Molecular Genetic Studies of Vertebrate Ecology: The Analysis of Senescence, Offspring Sex Ratio Variation and Population Diversity

Helen Whitaker

This thesis is submitted for the degree of Doctor of Philosophy.

Division of Environmental and Evolutionary Biology,

Institute of Biomedical and Life Sciences,

University of Glasgow

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Contents

Summary 1

List of Tables 4

List of Figures 5

Chapter 1: General Introduction 7

1.1 Genetic markers in ecological study 8

1.2 Classification 9

1.3 Population biology 11

Phylogeography 11

Intra-specific adaptation 12

Conservation genetics 13

1.4 Variation between individuals 14

Paternity analysis 14

Sex determination 14

Senescence 16

1.5 Genetic function and ecological interaction 17

1.6 Outline of thesis 18

Chapter 2: Molecular Correlates of Ageing in Birds: Telomeres and Senescence 23

2.1 Introduction 24

Telomere structure and function 24

Telomeres and senescence 26

Telomerase 30
Avian telomeres 31
2.2 Methods 32
DNA extraction: phenol/chloroform method 32
Restriction enzyme digestion and agarose gel electrophoresis 34
Southern hybridisation 35
Non-denaturing hybridisation 38
Densitometry 40
DNA quantification 42
2.3 Discussion 45
Protocol 45
Correlation of blood cell telomere length with age 45

Chapter 3: Variation of Offspring Sex Ratio in the Herring Gull, *Larus argentatus*: The Effects of Season and Hatching Sequence **50**

3.1 Introduction 51
Sex allocation 52
Avian sex ratio variation 52
3.2 Methods 57
DNA extraction - Chelex protocol 59
Sex determination by PCR 59
Statistical analysis 62
3.3 Results 64
3.4 Discussion 65
Seasonal variation of offspring sex ratio in the Herring gull 67
Hatch order and sex ratio in the Herring gull 72

Further study 75

Mechanisms of sex ratio adjustment 76

Adaptive sex ratio manipulation: a matter of opinion? 79

Chapter 4: Differences in Offspring Sex Ratio in the Three-spined Stickleback (*Gasterosteus aculeatus*): The Relationship with Paternal Attractiveness **81**

4.1 Introduction 82

The three-spined stickleback 82

Section I: The Relationship of Offspring Sex Ratio with Paternal Attractiveness 83

4.2 Introduction 83

Reproductive biology of the three-spined stickleback 84

Sexual selection 85

Reproductive costs and benefits 89

4.3 Methods 92

Husbandry 92

Mate choice trials 93

Split clutch *in vitro* fertilisation 94

Sex determination by PCR 96

4.4 Results 99

Mate choice and IVF 99

Hatching success, male colouration and clutch sex ratio 102

4.5 Discussion 105

Female preference for redder males 105

Male quality and offspring sex ratio 109

Sex ratio manipulation 111

Section II: Determination of Sex from Fertilised Eggs 113

4.6 Introduction 113

4.7 Methods 113

4.8 Results 115

DNA yield 115

PCR efficiency 116

Sex ratio 116

4.9 Discussion 118

Sexing stickleback embryos 118

Stickleback sex ratio 118

Chapter 5 Post-glacial Microsatellite Variation amongst Three- spined Sticklebacks from North East Scotland 121

5.1 Introduction 122

Stickleback speciation 124

Microsatellites 129

Mutation 130

Measuring population differentiation 132

5.2 Method 134

DNA extraction: salt method 134

Microsatellite selection 135

PCR 135

Microsatellite measurement 138

5.3 Results 141

Variability within populations:

Hardy-Weinberg equilibrium 141

Variability between populations:

Population pair-wise comparisons 146

Possible evolutionary correlates of genetic differentiation 148

Assignment test 151

5.4 Discussion 153

Hardy-Weinberg proportions 153

Genetic distance measures 154

Speciation 155

Sympatric morphs 156

Gene flow 158

Stickleback evolution 159

References **161**

Appendix I: Reagents **196**

Appendix II: Stickleback colour chart **200**

List of Tables

3.4 Observed and expected numbers of males and females within Herring gull broods

4.4 Results of stickleback mate choice trials and structure of the experimental crosses

4.6 Hatching success and the sex ratio of experimental stickleback clutches

5.3 Microsatellite loci used for the analysis of genetic variation in the Three-spined stickleback

5.6 Hardy-Weinberg test: Loch Ruthven: spineless morph sample

5.7 Hardy-Weinberg test: Loch Ruthven: spined morph sample

5.8 Hardy-Weinberg test: salt marsh sample

5.9 Hardy-Weinberg test: Loch a' Choire sample

5.10 Hardy-Weinberg test: Loch Ruthven, spined morph, sampling site A

5.11 Hardy-Weinberg test: Loch Ruthven, spined morph, sampling site B

5.12 Population pair-wise F_{ST} values with p-values denoting the probabilities, associated with each statistic, that the genetic difference between each pair of populations is zero.

5.13 Population pair-wise R_{ST} values with p-values denoting the probabilities, associated with each statistic, that the genetic difference between each pair of populations is zero.

