of the midgut epithelium accompanied by pathological changes to invaded midgut epithelial cells (although not extrusion of the latter) using the same malaria parasite-mosquito vector combination. In the current context, the following comments by Huff are of particular interest: "Their [normal and degenerate zygotes] staining is remarkably similar to the cells of the stomach epithelium, in both cytoplasm and nucleus. Degenerating zygotes can be distinguished by their angular outlines, the dissolution or disintegration of the nucleus, the appearance of bubble-like vacuoles, and most of all, by their staining which is almost always darker than that of the adjacent stomach wall... The depth of staining was found to be a very delicate criterion for degeneracy." (Huff, 1934). The *camera lucida* drawings given by Huff (reproduced in Figure 6.8) also reveals a striking resemblance between "zygotes" associated with the midgut epithelium and the extruding *An. stephensi* midgut epithelial cells described in Chapter 4, Section 4.3.4.[2] The strong similarities between "zygotes" and extruding midgut epithelial cells suggests Huff mistakenly interpreted morphologically-abnormal midgut epithelial cells as malaria parasites. Although this re-interpretation does not prove that *P. cathemerium* and *P. relictum* ookinete invasion causes extrusion of midgut epithelial cells from the midgut epithelium of *Cu. pipiens*, taken together with the previous study by Maier (1973), it provides good circumstantial evidence.

Although the unified model of the route of ookinete migration across the midgut epithelium seems applicable to a wide range of evolutionarily divergent malaria parasite-mosquito vector combinations, it is not certain that this model accurately describes the route of migration taken by *all* ookinetes within any given malaria

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[2] Huff's (1934) diagram of zygote penetration of the midgut epithelium (his Text Figure 2) also depicts malaria parasites with a striking resemblance to the extruding midgut epithelial cells observed here in histological sections from *An. stephensi* (his 2b to 2d).

parasite-mosquito vector combination. As noted in Chapter 4, a small proportion of *P. falciparum* ookinetes (and a large proportion of oocysts) were not obviously associated with morphologically-abnormal midgut epithelial cells. In most instances, there are reasonable arguments that suggest these malaria parasites did initially enter the midgut epithelium *via* an intracellular route (Chapter 4, Section 4.4.2.1). If these arguments are correct it may be difficult to prove formally that all ookinetes initially enter the midgut epithelium *via* an intracellular route. Other studies have also reported that a small proportion of malaria parasites invading the midgut epithelium are not associated with morphologically-abnormal midgut epithelial cells (Han *et al.*, 2000; Danielli *et al.*, 2005; Gupta *et al.*, 2005). Therefore, the (unanswered) question arises as to whether these particular malaria parasites have invaded the midgut epithelium *via* a solely intercellular route (Chapter 4, Section 4.4.2.1). Recent studies have apparently shown that tachyzoites of the related apicomplexan parasite *Toxoplasma gondii* cross polarised epithelial cell monolayers *via* a solely intercellular route (Barragan & Sibley, 2002; Barragan, Brossier & Sibley, 2005). There is no known theoretical reason why *Plasmodium* ookinetes could not migrate across the mosquito midgut epithelium *via* a similar intercellular route. Alternatively, given the previous confusion and controversy surrounding the route of ookinete migration across the midgut epithelium, perhaps the route taken by *Toxoplasma* tachyzoites is not quite what it seems (Section 6.4.4).

In contrast to the unified model of the route of ookinete migration across the midgut epithelium outlined here, the Ross Cell model states that ookinetes invade a morphologically and biochemically distinct subgroup of midgut epithelial cells without causing pathological changes to the latter (Shahabuddin & Pimenta, 1998; Cociancich *et al.*, 1999; Shahabuddin, 2002). As discussed above, there was no evidence that the Ross cell model applied to *P. falciparum* 3D7A in *An. stephensi*. Recently published studies using the *P. gallinaceum-Ae. aegypti* malaria parasite-mosquito vector combination also found no evidence for the existence of Ross cells (Zieler & Dvorak, 2000; Gupta *et al.*, 2005). In these studies, ookinetes invaded morphologically-normal midgut epithelial cells, which subsequently underwent pathological changes as a result of malaria parasite infection. The extruding midgut epithelial cells observed, in the work presented in this thesis, in uninfected and infected *An. stephensi* also possessed some of the characteristics previously ascribed to Ross cells, providing further support for the view that, rather than being a specific type of midgut epithelial cell, Ross Cells are regular midgut epithelial cells exhibiting morphological abnormalities resulting

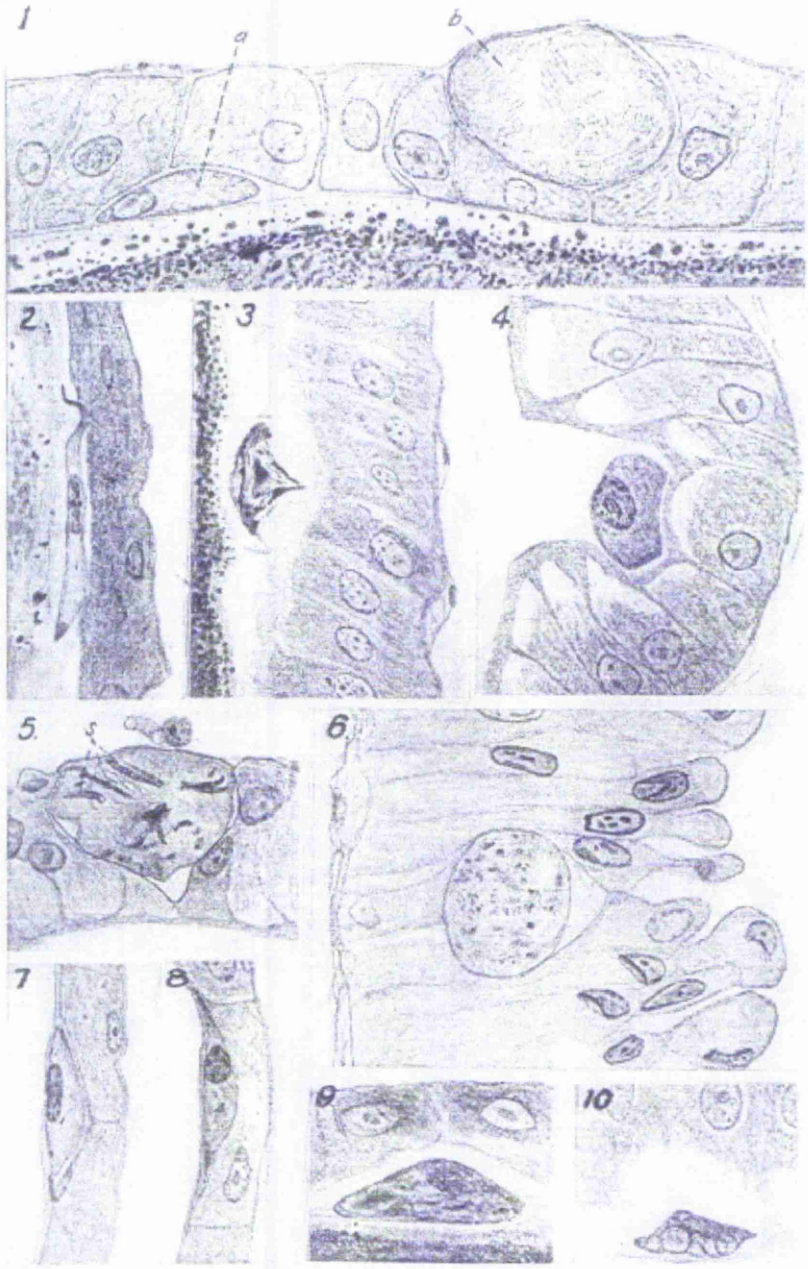


Figure 6.8 ***P. cathemerium*** **and** ***P. relictum*** **"zygotes" penetrating the midgut epithelium of** ***Cu. pipiens*****.**

Reproduction of Plate I from Huff (1934) showing camera lucida drawings of histological sections derived from blood-fed *Cu. pipiens* midguts. Images **1b**, **5** and **6** unquestionably show late oocyst stages of *P. cathemerium* and *P. relictum*. More contentiously, Huff claimed images **1a**, **2**, **4**, **7** and **8** showed normal zygotes, while **3**, **9** and **10** showed degenerating zygotes, of these two avian malaria parasite species. Images **1 to 10**: 16, 14, 14, 27, 22, 180, 45, 16, 12 and 30 hours pbf, respectively.

from ookinete invasion or *in vitro* manipulation of the midgut epithelium (Han *et al.*, 2000; Zieler & Dvorak, 2000; Gupta *et al.*, 2005). Furthermore, as currently formulated, the Ross Cell model provides no basis for understanding the intercellular location of ookinetes, the occurrence of stalk-form ookinetes, ookinete migration through multiple adjacent midgut epithelial cells and the presence of oocysts immediately above the lateral plasma membranes of midgut epithelial cells. All of these phenomena have been observed in the malaria parasite-mosquito vector combination for which the Ross Cell model was initially proposed (Stohler, 1957; Mehlhorn *et al.*, 1980; Torii *et al.*, 1992; Vernick *et al.*, 1999; Zieler & Dvorak, 2000).