5.18 Assignment test

List of Figures

2.1 Telomere structure

2.2 The end replication problem

2.3 Agarose gel electrophoresis

2.4 Southern blot

2.5 Autoradiograph showing hybridised telomeric fragments from Zebra finch DNA

2.6 Density profile of telomeric smear

3.1 Herring gull sexing gel, showing PCR products from male and female genomic DNA

3.2 Relationship between mean Herring gull clutch sex ratio and mean date of hatching

3.3 Relationship between Herring gull offspring sex ratio and hatch order

4.1 Arrangement of aquaria during stickleback mate choice trials

4.2 Split clutch *in vitro* fertilisation

4.3 Stickleback sexing gel, showing PCR products from male and female genomic DNA

4.5 Relationship between male colouration and female attention during mate choice trials

4.7 Relationship between stickleback embryo mortality during incubation and clutch sex ratio

4.8 Mean DNA yield from extraction from stickleback embryos, sampled daily during incubation

4.9 The percentage of embryos that could be sexed by PCR, from DNA extracted from stickleback embryos, sampled daily during incubation

4.10 Sex ratio of stickleback embryos, sampled daily during incubation

5.1 Map of Scotland with an expanded view of the Moray Firth coastal region, showing sampling sites

5.2 The phylogenetic raceme of the *Gasterosteus aculeatus* species complex

5.4 GeneScan gel image showing microsatellites amplified from two loci in the stickleback

5.5 GeneScan fluorescence profile showing allele lengths at two microsatellite loci in the stickleback

5.14 Linearized F_{ST} between each stickleback sub-population and the marine form plotted against population altitude

5.15 Linearized F_{ST} between each stickleback sub-population and the marine form plotted against the distance from the salt marsh sampling site.

5.16 Linearized F_{ST} between each sub-population and the marine form plotted against the mean length corrected body mass for each sub-population

5.17 Linearized F_{ST} between each stickleback sub-population and the marine form plotted against the mean length corrected body depth for each sub-population

Chapter 1

General Introduction

Techniques that reveal the molecular variation between individuals and populations have become invaluable tools in the study of ecology and evolution. The research described in this thesis, encompassing the fields of behavioural ecology and population genetics, illustrates a range of the applications of DNA analysis. Molecular genetic methods are utilised, here, to investigate avian senescence, offspring sex ratio variation in the Herring Gull (*Larus argentatus*) and the Three-spined stickleback (*Gasterosteus aculeatus*) as well as the genetic structure of stickleback populations.

In this introduction I shall provide an overview of the insight that exploitation of genetic markers has lent to a wide variety of ecological research areas. A comprehensive review of the applications of molecular genetics to ecological and evolutionary investigation would occupy many volumes of text. The examples used represent a cross section of research - spanning analyses at the level of species, populations within species and comparisons among individual organisms. Within this framework of levels of organisation, I shall also present the areas of study that are focused upon in later chapters. Finally, I shall outline the aims of each investigation described in this thesis and the purpose of the molecular analysis used in each.

1.1 Genetic markers in ecological study

The development of molecular ecology has been led by advances in DNA-based techniques for the analysis of genetic diversity. The first technique to provide co-dominant genetic markers (with which heterozygotes can be distinguished from homozygotes) that could be easily assayed, was allozyme electrophoresis (Lewontin and Hubby, 1966). This procedure separates the different proteins (allozymes) that result from allelic variation at protein coding

loci and, although offering relatively low resolution, remains an economic and convenient method for investigating genetic differences between populations and individuals (May, 1998).

Subsequent advances have been made in the direct measurement of DNA variation, covering a range of levels of resolution, difficulty and expense. The discovery of restriction endonucleases, which cleave DNA at specific sequences (e.g. Kelly and Smith, 1970) has allowed the development of restriction fragment length polymorphism (RFLP) analysis and revolutionised DNA manipulation techniques. The ability to sequence DNA rapidly (Sanger and Coulson, 1975) has since made important impact upon molecular biology and has culminated recently in the development of polymerase chain reaction (PCR) (Saiki *et al.*, 1985; Mullis and Faloona, 1987), which amplifies specified stretches of DNA to concentrations that can be easily analysed. A wide range of PCR primers are now available that can be used to amplify highly conserved genes (such as the genetic material of the mitochondria and ribosomes) across varying taxonomic ranks (e.g. Simon *et al* 1994). At the level of shallow phylogeny and individual variation, considerable progress has been made using the analysis of microsatellite arrays - common repetitive DNA sequences, which have a high mutation rate that allows the investigation of molecular variation with high resolution (Hancock, 1999).

1.2 Classification

The development of molecular systematics over the past 20 years has significantly advanced the study of evolution. The issue of circularity often flaws traditional analyses of adaptive radiation, based on morphological variation in response to ecological selection pressures. That is to say that the phenotypic traits undergoing selection are likely to be those that are used by the researcher to

classify the organisms in question. Givinish (1997) states that "any rigorous, non-circular study of adaptive radiation must be based on a phylogeny that has been derived independently of the traits involved in that radiation" (Givinish, T.J.,1997, page 6.). Excluding such traits from morphological analysis can lead to problems, if the remaining traits have converged or are subject to evolutionary constraint (Givinish, 1997).

Nevertheless, molecular phylogenies are sometimes consistent with those based upon morphology. For example, Titus and Larson (1995) found that morphology and mtDNA sequence data, relating to the evolutionary radiation of salamanders, showed a high degree of congruence (97.2%). In contrast, studies of cichlids, which show adaptive radiation to the extent of hundreds of species within each of the three East African Great Lakes, have revealed striking examples of parallel evolution in form and ecology. Morphological analyses could place two species of fish, which are adapted to the same ecological niches in separate lakes, within the same genus (Greenwood 1993). However, genetic data suggests that the species flocks evolved independently within each lake. For example, Reinthal and Meyer (1997) investigated the evolution four pairs of cichlid species from two localities in Lake Malawi. Their analysis of DNA sequence data indicated that all species from the same locality, irrespective of ecological or morphological similarity, shared a recent common ancestor, so were unlikely to have arisen following an invasion of multiple specialised lineage's.

1.3 Population biology

Factors that effect populations, within species, can be investigated using an extensive range of molecular tools (Hoelzel, 1998). Genetic variance can be measured between and within populations and used in population genetic modelling to answer questions relating to population size, migration, selection and historical events. The attributes of various genetic markers and their applicability to the study of different aspects of population biology are reviewed by Sunnucks (2000).

Phylogeography

Population genetic markers are often used in the study of phylogeography, the geographical distribution of genealogical lineages. Hewitt (2001) discusses the use of DNA sequence analysis in studying the effects of climatic shifts upon colonisation, range expansion, and within-species divergence of plant and animal populations. European biogeographic history is particularly well studied, the Pleistocene glaciations having restricted the range of most species to southern refugia. For example, analysis of mtDNA differentiation in Pine martens (*Martes martes*) and Polecats (*Mustela putorius*) revealed that present day central and northern European populations of both species have colonised their current range following post-glacial population expansion from a single European refugium (Davison *et al.* 2001). The authors also detected mtDNA introgression between Scandinavian Pine martens and the Sable marten (*Martes zibellina*), indicating that inter-specific hybridisation has occurred.

Intra-specific adaptation

The investigation of shallow phylogeny is often applied to populations, within species, that show diverse phenotypic variation. For example, the adaptive radiation of fish in post-glacial lakes, through the evolution of trophic polymorphism, has led to clusters of putative sub-species which often retain the ability to interbreed (reviewed by Schluter, 1996). The question of whether different phenotypes within lakes represent true, reproductively isolated, species as defined by the biological species concept (Mayr, 1963) can be addressed via the investigation of genetic differentiation.

Studies of the Arctic Charr, *Salvelinus alpinus*, illustrate the complexity of inter-specific variation. Although morphs within lakes often show extreme differences in trophic morphology, behaviour and ecology, levels of reproductive isolation between morphs is variable (reviewed by Jonsson and Jonsson, 2001). Certain aspects of polymorphism can be linked to phenotypic plasticity, the variable expression of genes in response to environmental stimuli (Scheiner, 1993). For example, Arctic charr morphs often show differences in age and size at maturity that might result from genetic variation. However, maturation rate can also be influenced directly by food quality during development (Svädeng, 1991).

Investigation of the genetic and morphological variation of charr species has revealed different levels of coupled genetic and morphological divergence (Gislason *et al.*, 1999). Additional studies of polymorphic fish populations have found similar relationships between gene flow and the extent of phenotypic diversification in relation to differential resource use. Examples include Coregonid species, such as the Whitefish, *Coregonis clupeaformis,* (Lu and Bernatchez, 1999) and the Three-spined stickleback (Taylor, 2000).

Stickleback genetic variation (measured at microsatellite loci) is investigated in relation to differences in morphology between populations from lochs in the North East of Scotland, in Chapter 5 of this thesis. The evolutionary history of the Three-spined stickleback is characterised by substantial diversification of the ancestral marine form, following repeated invasions of freshwater habitats (Bell and Foster, 1994). Post-glacial population divergence (both morphological and genetic) has been widely studied in populations from British Columbia (McPhail, 1984; Schluter, 1993; Taylor and McPhail, 1999; Rundle *et al.*, 2000, Taylor, 2000) and in populations across Germany (Reusch *et al.*, 2001).

Conservation genetics

Molecular analysis often has important applications in conservation biology, including the genetic management of captive breeding programmes to avoid inbreeding (Tudge, 1993). Genetic sampling is also useful to the management of wild populations. For example, microsatellite variation has been used to assess the impact of hybridisation with domestic cats upon populations of the Scottish wildcat, *Felis silvestris* (Daniels *et al.*, 2001). Analysis of parent-offspring and sibling-sibling relationships showed recent inbreeding between tabby (wildcat phenotype) and non-tabby wild-living cats, suggesting a need for controls on domestic cat populations to prevent further introgression.

1.4 Variation between individuals

Paternity analysis

DNA based identification of parent-offspring relationships provides interesting avenues of research in behavioural ecology; for example, the study of extra-pair fertilisation in birds (Moller and Birkhead, 1993). Analysis of extra-pair offspring in Bluethroats (*Luscinia svecica*) has shown that nestlings sired by extra-pair males had improved immunocompetence relative to their maternal half-siblings (Johnsen *et al.* 2000). In addition, the paternal half-siblings (in the genetic father's nest) showed lower immunocompetence than the extra-pair young. These results suggest that females engage in extra-pair copulation to obtain compatible genes that increase offspring viability.

Sex determination

The development of sex-linked genetic markers that allow the sex-determination of newly hatched birds (Griffiths, 1998) has stimulated research into the factors that govern the differential allocation of offspring sex. The production of equal numbers of male and female offspring in sexually reproducing diploid species was theoretically discussed by Fisher in 1958. Fisher states that frequency-dependant selection stabilises the sex ratio near unity so that the average investment of parents in their sons and daughters should be equal; higher investment in one sex would be expected to be offset, evolutionarily, by higher mortality in that sex (Fisher, 1999). Fisher's theory assumes a linear net relationship between reproductive investment and return. However, Charnov (1982) has argued that investment may not always be proportional to return and

that if marginal returns in offspring reproductive success vary with offspring sex, then an equal investment in male and female offspring is not always expected.

Parental manipulation of offspring sex ratio has been demonstrated in eusocial insects (Trivers and Hare, 1976), in which sex is determined by haplodiploidy. That is, females develop from fertilised eggs and are diploid whilst males develop from unfertilised eggs and are haploid. Similarly, adaptive sex ratio biases have been found in reptiles with temperature dependent sex determination (e.g. Freedberg and Wade, 2001). However, allocation of sex in many vertebrate species, including birds, is potentially constrained by chromosomal sex-determining mechanisms and male and female gametes are expected to be produced in a 1:1 sex ratio (Fisher, 1999).

Trivers and Willard (1973) predicted that where vertebrate species show extensive parental investment, the sex and number of offspring produced should be adaptively varied in response to maternal condition. Natural selection should favour a biased sex ratio when there is a difference between the cost and benefit of producing males and females, such that a female in poor condition is likely to overproduce the cheaper sex so that offspring quality and survival is maximised.