6.4.4 Explaining the century of controversy surrounding the route of ookinete migration across the midgut epithelium

> "...the pitfalls inherent in drawing conclusions about the nature of a dynamic process from evidence obtained in still pictures are obvious..."
> - Vanderberg, Rhodin & Yoeli (1967) *J. Protozool.* **14**: 82-103.

If the unified model of ookinete invasion of the mosquito midgut epithelium described in Section 6.4.1 is generally and, perhaps, universally applicable, this raises the curious question of why despite numerous previous studies no other investigators have fully described the same phenomena (Shahabuddin, 2002). In particular, why have some investigators observed malaria parasites in either only intra- or intercellular positions (and not in both locations within the midgut epithelium) and others not observed pathological changes in ookinete-invade midgut epithelial cells?

6.4.4.1 Small sample sizes

Previous electron microscopy studies have necessarily been based upon a small number of observations: ookinete invasion of the midgut epithelium is a rare event and hundreds of ultrathin sections must be examined to find just a few invading malaria parasites. For example, Canning & Sinden (1973) apparently observed only a single *P. berghei* ookinete in the midgut epithelium of *An. stephensi* while Syafruddin *et al.* (1991) observed 15 *P. yoelii nigeriensis* ookinetes within the midgut epithelium of *An. omorii*. Unfortunately, most electron microscopy studies have not indicated exactly how many malaria parasites were observed and what their relative distribution was within the midgut epithelium. Consequently, the representativeness (and robustness) of the observations made in these latter studies is difficult to judge. Despite this, sampling error resulting from small sample sizes has probably played a significant role in the

conflicting descriptions of ookinete invasion of the midgut epithelium (Torii *et al.*, 1992). However, several light microscopy studies, where many relatively thick histological sections could be examined, have made very large numbers of observations. Therefore, small sample sizes alone cannot be the explanation for the failure of some investigators to observe in full detail the route of ookinete invasion across the midgut epithelium and the occurrence of pathological changes in ookinete-invaded midgut epithelial cells. For example, several light microscopy studies present images that appear to show extruded midgut epithelial cells associated with invading malaria parasites but these were not commented upon by the investigators (for a more detailed discussion see Footnote 1 and Section 6.4.3 above) (Huff, 1934; Omar, 1968b).

6.4.4.2 Time of sampling

The time at which malaria parasites have been sought has also been proffered as an explanation for the contradictory reports on the nature of ookinete invasion of the midgut epithelium (Torii *et al.*, 1992; Han *et al.*, 2000). However, malaria parasite infection of the midgut epithelium is asynchronous; ookinetes can be found in all stages of invasion throughout an extended period of time, ranging from approximately 20 to 36 hours post-bloodfeeding (Figure 4.44) (Omar, 1968b). Consequently, the asynchrony of ookinete migration across the midgut epithelium probably makes the exact time of sampling of relatively little importance (Chapter 4, Section 4.4.2.1).

6.4.4.3 Method of sample preparation and examination

Bias from small sample sizes is compounded by technical limitations inherent with examination of conventionally-stained *sectioned* material. Unless great care is taken to follow specific midgut regions in multiple consecutive serial sections, biased and misleading observations can easily result. Frequently, only single aspects of a given ookinete invasion event are present within each histological section. For example, malaria parasites were observed in intercellular locations between morphologically-normal midgut epithelial cells (e.g. Figure 4.27). However, in subsequent adjacent histological sections, in which the intercellular ookinetes were no longer apparent, midgut epithelial cells exhibiting altered morphology were found in the same region of the midgut epithelium as the intercellular ookinete observed in the earlier sections. Similarly, intracellular ookinetes were seen within apparently morphologically-normal midgut cells with clear signs of abnormality in subsequent sections (e.g. Figure 4.13C and Figure 4.25). In these examples, if the sections

containing ookinetes were the only ones to be examined, it would be understandable if it was concluded that ookinete invasion is either solely intercellular or is intracellular but does not induce pathological changes in invaded midgut epithelial cells.

Notably, the most cogent and comprehensive studies of ookinete migration across the mosquito midgut epithelium have examined *whole* midguts (or midgut epithelial sheets) following ookinete invasion through the midgut epithelium in real time and/or using specific fluorescent markers (Han *et al.*, 2000; Zieler & Dvorak, 2000; Vlachou *et al.*, 2004). Such studies unambiguously observed intracellular ookinete invasion of the midgut epithelium and pathological changes in the parasite-invaded midgut epithelial cells. However, even in these studies, there were technical limitations that prevented characterisation of ookinete entry into, or exit from, the midgut epithelium

6.4.4.4 The complexity and specificity of the route of ookinete migration across the midgut epithelium

Combined with small sample sizes and use of sectioned material, the unexpected complexity of the route of ookinete migration is probably a significant factor in the contradictory observations and theories surrounding this stage of the malaria parasite life cycle. In particular, the sites of ookinete entry and exit from invaded midgut epithelial cells, and the relative speed of ookinete migration through different regions of the midgut epithelium, seem uniquely and deviously designed by Mother Nature to catch out unwary investigators. If ookinete entry into the midgut epithelium occurred through the central region of the apical plasma membrane of midgut epithelial cells, the controversy over whether ookinete invasion was intra- or intercellular would presumably have never have occurred. Additionally, in fixed material, ookinetes in the process of entering the midgut epithelium are more frequently observed in extracellular locations within the midgut lumen rather than within the apical region of invaded midgut epithelial cells (although both events are rare) (Chapter 4). This suggests that relative to the process of breaching the lateral apical plasma membrane of invaded midgut epithelial cells, internalisation of the ookinete is more rapid, an inference supported by observations of live malaria parasites *in vitro* (Zieler & Dvorak, 2000). Consequently, ookinetes in the process of entering the midgut epithelium are more likely to be observed in a location suggesting intercellular migration across the midgut epithelium. The significant invagination of the apical surface of the midgut epithelium that occurs during ookinete entry into midgut epithelial cells may also create the appearance that ookinetes are in intercellular locations within the midgut epithelium

when they are actually in extracellular locations within the midgut lumen (Figure 6.4) (Baton & Ranford-Cartwright, 2005a). Similarly, if invaded midgut epithelial cells were never extruded from the midgut epithelium, and intracellular ookinetes could always directly attain the basal surface of the midgut epithelium, there would be no (apparent) requirement for ookinetes entering the midgut epithelium intracellularly to subsequently adopt an intercellular route of migration across the midgut epithelium.

6.4.4.5 "Eyes without feeling, feeling without sight" (or conceptual blindness)

A final factor contributing to incomplete descriptions of ookinete invasion of the midgut epithelium would appear to be "conceptual blindness". The failure of *any* studies to reveal the presence of intercellular ookinetes *within* the apical third of the midgut epithelium should perhaps have raised suspicions about whether malaria parasite migration occurred *via* a solely intercellular route. However, the apparent failure of several researchers (and possibly others) to recognise the existence of extruding midgut epithelial cells, despite evidence that such phenomena were present within the specimens they examined, can presumably only be accounted as conceptual blindness: the subjective patterns of thought of the brain preventing objective perception of reality.