Studies of sex ratio variation in birds, many based upon expectations of adaptive manipulation derived from Trivers and Willards' (1973) hypothesis are reviewed by Clutton-Brock (1986) and Sheldon (1998). The study described in Chapter 3 of this thesis used molecular sexing techniques to investigate offspring sex ratio variation between individuals in a wild population of the Herring gull, in relation to maternal quality.

Sex-linked markers for the determination of sex from DNA of the Three-spined stickleback have also been recently developed (Griffiths *et al.*, 2000). The stickleback provides a good model organism for the study of sex ratio variation; It

can be maintained and bred in relatively large numbers under controlled conditions in aquaria. Brood sizes are large, with clutches from a female of approximately fifty eggs being common. In addition, the utilisation of split-clutch *in vitro* fertilisation techniques (Barber and Arnott, 2000) allows any potentially confounding effects of maternal and paternal variation to be separated in the analysis of offspring sex ratio. The variability of offspring sex ratio, in relation to paternal quality, in the stickleback is examined in Chapter 4 of this thesis.

Senescence

Senescence is suggested to have evolved via trade-offs between age-specific fecundity and survival. Medawar (1952) first proposed the idea that selection might be absent against degeneration with age. Williams (1957) expanded upon this, adding that genetic mutations, which increase fecundity early in life, could be maintained by positive selection, even when the same mutations cause negative effects upon fecundity and survival later in life. The expected life span of individuals within species and the consequences of senescence have significant effects upon the evolution of life histories (Harvey *et al.*, 1989). Age-sensitive events are subject to selection and the likelihood of survival to a given age, coupled with age-specific variation in the ability of individuals to produce offspring, controls the age structure of populations (Pianka, 2000).

Individuals of some species can be aged by researchers using morphological features. For example, sclerochronology of reptiles (seasonal growth cycles recorded in hard tissues) can be used to estimate age *post mortem* (Castanet, 1994). There are also methods of age determination for ungulates, based upon tooth wear, although these appear fairly imprecise (Richardson *et al.*, 1995; Hewison *et al.*, 1999). Investigation relating to the age of individuals in

free-living bird and mammal populations is largely restricted to the examination of tagged individuals and requires longitudinal monitoring. Field studies upon known-age individuals include research into the effects of age upon reproductive performance in deer (Clutton-Brock 1984), seabirds (Daunt *et al.*, 1999) and swallows (Robertson and Rendell, 2001). The gathering of age-related information would be greatly assisted if molecular markers from DNA samples could be conveniently used to estimate the age of an individual.

Genetic research in humans has shown that telomere length (regions of repetitive DNA at the end of each arm of a linear chromosome) correlates with the age of an individual (Hastie *et al.*, 1990; Harley. *et al.*, 1990; Christafalo *et al.*, 1998; Iwama and Toyama, 1998). It was therefore decided to investigate the possibility of applying telomere measurement techniques to samples of genomic DNA from birds. Analysis of telomere length in birds, as a method of age determination, was attempted and is described and discussed in Chapter 2.

1.5 Genetic function and ecological interaction

Although genetic analysis is used in a wide range of ecological disciplines to examine variation between species, populations and individuals, little is known about the complex relationships between the genotype, phenotype and ecological circumstances of most wild species. Jackson *et al.* (2002) highlight the need for functional approaches, such as the identification of genes that control ecologically relevant traits and their interaction with the environment. Much recent research has been devoted to the analysis of mircorarrays, in which the expression of hundreds to thousands of genes can be assayed to measure the response of an organism to its environment (Brown and Botstein, 1999). The mapping of genes that undergo ecological selection, such as the loci that control continuous variation

of skeletal morphology in the stickleback (Peichel *et al.*, 2001) is also extremely important to the understanding of adaptation. However, the few model species that have been genetically well characterised in the laboratory (e.g. *Drosophila* and *Arabidopsis*) currently limit the range of investigation of genetic interaction with ecological processes. Jackson *et al.* (2002) suggest that, in order to study gene function in a natural context, a larger set of model organisms is needed.

1.6 Outline of thesis

The subjects studied are diverse from chapter to chapter, and the methodology and results are, therefore, described and discussed in their entirety within each chapter. The first study described in Chapter 2 concerns molecular correlates of ageing in birds. The aim of this research was to investigate the possibility of using telomere length as a marker of ageing in birds and to develop a procedure that could be applied to research in avian ecology in general. Telomere length was examined by extraction of DNA from blood samples of known age individuals which, in birds, should be facilitated by the fact that, unlike those of humans, avian red blood cells are nucleated. Analysis was also attempted using DNA extracted from a range of avian tissue samples. Telomeres were isolated by restriction digestion of DNA, using enzymes that cut genomic DNA frequently, with the exclusion of the (known) telomeric sequence. The resulting telomeric fragments were then resolved on agarose gels, for subsequent analysis of mean telomere length.

Chapters 3 and 4 describe two studies that utilise PCR analysis of offspring sex ratio. In Chapter 3, I investigate primary offspring sex ratio variation in the Herring gull. A biased offspring sex ratio has been shown to occur in response to maternal condition in gulls by Nager *et al.* (2000). Female Lesser Black-backed

gulls (*Larus fuscus*) were induced to lay extended clutches by removing and cross-fostering newly laid eggs. The sex of chicks hatching, that were laid towards the end of an extended sequence, when female condition has declined, was strongly female biased. In gulls, males are of a similar mass to females at hatching but are skeletally larger and are so assumed to carry less nutritional reserves (Nager *et al.*, 2000). This difference may explain a sex-related increase in post-hatching mortality found in male Lesser Black-backed gull chicks (Griffiths, 1992).