6.4.5 Summary

Previous observations, even those that are seemingly contradictory and whose significance has been alternatively interpreted, are consistent with a single unified model of the route of ookinete invasion across the mosquito midgut epithelium. Most studies have used *conventionally-stained sectioned* material in which it can be difficult to observe spatial associations between malaria parasites and invaded midgut epithelial cells exhibiting altered morphology. Combined with small sample sizes and mind-sets not necessarily expecting ookinete invasion of the midgut epithelium to be as intricate and complicated as it has been revealed to be, perhaps this is the main reason why other researchers have failed to report in full detail observations consistent with the unified model of the route of ookinete invasion.

6. 5 The cellular composition of the mosquito midgut epithelium

The Ross Cell model placed renewed emphasis on characterising the different cell types comprising the mosquito midgut epithelium and the possible of involvement of these cell types in ookinete invasion (Shahabuddin & Pimenta, 1998; Cociancich *et al.*, 1999; Shahabuddin, 2002). Particular attention was paid to the cellular composition of

the mosquito midgut epithelium during the current histological investigation. Only three morphologically-distinct cell types were observed, consistent with previous descriptions of the mosquito midgut epithelium. However, other morphological forms, apparently intermediate between regenerative/midgut epithelial cells, were also seen.

6.5.1 Midgut epithelial cells

Previous studies of intact midguts from anopheline and culicine mosquitoes concluded that columnar midgut epithelial cells were morphologically similar (Bertram & Bird, 1961; Freyvogel & Stäubli, 1965; Hecker *et al.*, 1971b; Hecker, 1977; Houk, 1977; Weaver & Scott, 1990b; Han *et al.*, 2000; Zieler & Dvorak, 2000). Although minor variation in electron density was sometimes apparent, and the existence of distinct midgut epithelial cell types was not precluded by some investigators (Bertram & Bird, 1961), midgut epithelial cells were assumed to be functionally equivalent. In *An. albimanus* and *An. stephensi*, columnar midgut epithelial cells also appeared morphologically indistinguishable: with the possible exception of the intermediate regenerative/midgut epithelial morphologies described in Chapter 5, there was no evidence for the existence of (morphologically) distinct types of midgut epithelial cell.

No (normal) midgut epithelial cells were observed in *An. albimanus* and *An. stephensi* that possessed the morphological characteristics previously ascribed to Ross cells (Shahabuddin & Pimenta, 1998; Cociancich *et al.*, 1999; Shahabuddin, 2002) suggesting that this cell type does not exist in anopheline mosquitoes. Cociancich *et al.* (1999) proposed that the non-random distribution of malaria oocysts on the midguts of *An. gambiae* and *Ae. aegypti* resulted from the spatial distribution of Ross cells within the midgut epithelium, as the latter type of midgut cells were preferentially invaded by ookinetes and exhibited a similar distribution to oocysts (Shahabuddin & Pimenta, 1998; Cociancich *et al.*, 1999). If Ross cells do not exist then other explanations are presumably required to account for the biased distribution of oocysts over the midgut.

6.5.2 Endocrine cells

The pale triangular cells with long narrow apical extensions observed in *An. albimanus* and *An. stephensi* have not been previously reported. However, these cells resemble the "clear" "open" endocrine cells previously described in *Ae. aegypti* (Brown, Raikhel & Lea, 1985; Brown, Crim & Lea, 1986; Brown & Lea, 1989) and are presumed to be an equivalent cell type of anopheline mosquitoes. The apical surface of aedine "open" endocrine cells has been shown using electron microscopy to possess

microvilli. Light microscopy, however, does not allow sufficient resolution to be certain that the corresponding cells in *An. albimanus* and *An. stephensi* also possess microvilli, although such structures appear to be present on the endocrine cells of these mosquito species. The number of "open" endocrine cells observed in the posterior midgut of the two anopheline mosquito species was similar to the several hundred previously reported for *Ae. aegypti* (Brown *et al.*, 1985; Veenstra *et al.*, 1995).

Although previous authors have stated that endocrine cells are present in the midgut epithelium of *An. stephensi* (Hecker, 1977; Glättli, Rudin & Hecker, 1987), the overall morphology of these cells was not described. Hecker (1977) cursorily described the presence of "clear cells" containing secretory granules in the midgut epithelium of both anopheline and culicine mosquitoes promising a fuller description in a subsequent manuscript that has not been published. In contrast to anopheline mosquitoes, endocrine cells in aedine mosquitoes are relatively well-characterised (Brown *et al.*, 1985; Brown *et al.*, 1986; Brown & Lea, 1989; Veenstra *et al.*, 1995; Brown & Cao, 2001). In *Ae. aegypti*, four morphologically distinct types of midgut endocrine cell have been described: "closed" and "open" cell types (either terminating within the midgut epithelium or extending to the luminal surface, respectively) that could both be found with either "clear" (electron lucent) or "dark" (electron dense) cytoplasm (Brown *et al.*, 1985; Brown *et al.*, 1986; Brown & Lea, 1989). Unless the dark-staining triangular cells observed in *An. albimanus* and *An. stephensi* are assumed to represent a heterogeneous population of distinct cell types (i.e. closed endocrine and regenerative cells), only one type of morphologically distinct endocrine cell was present in these two anopheline mosquito species. This may reflect genus-specific differences in the types of endocrine cell in aedine and anopheline mosquitoes or reflect differences in interpretation. As noted in Chapter 4, the apical and basal regions of individual open endocrine cells were most frequently present in different adjacent histological sections. When the basal body of an open endocrine cell alone is present within a given section, it looks like a closed endocrine cell. However, Brown & Lea (1989) stated that consecutive serial sections were followed for both open and closed endocrine cell types, so it is unlikely the closed endocrine cells observed by these authors did not genuinely terminate within the midgut epithelium. Brown *et al.* (1985) noted that endocrine and regenerative cell types were often found together and suggested that "endocrine cells may differentiate from regenerative cells as dark closed cells; some of which may grow to the lumen and become open cells". Alternatively the dark closed "endocrine" cells

might be regenerative cells differentiating into normal columnar midgut epithelial cells (see Chapter 5). Brown *et al.* (1985) also observed the presence of unusual, and sometimes quite large, lamellar bodies in the apical and lateral regions of some endocrine cells. Such structures might represent nascent forms of the apical microvilli-lined cavities observed within the unusual midgut epithelial cells interpreted here as differentiating into normal columnar midgut epithelial cells (Chapter 5; cf. Figure 5.1E).

Although open endocrine cells are potentially accessible to ookinetes as initial sites of entry into the midgut epithelium, for *P. falciparum* in *An. stephensi*, no malaria parasites were found within, or in close proximity to, these cells (Chapter 4). Similarly, there was also no evidence that endocrine cells were invaded by *P. gallinaceum* ookinetes in *Ae. aegypti* (Shahabuddin & Pimenta, 1998). The relative paucity of the endocrine cells within the midgut epithelium may mean that ookinetes only rarely encounter and, hence, invade these cells.

6.5.3 Regenerative cells

The dark triangular cells observed in the basal region of the midgut epithelia of *An. albimanus* and *An. stephensi* are assumed to be regenerative cells, as previously described for anopheline mosquitoes (Hecker, 1977), although there still remains no formal proof for this function (Chapter 5). As discussed above (Section 6.5.2), some dark triangular cells observed here may be endocrine cells of the "closed" type similar to those previously described in *Ae. aegypti* (Brown *et al.*, 1985; Brown *et al.*, 1986). Given the variability in size of the dark triangular cells, they may represent a heterogeneous mixture of more than one distinct cell type, which cannot be distinguished in Giemsa-stained histological sections. The alternative explanation is that this variability results from regenerative cells undergoing division and differentiation into midgut epithelial cells (Chapter 5). The dark triangular cells observed here in *An. albimanus* and *An. stephensi* presumably correspond to the small, flat triangular cells, previously reported in *An. stephensi*, which terminate within the basal region of the midgut epithelium and overexpress V-ATPase (Han *et al.*, 2000).

P.falciparum parasites in the basal region of the midgut epithelium were occasionally observed adjacent to the dark triangular cells in *An. stephensi*. However, the location of regenerative cells within the basal region of the midgut epithelium makes them improbable sites for initial entry of ookinetes into the midgut epithelium (Shahabuddin & Pimenta, 1998; Han *et al.*, 2000).