Laying date is accepted to be an indicator of maternal quality and reproductive success in many species of seasonally breeding birds (Perrins, 1970), including gulls (Nisbet and Drury, 1972). It was decided to use hatching date of Herring Gull clutches, at a breeding colony on Walney Island, Cumbria, as a correlate of maternal condition to investigate whether sex ratio manipulation occurs in gulls, under natural conditions. It is hypothesised that clutches hatching later in the season, laid by poorer quality females, should be female-biased. In order to test this, blood samples were collected from newly hatched chicks, from complete clutches of three eggs, throughout the hatching period. Molecular analysis of sex was carried out using DNA extracted from each blood sample (Griffiths *et al.*, 1998) and the sex ratio of each clutch analysed in relation to hatching date.

Molecular sexing techniques were also applied to the analysis of offspring sex ratio in sticklebacks, detailed in Chapter 4. The Three-spined stickleback provides an interesting subject for the study of offspring sex ratio. During breeding, males develop nuptial colouration (including red, carotenoid-based, ventral skin pigmentation), build a nest and compete for mates (Wootton, 1976; Wootton *et al.*, 1995). Females lay their eggs in the nest of the chosen male, who then provides all of the parental care. Red colouration has been shown to indicate

male body condition in the stickleback with good quality males having more intense red colouration (McLennan and McPhail, 1989b; Frischknecht, 1993; Bakker, 1999). This relationship would appear to form a basis for female mate choice, with females generally preferring to mate with redder males (Milinski and Bakker, 1990; Bakker, 1993).

The aim of the study (described in Chapter 4) was to investigate the effect of paternal quality upon offspring sex ratio, in the stickleback. Paternal quality was assessed by the intensity of red nuptial colouration and its relationship with female choice analysed in a captive population sample, using mate choice trials. Clutches of eggs were fertilised using a split clutch *in vitro* fertilisation protocol (Barber and Arnott, 2000). Pairs of males of differing redness were used to fertilise clutches of eggs from pairs of females. Half of each of two females clutch was fertilised by each of two males and the eggs incubated artificially (Barber and Arnott, 2000). The resulting fry were then sexed at hatching using a PCR based technique (Griffiths *et al.*, 2000). This split-clutch design should allow the comparison of offspring sex ratio, at fertilisation, between males of differing quality, whilst controlling for maternal effects.

During the incubation of the fertilised stickleback clutches, many of the embryos were destroyed by fungal infection. This setback prompted the analysis described in the second section of Chapter 4, which assessed the efficacy of sexing stickleback embryos at earlier stages of development. DNA was sampled from stickleback embryos daily between fertilisation and hatching (a period of approximately ten days). The amount of DNA extracted was quantified and the effectiveness of the PCR-based sexing technique monitored in relation to embryo age.

In Chapter 5, microsatellite variation, between populations of the Three-spined stickleback, was analysed. The use of microsatellites as genetic markers is growing rapidly and covers a wide variety of applications (Goldstein and Schlötterer, 1999). Although several alternative approaches to measuring molecular variation between groups of individuals are available (Sunnucks, 2000), microsatellite analyses have a number of useful attributes, when used in the investigation of shallow (intra-genus or intra-species) phylogeny.

The term microsatellite is now commonly used to refer to sequences in the genome, consisting of a motif of less than six base pairs in length that is repeated head-to-tail, without interruption (Hancock, 1999). Microsatellites tend not to be highly conserved between species - only 30% of human microsatellites are present in rodents (Stallings, *et al.*, 1991). It is this variability, a product of a high mutation rate that allows sequence length variation, at specific microsatellite loci, to be utilised in the investigation of genetic differentiation within species. In addition, microsatellites are numerous and codominant (Sunnucks, 2000), meaning that heterozygotes for a given locus can be distinguished from homozygotes.

The main drawback of microsatellites is that extreme inter-specific variation means that they often need to be characterised by researchers of species that are being examined for the first time (Zane *et al.*, 2002). However, PCR primers that amplify microsatellite loci in the Three-spined stickleback have already been published by several authors (Rico *et al.*, 1993; Taylor, 1998; Largiadèr *et al.* 1999).

The analysis of microsatellite variation from populations from North East Scotland, detailed in Chapter 5, was carried out in collaboration with an investigation of morphological divergence by Arnott, Barber and Pagnon (unpublished). The analysis of microsatellite variation is based upon tissue

samples from stickleback collected from a loch containing two distinct morphs, one group with pelvic spines and another lacking pelvic spines. These morphs were compared at five microsatellite loci along with a local salt marsh population (representing the marine ancestral form) and a population situated upstream, in a neighbouring loch.

Chapter 2

Molecular Correlates of Ageing in Birds:

Telomeres and Senescence

2.1 Introduction

The age structure of a population is an important ecological parameter. However, research relating to the life-spans of higher animals often requires an extensive longitudinal study. For example, ornithologists might be able to recognise seasonal cohorts of birds that exhibit distinctive plumage characteristics over their first few years of life, whilst many long-lived species are only recognisable as juveniles or adults. In order to be more precise, birds can be ring-marked as hatchlings. Those that are encountered in subsequent years may then be used as a sample of known age individuals.

A useful alternative might be found in the use of molecular markers. Simple changes to DNA occur within an organism each time that a cell divides. Telomeres (the extensive sequences found at the ends of chromosomes) have become a focus of substantial research and it is the object of this study to examine the telomeres of birds. It is hoped that this investigation will allow new techniques to be applied within an ecological context.

Telomere structure and function

In eukaryotes, a copy of the genome is contained in the nucleus of each cell, as a set of linear chromosomes. Telomeres form the termini of each arm of a linear chromosome. The chromosome consists of a matching pair of DNA molecules, folded and packaged with protein to form sister chromatids (Figure 2.1). DNA is composed of nucleotides, which in turn are each composed of a nitrogen base, deoxyribose sugar and phosphate group. The nucleotides bond together, via the sugar and phosphate groups, in series, to form two strands of a DNA molecule. Each nucleotide is identical except for its base which is either adenine, thymine, guanine or cytosine (denoted A, T, G and C). These form base

pairs, joined by hydrogen bonds, which hold together the two strands of a DNA molecule (T bonds preferentially with A and G with C). When the two stands of a DNA molecule are separated, exposed bases will usually only pair with its complimentary base. Thus each single strand of a pair is the template of the other (Watson and Crick, 1953). Telomeric DNA consists of a long, repeated sequence of bases, rich in guanine. The vertebrate telomeric sequence is highly conserved (Meyne *et al.*, 1989) and is represented in Figure 2.1 (expanded view). The unit (TTAGGG) is repeated several thousand times within a single telomere.

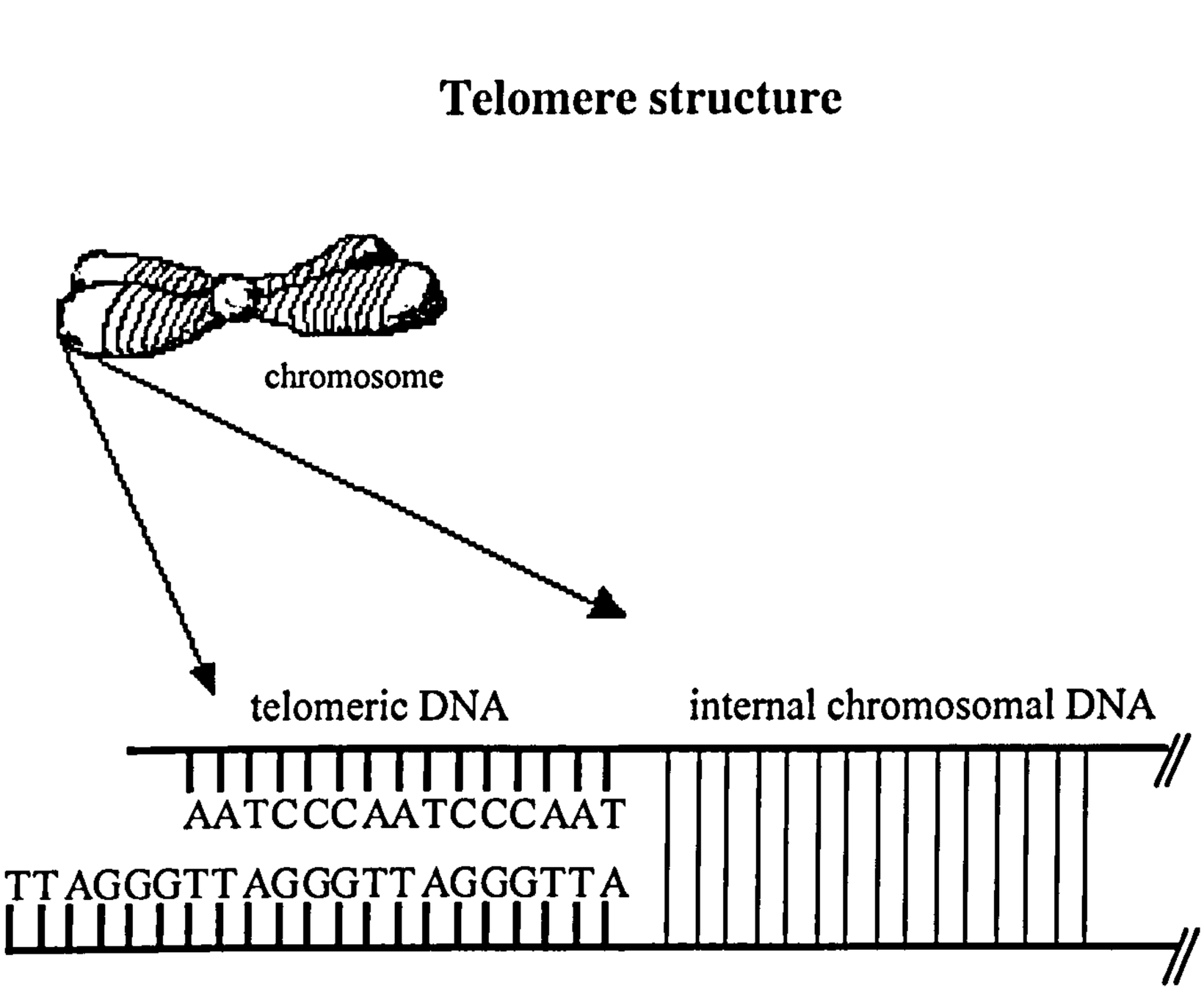


Figure 2.1: Telomere structure: The expanded view represents the 10-15kb telomeric sequence, with the repeating unit $(TTAGGG)_n$, as it borders the internal chromosomal DNA.

One of the functions of telomeres is to act as binding sites for the proteins that cap and protect chromosomes from degradation and end-to-end fusion's. This role was identified by McLintock (1941) who recognised that intact chromosomes differed from those that were broken by possessing a structure that stabilised the ends, preventing end-to-end fusion. This telomeric sequence does degrade, through oxidative stress (von Zglinicki *et al.*, 1995), possible exonuclease activity (Makarov *et al.*, 1997) and as a result of mitosis. Because the telomere does not contain codons for protein synthesis, it may, therefore, serve as a buffer that protects internal chromosomal genes from deletion. Telomeres also play a role in meiosis, transcriptional silencing and interact with cell cycle checkpoint controls (McEachern *et al.*, 2000).

Telomeres and senescence

Hayflick and Moorehead (1961) demonstrated that the replicative capacity of cells in culture decreased with increasing age of the donor tissue. In addition, Olovnikov (1973) noted that incomplete copying of the ends of chromosomes led to shortening of so-called "telogenes", with each round of mitosis.