6.5.4 Unusual midgut epithelial cell morphologies

The unusual midgut epithelial cells possessing apical cavities lined by microvilli-like structures described in Chapter 5 appear to correspond to morphologically similar midgut cells of unknown function previously reported in the anterior and posterior midguts of two culicine mosquito species (*Culex tarsalis* and *Culiseta melanura*) (Houk, 1977; Weaver & Scott, 1990b). Light microscopical examination of histological sections does not allow sufficient magnification to ascertain definitely the identity of the structures lining the apical cavities of these unusual midgut epithelial cells. The previous studies of culicine mosquitoes used transmission electron microscopy and clearly demonstrated that the apical cavities of these midgut epithelial cells were lined by microvilli (Houk, 1977; Weaver & Scott, 1990b). Similar to the observations reported in Chapter 5, these electron microscopy studies also reported the presence of extraneous material within the central region of the apical cavity (Weaver & Scott, 1990b), providing further corroborative evidence that the observations made in these studies relate to the same population of midgut cells described here. However, the unusual midgut epithelial cells possessing apical microvilli-lined cavities were not previously reported to be located immediately adjacent to regenerative cells and their significance was unknown (Houk, 1977; Weaver & Scott, 1990b). The unusual midgut epithelial cells could represent a distinct, terminally-differentiated, cell type with specialised functions, or normal columnar midgut epithelial cells undergoing some particular transformational event. Based upon the observations reported in Chapter 5, these unusual midgut epithelial cells are proposed to be intermediate forms in the differentiation of regenerative cells into normal columnar midgut epithelial cells, although proof of this interpretation of the morphological evidence is still required.

Previous studies have found little evidence of regenerative cell division and differentiation in the midguts of either uninfected or virus-infected mosquitoes (Hecker *et al.*, 1971a; Hecker *et al.*, 1971b; Hecker, 1977; Weaver *et al.*, 1988; Weaver & Scott, 1990a). Consequently, regenerative cell division and differentiation has been assumed to either be rare or absent in the midguts of mature adult female mosquitoes (Hecker *et al.*, 1971b; Hecker, 1977; Billingsley, 1990b; Weaver & Scott, 1990b). However, Day & Bennetts (1953) reported "proliferation" and "regeneration" of the midgut epithelium of adult female *Ae. aegypti* following mechanical rupture of the midgut, although the exact processes involved were not defined. Stueben (1978) also claimed that invasion of the mosquito midgut epithelium by microfilariae of the dog heartworm *Dirofilaria*

immitis resulted in "extensive cellular proliferation and a high level of mitotic activity [of the midgut epithelium]". The most convincing previous description of regenerative cell proliferation was from the midgut epithelia of mature adult *Ae. aegypti* males (Hecker *et al.*, 1971a). However, these studies did not describe in detail the differentiation of regenerative cells into columnar midgut epithelial cells nor report the presence of unusual midgut epithelial cells possessing microvilli-lined apical cavities.

A precedent for the model of proliferative regeneration of the adult mosquito midgut epithelium presented in Chapter 5 is the differentiation of regenerative cells into goblet cells in the larval midguts of the tobacco hornworm *Manduca sexta* (Hakim, Baldwin & Bayer, 1988; Baldwin & Hakim, 1991). In *M. sexta*, regenerative cells differentiating into goblet cells develop large, apical, internally closed, microvilli-lined cavities known as "goblets". As differentiation proceeds, the goblets eventually open through, and become contiguous with, the apical surface of the midgut epithelium *via* a "valve" structure created by interdigitation of the cytoplasmic projections lining the internal cavity. However, unlike the model proposed in Chapter 5 for adult mosquitoes, mature goblet cells in larval lepidoptera apparently retain their apical cavities and are thought to be a distinct and functionally specialised type of midgut epithelial cell mediating ion transport across the midgut epithelium (Anderson & Harvey, 1966).

As previously noted, differentiating lepidopteran goblet cells are morphologically remarkably similar to the unusual midgut epithelial cells possessing apical microvilli-lined cavities seen in mosquitoes (Weaver & Scott, 1990b). As well as apical microvilli-lined cavities, lepidopteran goblets also contain "a flocculent precipitate", referred to as goblet matrix (Baldwin & Hakim, 1991), which is seemingly equivalent to the refractory fibrotic material observed within the apical cavities of the corresponding unusual midgut epithelial cells seen in mosquitoes (Chapter 5) (Weaver & Scott, 1990b). Does the morphological similarity of these midgut cells from different insects reflect a functional equivalence? Ross cells have previously been proposed to be a type of goblet cell present within the mosquito midgut epithelium (Shahabuddin & Pimenta, 1998; Cociancich *et al.*, 1999). However, earlier studies did not report the presence of goblet cells within the posterior midgut epithelium of adult female mosquitoes (Bertram & Bird, 1961; Freyvogel & Stäubli, 1965; Hecker *et al.*, 1971b; Hecker, 1977). And more recent studies have also been unable to confirm the existence of Ross cells in mosquitoes, which are now regarded as ordinary midgut epithelial cells exhibiting morphological abnormalities as a result of ookinete invasion (Section 6.4.3 and 6.5.1)

(Han *et al.*, 2000; Zieler & Dvorak, 2000; Gupta *et al.*, 2005). Weaver & Scott (1990b) suggested that the unusual midgut epithelial cells possessing apical microvilli-lined cavities seen in mosquitoes might be goblet cells, although they believed further work was required to confirm the functional significance of these cells. However, the evidence presented in Chapter 5 seems to argue against the unusual midgut epithelial cells of mosquitoes being a specialised type of midgut epithelial cell: if the unusual midgut epithelial cells possessing apical microvilli-lined cavities were goblet cells, why should they always be located immediately adjacent to regenerative cells and why should their number increase following ookinete invasion of the midgut epithelium?

Perhaps the converse, if heretical, possibility that lepidopteran "goblet cells" are intermediate forms in the differentiation of regenerative cells into normal columnar midgut epithelial cells (rather than the terminally-differentiated cell type currently believed) should be considered. In an early study, Anderson & Harvey (1966) stated that an unanswered fundamental question regarding lepidopteran goblet and columnar midgut epithelial cells was if "one could be a physiological phase of the other?". Despite an awareness of this possibility, these authors concluded that these different morphologies were indicative of developmentally and functionally distinct cell types. Subsequent investigators have accepted and extended the interpretation of Anderson & Harvey (1966), and the possibility that goblet cells might be a physiological phase in the development of normal columnar epithelial cells has no longer been considered. However, observations from more recent studies reporting the existence of regenerative cells and describing their differentiation into mature midgut cells are consistent with the interpretation that lepidopteran goblet cells are regenerative cells differentiating into normal columnar epithelial cells: in *M. sexta*, goblet cells are also preferentially located immediately adjacent to regenerative cells and clear pathways are occasionally observed through the apical valves of interdigitating microvilli of mature goblet cells (Hakim *et al.*, 1988; Baldwin & Hakim, 1991). The "clear pathways" have been interpreted as a fixation artefact, such that the apical valves are believed to be tightly sealed *in vivo* (Baldwin & Hakim, 1991). Perhaps these pathways represent apical cavities everting and opening out to the midgut lumen to become the apical brush border of normal columnar midgut epithelial cells, as proposed in Chapter 5. It is also unclear if previous studies of *M. sexta* actually observed, or just inferred the existence, of intermediate forms representing regenerative cells differentiating into normal columnar midgut epithelial cells, as distinct from differentiating goblet cells (Hakim *et al.*, 1988; Baldwin

& Hakim, 1991). Jager *et al.* (1996) were unable discriminate between regenerative cells differentiating into goblet cells and normal columnar epithelial cells, consistent with the existence of a single population of differentiating regenerative cells.

The aforementioned "Ross cells" express high levels of V-ATPase, a characteristic shared with lepidopteran goblet cells (Jager *et al.*, 1996; Wieczorek *et al.*, 2000), which led to the initial suggestion that the former might be examples of the latter in mosquitoes (Shahabuddin & Pimenta, 1998). As already mentioned, the unusual morphological characteristics of "Ross cells", including over-expression of V-ATPase, are now thought to result from ookinete invasion (Han *et al.*, 2000; Zieler & Dvorak, 2000). The original studies describing Ross cells did not formally demonstrate that the morphologically unusual midgut epithelial cells invaded by ookinetes were also the midgut cells expressing high levels of V-ATPase (Shahabuddin & Pimenta, 1998; Cociancich *et al.*, 1999). Therefore, it is possible that ookinete-invaded midgut epithelial cells *do not* over-express V-ATPase and that over-expression of V-ATPase is a characteristic of a *different* population of uninvaded midgut cells. Indeed, as previously noted (Section 6.5.3), Han *et al.* (2000) reported that uninvaded regenerative cells were the only V-ATPase positive cells apparent in the midguts of *An. stephensi* infected with *P. berghei*, leaving the V-ATPase positive cells associated with *P. gallinaceum* ookinetes invading *Ae. aegypti* and *An. gambiae* unexplained. An intriguing possibility is that the highly V-ATPase positive cells associated with *P. gallinaceum* ookinete invasion in the latter mosquito species are regenerative cells differentiating into normal columnar midgut epithelial cells. According to the model of proliferative regeneration of the mosquito midgut epithelium and other conclusions presented in Chapter 5, malaria parasites infecting the midgut epithelium are predicted to be associated with regenerative cells differentiating into normal columnar midgut epithelial cells as a consequence of the destruction of invaded midgut epithelial cells accompanying ookinete invasion. Previous observations from *M. sexta* are again consistent with this interpretation and provide further circumstantial evidence that lepidopteran goblet cells might be intermediate forms rather than terminally differentiated midgut cells. In lepidoptera, V-ATPase expression markedly increases during the period of regenerative cell differentiation, such that mature goblet cells express high levels V-ATPase throughout the apical plasma membrane of the "goblet" (Jager *et al.*, 1996). V-ATPase positivity in Ross cells also localised to the apical membrane and undefined internal tubular structures (Shahabuddin & Pimenta, 1998).

Furthermore, if goblet cells are intermediate forms differentiating into normal columnar epithelial cells, this could explain the otherwise "enigmatic" presence of high levels of *V-ATPase* mRNA and cytosolic V-ATPase observed in mature columnar epithelial cells of *M. sexta* (Jager *et al.*, 1996): these molecules could be "carried-over" from the previous "goblet" phase of cell development and differentiation.

Although there are morphological similarities between unusual midgut epithelial cells possessing microvilli-lined apical cavities in mosquitoes and goblet cells of lepidoptera, there are differences between these midgut cells from different insect orders. Weaver & Scott (1990b) noted that the microvilli lining the apical cavities of the unusual midgut epithelial cells seen in mosquitoes were not associated with, and did not contain, elongated mitochondria as sometimes observed for the microvilli lining the cavities of goblet cells (Anderson & Harvey, 1966). Furthermore, goblets often extend deeply into the basal region of mature goblet cells, and the "depth" of the goblets varies systematically along the length of the larval lepidopteran midgut (Cioffi, 1979). Lepidopteran goblet cells and the unusual midgut epithelial cells possessing microvilli-lined apical cavities of mosquitoes are probably distinct cell types and functionally unrelated to one another: the morphological similarity of these midgut cells might simply be coincidental or reflect a shared ancestral character that has subsequently undergone divergent evolution and become exapted for different purposes.

If lepidopteran goblet cells and the unusual midgut epithelial cells possessing microvilli-lined apical cavities of mosquitoes are distinct cell types, we should still ask why the differentiation of columnar midgut epithelial cells from regenerative cells has apparently not been observed in lepidoptera. Given the high rate of growth of the midgut epithelium of larval lepidoptera, it is surprising this differentiation has not (apparently) been observed: unlike mature adult mosquitoes, regenerative cell division and differentiation are abundant in the larval lepidopteran midgut. Proliferation and differentiation of regenerative cells within the midgut epithelium is a fundamental and, presumably, evolutionarily ancient process originated in the common ancestor of extant insect orders. Regenerative cell proliferation and differentiation might be expected to proceed *via* a similar manner in both mosquitoes and lepidoptera. If the unusual midgut epithelial cells possessing microvilli-lined apical cavities observed in mosquitoes are nascent midgut epithelial cells, it might be surprising if new midgut epithelial cells were not formed in a similar manner in lepidoptera. Conceivably, the differentiating "goblet cells" of lepidoptera could represent a heterogeneous population of midgut cell types,

some of which develop into normal columnar midgut epithelial cells (as proposed for mosquitoes) while others terminally-differentiate into mature goblet cells.

Zieler *et al.* (2000) reported several novel midgut cell morphologies in the midgut epithelium of *Ae. aegypti*, using scanning electron microscopy, which were suggested to be a variety of previously unrecognised distinct cell types of unknown function. A characteristic feature of these novel midgut cell morphologies was the possession of an apical surface lacking or relatively denuded of microvilli. Some of these so-called "bare" cells protruded from the apical surface of the midgut epithelium and probably correspond to damaged columnar midgut epithelial cells extruding into the midgut lumen (Han *et al.*, 2000; Gupta *et al.*, 2005). Other bare cells were deeply recessed within midgut epithelium, beneath the plane of the apical microvillar brush border surface of the midgut epithelium. The significance of these latter midgut cell morphologies has not been explained. Previous ultrastructural studies of *Ae. aegypti* failed to find any midgut epithelial cell types to which these recessed cells might correspond (Bertram & Bird, 1961; Freyvogel & Stäubli, 1965; Hecker *et al.*, 1971b; Hecker, 1977; Zieler *et al.*, 2000). Following the model of midgut regeneration presented in Chapter 5 for anopheline mosquitoes, perhaps this population of bare cells in *Ae. aegypti* corresponds to differentiating regenerative cells in the process of emerging through the apical surface of the midgut epithelium prior to and/or during eversion of the internal microvilli-lined cavity (Figure 5.6 and Figure 5.7). The apical surface of one of the bare cells recessed within the midgut epithelium observed by Zieler *et al.* (2000) appeared to possess a prominent, central, elevated pore-like structure. Perhaps this structure derives from the fusion of the internal microvilli-lined cavity with the apical plasma membrane of a differentiating regenerative cell that had emerged through the microvillar brush border of the midgut epithelium.

6. 6 Conclusions

The studies presented in this thesis have made a number of important and novel findings, including identifying the when the *P. falciparum* 3D7A clone fails to infect *An. albimanus* and describing the route of ookinete invasion for this malaria parasite clone across the midgut epithelium of *An. stephensi*.

Appendix 1 - Media / Solutions

Thawing Solution I: in ddH_20 and 0.22 μm sterile-filtered; stored at 4°C.
12 % (w/v) NaCl (BDH Laboratory Supplies, Cat. No. 102415K).

Thawing Solution II: in ddH_20 and 0.22 μm sterile-filtered; stored at 4°C.
1.6 % (w/v) NaCl (BDH Laboratory Supplies, Cat. No. 102415K).

Thawing Solution III: in ddH_20 and 0.22 μm sterile-filtered; stored at 4°C.
0.9 % (w/v) NaCl in ddH_2O (BDH Laboratory Supplies, Cat. No. 102415K);
0.2 % (w/v) dextrose.

Freezing Solution: in ddH_20 and 0.22 μm sterile-filtered; stored at 4°C.
0.65% (w/v) NaCl (BDH Laboratory Supplies, Cat. No. 102415K);
3.0 % (w/v) D-sorbitol (Koch Light Laboratories Ltd, Cat. No. 5063-00);
28.0 % (v/v) glycerol (BDH Laboratory Supplies, Cat. No. 101186M).

Incomplete RPMI 1640 medium: in ddH_20, adjusted to pH 7.2 and 0.22 μm sterile-filtered; used within four weeks; stored at 4°C.
10.4 g/l GIBCO® RMPI 1640 (+ L-glutamine, - $NaHCO_3$) (Invitrogen, Cat. No. 51800-019);
5.94 g/l (25 mM) 1-piperazineethane sulfonic acid (HEPES) (Sigma, Cat. No. H3375);
50 mg/l (0.37 mM) hypoxanthine (Sigma, Cat. No. H9636).

Complete RPMI 1640 medium: used before turns pink / within 2 weeks; stored at 37°C.
500 ml incomplete RPMI 1640 medium;
21 ml of sterile-filtered 5 % (w/v) $NaHCO_3$ (Fisher Scientific, Cat. No. S/4240/60) in ddH_2O; used within 1 week; stored at 4°C;
50 ml (10 % v/v) sterile heat-inactivated (1 hour at 56°C) pooled human AB serum; stored at -75°C.