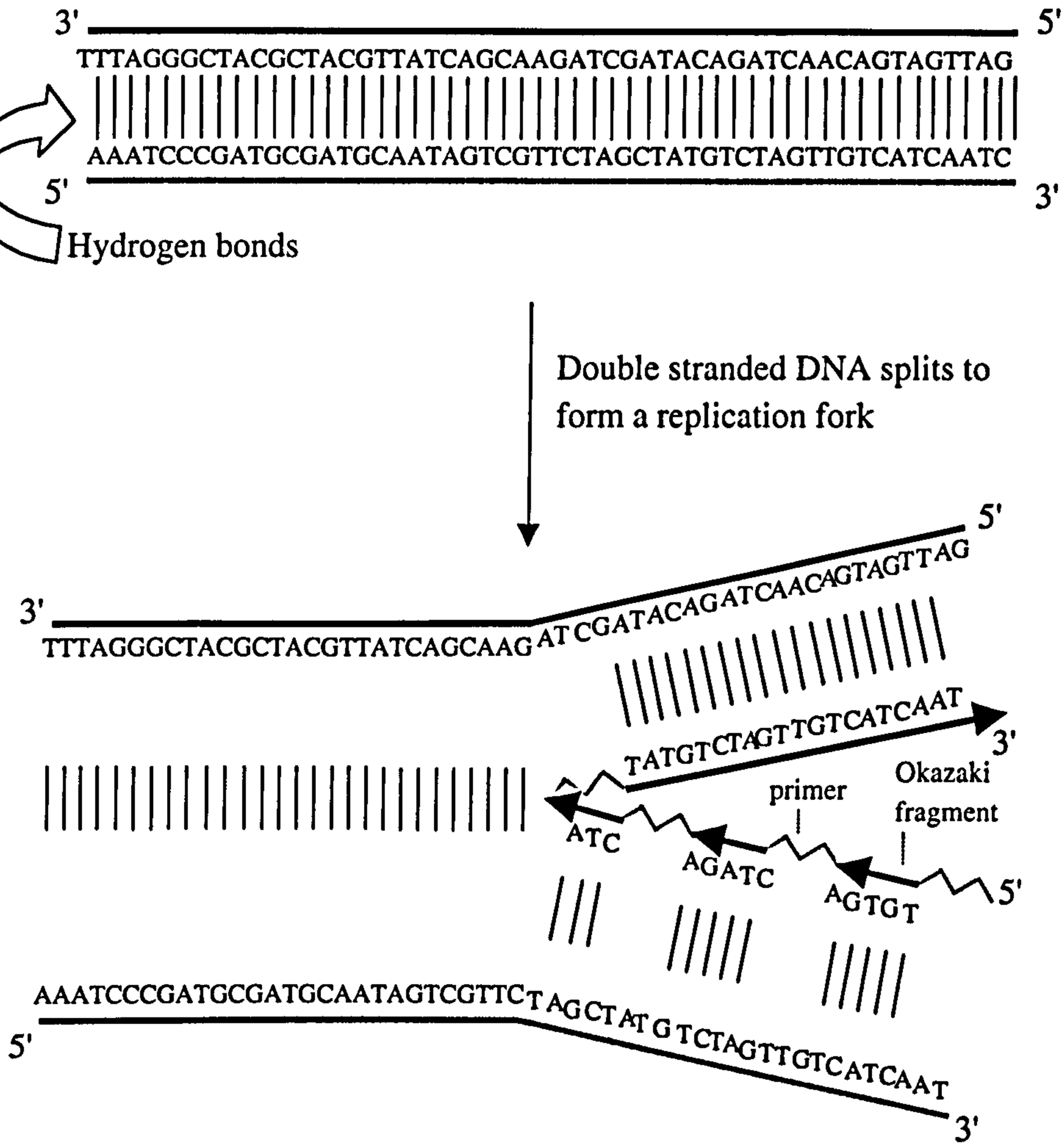
Telomere shortening occurs with cell division due to the failure of enzymes to copy the very end of a DNA strand during replication. Figure 2.2 represents the telomeric end of a DNA duplex, during replication. The nucleotide units that bond to form DNA molecules are asymmetrical and bind only in the 5' to 3' orientation. The upper and lower strands of the DNA duplex are antiparallel in terms of their 5' to 3' orientation. During cell division, the duplex separates to form a replication fork. Each strand from the parental molecule becomes a template for a complementary new strand so that replication is semi-conservative.

Replication is initiated by RNA primers, which bind to the parental strand and are then elongated by DNA polymerase enzymes to form the complimentary strand. On removal of the RNA primers, gaps are sealed in the 5' to 3' direction. At the 3' end of the new strand, filling cannot occur and a guanine rich, single stranded, overhang remains (Wright *et al.*, 1997). This structure has recently been shown to form a loop (Griffith *et al.*, 1999) that may function to increase the stability of the end of the telomere.

Telomere shortening has been shown to occur in a variety of human tissues with age (Harley, 1995 reviews). Length has been shown to decline *in vitro* and *in vivo*, until a critical length is reached during senescence. In humans, a mean telomere length of 1.5 kb is proposed to coincide with cell cycle exit (Allsopp and Harley, 1995). Chin *et al.* (1999) show that shortening of telomeres to critical length activates the production of p53 protein that, in turn, induces transcription of cell cycle regulatory genes. The expression of inhibitors, p21 and p16INK, accompany the phenotypic alteration associated with senescence (Kipling and Faragher, 1997; Kiyono *et al*, 1998).