Giemsa's buffer solution: in ddH_2O and adjusted to pH 7.2 to 7.4; stored at room temperature.
3.0 g/l (21.1 mM) Na_2HPO_4 (BDH Laboratory Supplies, Cat. No. 102494C);
0.6 g/l (4.4 mM) KH_2PO_4 (Fisher Scientific Ltd, Cat. No. P/4800/53).

Giemsa's stain: made fresh daily; stain methanol fixed blood smears for 35 mins for asexual parasites and 40 mins sexual parasites, and rinse slides in H_2O.
5 % (v/v) Giemsa's stain solution, Gurr® improved R66 (BDH Laboratory Supplies, Cat. No. 350864);
95 % (v/v) Giemsa's buffer solution.

Glucose solution for adult mosquitoes: in ddH_2O; stored at 4°C.
5.0 % (w/v) D-glucose (Fisher Scientific Ltd, Cat. No. G/0500/53);
0.05 % (w/v) para-amino-benzoic acid (PABA) (Fisher Scientific Ltd, Cat. No. A/2840/48).

Appendix 2 - Details of the multiple linear regression analysis presented in Chapter 5, Section 5.3.2.

Model	No. of predictor variables[a]	r^2	C_p[b]	BIC[c]	Intercept	Predictor variables[d]			
						Time post-bloodfeeding (hours)	No. of ookinetes within midgut epithelium	Number of oocysts	No. of abnormal midgut epithelial cell events
1	3	0.767	3.087	86.788	1.747	-	-0.991	0.367	0.552
2	4	0.768	5.000	89.113	3.399	-0.057	-1.023	0.384	0.558
3	2	0.723	5.804	88.505	1.122	-	-1.102	-	0.712
4	3	0.724	7.641	90.398	-0.981	0.073	-1.053	-	0.695
5	2	0.650	13.608	94.147	3.169	-	-	0.476	0.197
6	3	0.660	14.591	95.115	-2.216	0.180	-	0.412	0.214
7	2	0.610	17.947	96.848	-7.104	0.327	-	-	0.351
8	1	0.582	18.991	97.266	4.876	-	-	0.827	-
9	1	0.574	19.811	97.744	2.545	-	-	-	0.358
10	2	0.583	20.878	98.536	3.183	0.058	-	0.817	-
11	2	0.582	20.974	98.590	4.840	-	0.021	0.816	-
12	3	0.583	22.795	99.829	2.466	0.080	0.052	0.785	-
12	2	0.340	46.947	110.356	-10.225	0.530	0.695	-	-
14	1	0.246	55.039	113.162	5.775	-	0.650	-	-
15	1	0.062	74.853	119.239	-4.510	0.428	-	-	-

The table on previous page lists all possible models using the four predictor variables included in the multiple linear regression analysis described in Sections 5.2.5 and 5.3.2. The models are listed in order of superiority according to the statistical criteria employed (i.e. where the lowest values of C_p and BIC indicate the model with the best fit to the observed data).

[a] The number of predictor variables included in the specified model.

[b] C_p = Mallow's C_p.

[c] BIC = Schwarz Bayesian information criterion.

[d] The partial regression slopes (β) for the four predictor variables included in the multiple linear regression analysis. Where no value is given, the relevant predictor variable was not included in that particular model.

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Opinion

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Do malaria ookinete surface proteins P25 and P28 mediate parasite entry into mosquito midgut epithelial cells?

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Abstract

Background: P25 and P28 are related ookinete surface proteins highly conserved throughout the *Plasmodium* genus that are under consideration as candidates for inclusion in transmission-blocking vaccines. Previous research using transgenic rodent malaria parasites lacking P25 and P28 has demonstrated that these proteins have multiple partially redundant functions during parasite infection of the mosquito vector, including an undefined role in ookinete traversal of the mosquito midgut epithelium, and it has been suggested that, unlike wild-type parasites, Dko P25/P28 parasites migrate across the midgut epithelium *via* an intercellular, rather than intracellular, route.

Presentation of the hypothesis: This paper presents an alternative interpretation for the previous observations of Dko P25/P28 parasites, based upon a recently published model of the route of ookinete invasion across the midgut epithelium. This model claims ookinete invasion is intracellular, with entry occurring through the lateral apical plasma membrane of midgut epithelial cells, and is associated with significant invagination of the midgut epithelium localised at the site of parasite penetration. Following this model, it is hypothesized that: (1) a sub-population of Dko P25/P28 ookinetes invaginate, but do not penetrate, the apical surface of the midgut epithelium and thus remain within the midgut lumen; and (2) another sub-population of Dko P25/P28 parasites successfully enters and migrates across the midgut epithelium *via* an intracellular route similar to wild-type parasites and subsequently develops into oocysts.

Testing the hypothesis: These hypotheses are tested by showing how they can account for previously published observations and incorporate them into a coherent and consistent explanatory framework. Based upon these hypotheses, several quantitative predictions are made, which can be experimentally tested, about the relationship between the densities of invading Dko P25/P28 ookinetes in different regions of the midgut epithelium and the number of oocyst stage parasites to which these mutant ookinetes give rise.

Implications of the hypothesis: The recently published model of ookinete invasion implies that Dko P25/P28 parasites are greatly, although not completely, impaired in their ability to enter the midgut epithelium. Therefore, P25 and/or P28 have a novel, previously unrecognized, function in mediating ookinete entry into midgut epithelial cells, suggesting that one mode of action of transmission-blocking antibodies to these ookinete surface proteins is to inhibit this function.

Background

P25 and P28 are related major ookinete surface proteins under consideration as candidates for inclusion in transmission-blocking vaccines [1-4]. Consequently, the expression [5-18], localisation [8,12,17-24] and function [21,25-29] of these molecules, together with the effect on parasite development of specific antibodies against them [6,8,21,22,24,30-35], have been extensively studied in a range of malaria parasite species.

P25 and P28 are structurally similar proteins, highly conserved throughout the *Plasmodium* genus [11,12,31,35-43], which contain four epidermal growth factor-like domains [36], putatively involved in cell-cell and/or cell-matrix interactions [21,25,26,28,29], that are expressed throughout the early life-cycle stages of the malaria parasite within the mosquito vector – from the macrogamete through to the oocyst stage [8,12,17-24]. P25 and P28 are located on the parasite surface, from which they are shed during ookinete gliding motility and traversal of the mosquito midgut epithelium [19-21,44,45]. The conservation of sequence, expression and location suggests that P25 and P28 have functionally equivalent roles in diverse malaria parasite species.

Previous research using transgenic *Plasmodium berghei* rodent malaria parasites lacking P25 and P28 demonstrated that these proteins have multiple and partially redundant functions during parasite infection of the mosquito vector [26,27]. Although Dko P25/P28 *P. berghei* parasites exhibit greatly reduced levels of oocyst infection compared to wild-type or Sko P25/P28 parasites, ookinetes lacking both P25 and P28 are still able to cross the midgut epithelium and establish oocyst infections [27]. Wild-type *P. berghei* ookinetes migrate intracellularly through the midgut epithelium causing significant damage to invaded midgut epithelial cells [44-48], which subsequently exhibit distinct morphological abnormalities [44-48], including loss of microvilli [44,45], protrusion into the midgut lumen [44,45,48] and up-regulation of molecules implicated in mosquito immune responses such as NOS [44,49] and SRPN10 [45,50]. Furthermore, P28 is found on the apical surface, and within the cytoplasm, of these abnormal midgut epithelial cells suggesting release/secretion from penetrating parasites during their intracellular migration [44,45]. Dko P25/P28 ookinetes have also been found deep within the midgut epithelium [27,45]. Initially, these parasites were suggested to be retarded in their transit through the midgut epithelium and killed by the epithelial cell defence reactions triggered by wild-type parasites [27]. Recently, however, Dko P25/P28 parasites were observed apparently deep within the midgut epithelium between morphologically normal midgut epithelial cells [45]. These midgut epithelial cells did not exhibit the abnormal characteristics typically associated with invasion by wild-type ookinetes, such as protrusion into the midgut lumen and up-regulation of SRPN10 [44,45,48]. Consequently, these Dko P25/P28 parasites were proposed to be migrating through the midgut epithelium *via* a solely intercellular route [45]. However, a recently published model of ookinete invasion across the mosquito midgut epithelium [51] suggests an alternative interpretation for the previously published observations of Dko P25/P28 parasites.

Presentation of the hypothesis

A unified model of the route of ookinete invasion across the mosquito midgut epithelium

The route of ookinete migration across the midgut epithelium of the mosquito vector has long been controversial [51]. The major argument in the literature has been whether ookinete invasion is either solely intercellular between, or intracellular through, midgut epithelial cells [51]. Recently, an attempt has been made to unify the apparently conflicting literature and integrate it with other recent observations [44,47,52] in order to provide a single general model of the route of ookinete invasion across the midgut epithelium applicable to diverse malaria parasite and mosquito vector species [51]. Subsequent observations of ookinete invasion of the midgut epithelium *in vivo* support this model [48]. According to the model, ookinete entry into the midgut epithelium is initially intracellular, occurring through the lateral apical plasma membrane of midgut epithelial cells (Figure 1) [47,51,52]. Significantly, ookinete entry into midgut epithelial cells is often associated with substantial local invagination of the midgut epithelium [52], an observation supported by re-interpretation of previously published images (Figure 2 in Ref. [19] and Figure 5 in Ref. [53]). Ookinetes pass intracellularly through one or more midgut epithelial cells, causing significant damage similar to that described for wild-type *P. berghei* ookinetes [44-48,51,52,54,55]. Subsequently, ookinetes exit invaded epithelial cells into the basolateral extracellular space between adjacent midgut epithelial cells [48,52,56], migrate intercellularly to the basal surface of the midgut epithelium and transform into oocyst stage parasites [51].

Significance of the unified model for understanding Dko P25/P28 P. berghei ookinete invasion

Following the model of ookinete invasion of the midgut epithelium outlined above (and Figure 1), two hypotheses about Dko P25/P28 *P. berghei* parasite infection of the mosquito vector are proposed. First, some Dko P25/P28 ookinetes invaginate, but are unable to penetrate, the apical surface of the midgut epithelium. Second, other Dko P25/P28 parasites are able to successfully enter and migrate across the midgut epithelium *via* an intracellular route, in a manner similar to wild-type parasites.

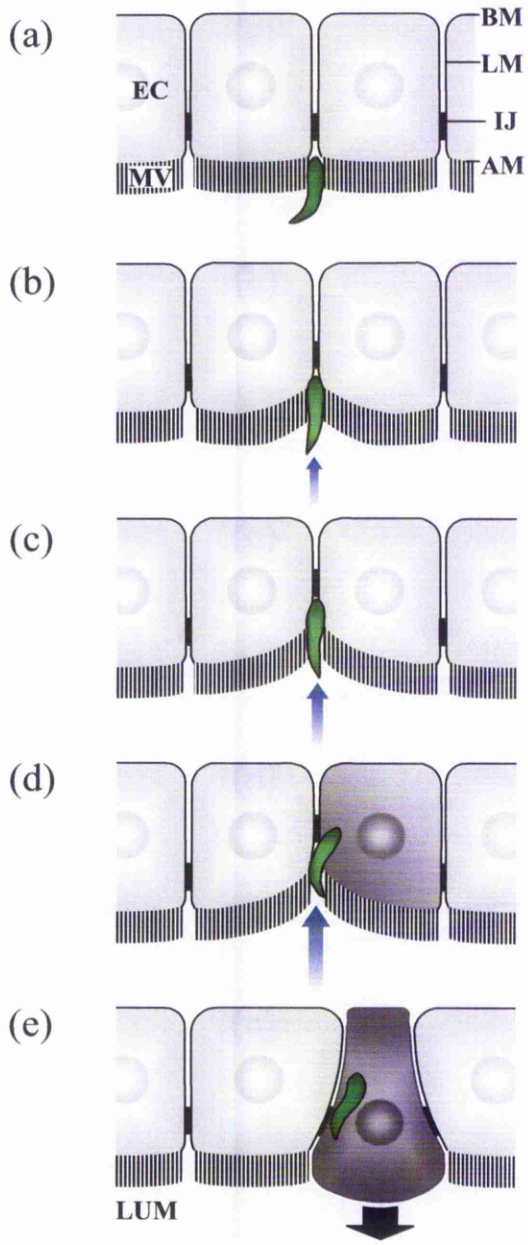


Figure 1
A general model of ookinete entry into the mosquito midgut epithelium. (a) Ookinetes (shown in green) enter the apical surface of the midgut epithelium, through the microvillar brush border (MV), where the lateral membranes (LM) of adjacent epithelial cells (EC) converge [47,51,52]. (b-c) Ookinete movement into the midgut epithelium causes substantial localized invagination of the latter (indicated by small blue arrows) [52,57]. (d) Ookinetes subsequently enter midgut epithelial cells through the lateral apical membrane immediately adjacent to the site where the intercellular junctions (IJ) begin [47,51,52]. (e) The ookinete proceeds intracellularly towards the basal membrane (BM) of the invaded midgut epithelial cell which exhibits morphological abnormalities including protrusion (indicated by large black arrow) into the midgut lumen (LUM) [44–48,52,54,55].

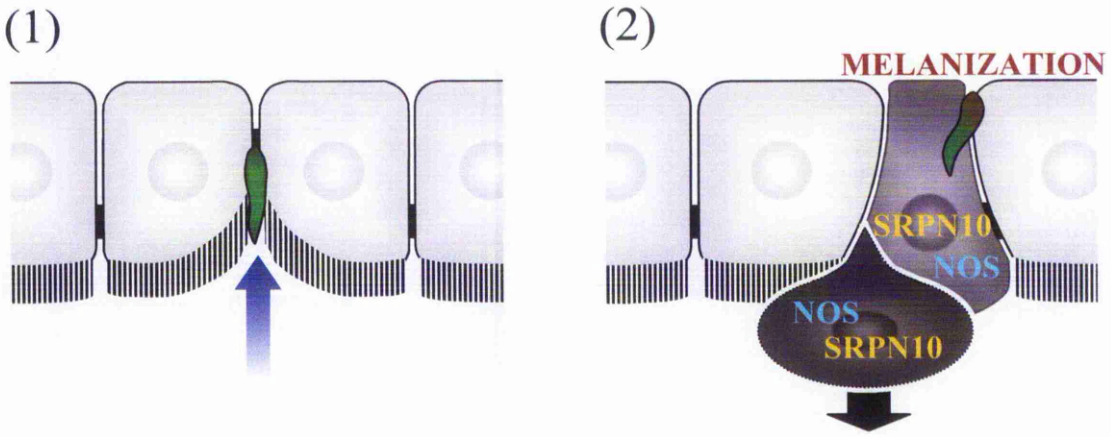


Figure 2
Dko P25/P28 *P. berghei* ookinete invasion of the midgut epithelium. The unified model of the route of ookinete invasion across the mosquito midgut epithelium (Figure 1) [51] implies that there are two sub-populations of Dko P25/28 parasites: (1) a major sub-population of Dko P25/28 ookinetes (shown in green) unable to penetrate midgut epithelial cells, which remain extracellular within the midgut lumen, embedded against the invaginated apical surface of the midgut epithelium (indicated by small blue arrow); and (2) a minor sub-population of Dko P25/28 ookinetes able to penetrate midgut epithelial cells, causing activation of mosquito immune responses and protrusion of invaded midgut cells, in a manner similar to wild-type parasites. Whether the latter parasites migrate through multiple adjacent midgut epithelial cells (as shown) is uncertain.

Testing the hypothesis

Re-interpretation of previously published observations of Dko P25/P25 P. berghei *parasites*

If the unified model of ookinete invasion is correct, the Dko P25/P28 *P. berghei* ookinetes observed deep within the midgut epithelium between morphologically normal midgut epithelial cells are actually extracellular parasites, outside the midgut epithelium and within the midgut lumen, attempting to enter the lateral apical membrane of midgut epithelial cells. The significant invagination of the midgut epithelium that occurs during parasite entry into midgut epithelial cells creates the appearance that these ookinetes are in intercellular locations within the midgut epithelium. This would be similar to the phenotype recently reported for *P. berghei* ookinetes in which the *maop* gene has been knocked out [57]. Ookinetes lacking MAOP are unable to rupture the apical plasma membrane of midgut epithelial cells [57]. Consequently, although MAOP-deficient ookinetes invaginate the midgut epithelium, these parasites are unable to enter into midgut epithelial cells and remain extracellular embedded against the apical surface of the midgut epithelium [57].

The actual extracellular location of Dko P25/P28 ookinetes apparently "within" the midgut epithelium is also suggested by the presence of unmelanized parasites in a refractory line of *Anopheles gambiae* mosquitoes [27]. Unmelanized parasites were observed apparently deep within the midgut epithelium exhibiting an abnormal gelatinous appearance suggested to result from exposure to either epithelial cell defence reactions or an early stage of the melanisation reaction [27]. However, as mentioned above, most Dko P25/P28 parasites do not appear to induce the epithelial cell defence reactions triggered by invading wild-type parasites [45]. Furthermore, the refractory *An. gambiae* line melanises wild-type parasites after their passage through midgut epithelial cells into the basolateral extracellular space between adjacent midgut epithelial cells [55,58,59]. Consequently, an alternative interpretation is that Dko P25/P28 ookinetes are unmelanized because of their extracellular location against the apical surface of the midgut epithelium, which fails to expose them to either epithelial cell or melanisation immune responses triggered by wild-type parasites. The gelatinous appearance of unmelanized parasites could be explained by prolonged exposure of ookinetes delayed in the process of midgut epithelium entry to the environment of the midgut lumen; for example, prolonged exposure to the mosquito digestive proteases secreted into the midgut lumen. Dko P25/P28 parasites have been shown

to be significantly more susceptible to protease digestion *in vitro* than wild-type parasites [27].

However, there is also evidence that some Dko P25/P28 ookinetes do enter the midgut epithelium. A minority of Dko P25/P28 ookinetes are found within midgut epithelial cells, which exhibit the re-distribution and up-regulation of SRPN10 associated with invasion by wild-type parasites [45]. Some Dko P25/P28 parasites are also melanized in the refractory *An. gambiae* line [27] implying entry into and passage through midgut epithelial cells to the basal surface of the midgut epithelium. Further, Dko P25/P28 parasites induce transcriptional up-regulation of mosquito immune response genes, defensin and GNBP, associated with midgut invasion by wild-type parasites [27]. These immune response genes are not induced by transgenic *ctrp*-disrupted *P. berghei* parasites that are unable to invade midgut epithelial cells [27,60]. Again, this implies that at least some Dko P25/P28 parasites successfully invade the midgut epithelium and trigger mosquito immune responses.

Experimentally testable predictions of our interpretation

There are several experimentally testable predictions that follow from the alternative interpretation for the previous observations of Dko P25/P28 *P. berghei* ookinete invasion of the midgut epithelium outlined above.

First, all melanized Dko P25/P28 parasites in the refractory *An. gambiae* line should be associated with morphologically abnormal midgut epithelial cells – cells through which these parasites have migrated intracellularly – exhibiting protrusion into the midgut lumen, and up-regulation of NOS and SRPN10. In contrast, unmelanized parasites should not be associated with any morphologically abnormal midgut epithelial cells, as these parasites have failed to enter the midgut epithelium and invade midgut epithelial cells. Unmelanized parasites are, however, expected to reside deep "within" the midgut epithelium in apparently intercellular locations between morphologically normal midgut epithelial cells (assuming that ookinetes on the apical surface of the midgut epithelium cannot be melanized). If Dko P25/P28 ookinetes do migrate across the midgut epithelium *via* a solely intercellular route there is no known reason why these parasites should not also be melanized in the basal region of the midgut epithelium. Consequently, if solely intercellular migration does occur melanized parasites should be found that are not associated with any morphologically abnormal midgut epithelial cells.

Second, there should be a quantitative relationship between the density of Dko P25/P28 ookinetes associated with morphologically abnormal midgut epithelial cells and the number of oocysts that subsequently develop on the basal surface of the midgut epithelium. Specifically, the number of oocyst stage parasites should be equal to or less than the number of Dko P25/P28 ookinetes associated with morphologically abnormal midgut epithelial cells, as only ookinetes migrating intracellularly are predicted to become oocysts. The Dko P25/P28 ookinetes located between morphologically normal midgut epithelial cells are not expected to transform into oocysts, as these parasites do not enter, and hence cross, the midgut epithelium. The number of ookinetes apparently migrating *via* a solely intercellular route greatly exceeds the number of intracellular ookinetes [45]. Consequently, if Dko P25/P28 ookinetes do migrate across the midgut epithelium *via* a solely intercellular route, the number of oocysts should greatly exceed the number of ookinetes migrating *via* an intracellular route (i.e. those associated with morphologically abnormal midgut epithelial cells).

Implications of the hypothesis

The re-interpretation presented here of previously published work on Dko P25/P28 *P. berghei* parasites implies that there are two sub-populations of Dko P25/P28 ookinetes, neither of which migrate across the midgut epithelium *via* a solely intercellular route (Figure 2). A major sub-population of Dko P25/28 ookinetes is unable to penetrate into midgut epithelial cells and remains extracellular within the midgut lumen, outside but embedded against the invaginated apical surface of the midgut epithelium. Consequently, these parasites appear to be in intercellular locations deep within the midgut epithelium, between the lateral membranes of adjacent midgut epithelial cells. It is proposed that these parasites fail to elicit mosquito immune responses triggered by intracellularly invading parasites, are not melanized in refractory *An. gambiae* and do not give rise to oocyst parasite stages. These parasites remain surrounded by morphologically normal midgut epithelial cells, which do not exhibit the morphological abnormalities associated with parasites invading intracellularly [45]. A second minor sub-population of Dko P25/28 ookinetes is able to penetrate into midgut epithelial cells, in a manner similar to wild-type parasites. These parasites are proposed to elicit mosquito immune responses, including up-regulation of defensin [27], GNBP [27], NOS and SRPN10 [45], undergo melanization in refractory *An. gambiae* [27], and form the few oocysts observed in Dko P25/P28 infections [27]. Accordingly, the latter parasite sub-population should be associated with midgut epithelial cells exhibiting morphological abnormalities associated with intracellular invasion by wild-type parasites [45]. However, if loss of P25 and/or P28 prevents entry into midgut epithelial cells, intracellular movement between multiple adjacent epithelial cells may also be inhibited in Dko P25/P28 parasites.

The reason for the existence of the two distinct sub-populations of Dko P25/P28 *P. berghei* ookinetes is unknown. One explanation is that loss of P25 and/or P28 impedes, but does not entirely prevent, penetration of the apical plasma membrane of midgut epithelial cells. Consequently, the entry of Dko P25/P28 ookinetes into the midgut epithelium may be protracted, prolonging the period of exposure to the hostile environment of the midgut lumen, which results in the death of most parasites before completion of midgut epithelial cell penetration. This interpretation is consistent with the observation of lysed Dko P25/P28 parasites on the luminal side of the midgut epithelium [45].

In summary, the unified model of the route of ookinete invasion across the mosquito midgut epithelium suggests a novel, previously unrecognized, function for P25 and/or P28 in mediating ookinete entry into the midgut epithelium. Specifically, the interpretation presented implies that the loss of P25 and/or P28 greatly impairs, but does not entirely abolish, ookinete entry into midgut epithelial cells and probably has relatively little effect on the ability of ookinetes to traverse through the cytoplasm of midgut epithelial cells. A role for P28 in parasite entry into the midgut epithelium is suggested by the deposition of this molecule at the site of ookinete penetration into midgut epithelial cells [44,45]. This interpretation contrasts with the original studies of Dko P25/P28 parasites, which concluded that P25 and P28 do not play a critical role in recognition, attachment or penetration of the luminal surface of the mosquito midgut epithelium [26,27] and suggests that one mode of action of transmission-blocking antibodies to these ookinete surface proteins is to inhibit parasite entry into midgut epithelial cells, as previously hypothesized [8].

List of Abbreviations

CTRP = circumsporozoite and thrombospondin-related anonymous protein-related protein; Dko P25/P28 = double knockout of P25 and P28; GNBP = gram-negative binding protein; MAOP = membrane-attack ookinete protein; NOS = nitric oxide synthase; Sko P25/P28 = single knockout of either P25 or P28; SRPN10 = serine protease inhibitor 10.

Authors' contributions

LAB wrote the manuscript and prepared the figures. LRC revised the manuscript. Both authors read and approved the final version of the manuscript.

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