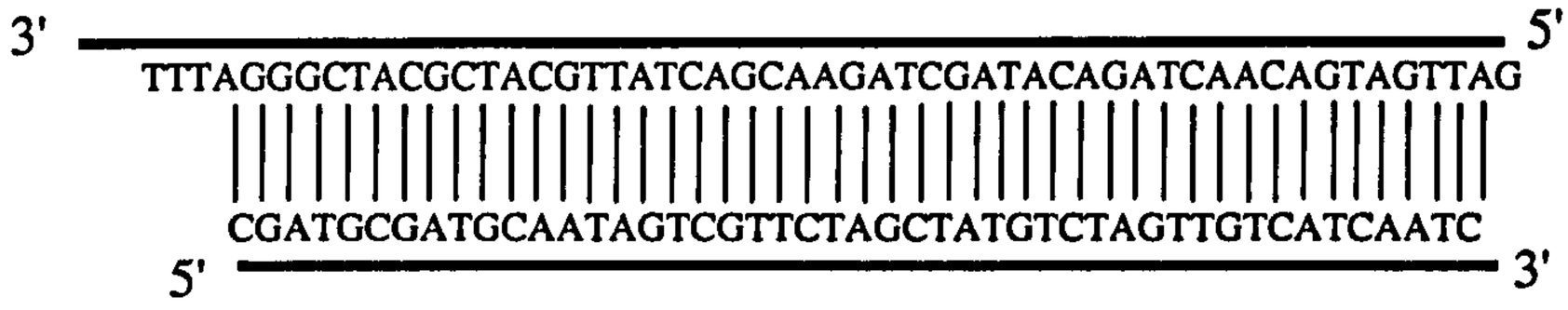
The decrease in telomere length of cells with increasing age gives an explanation for the Hayflick limit. This limit has also been characterised in a range of mammals by Rhome (1981), who shows that the replicative capacity of fibroblasts relates to the species longevity. It might, therefore, be expected that species telomere length would correlate to the limits found upon cell replicative capacity.

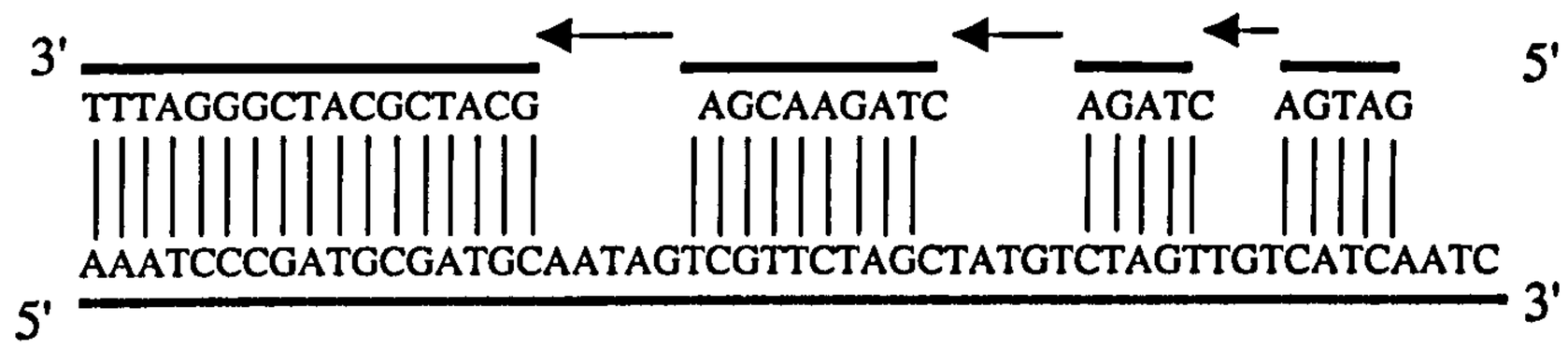
Figure 2.2: The end replication problem



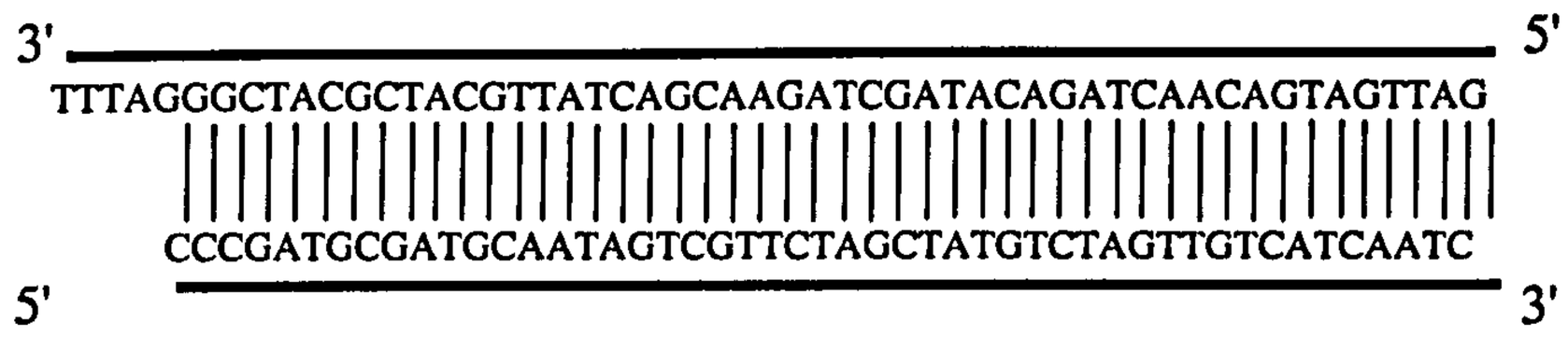
DNA strands are made by polymerase enzymes but these cannot start a new strand. Synthesis begins with a an RNA primer (⩘) which binds to the strand to be copied. DNA cannot be built in the 3' to 5' direction, so the lower strand (—) is copied as Okazaki fragments, synthesised 5' to 3', as the replication fork "unzips".

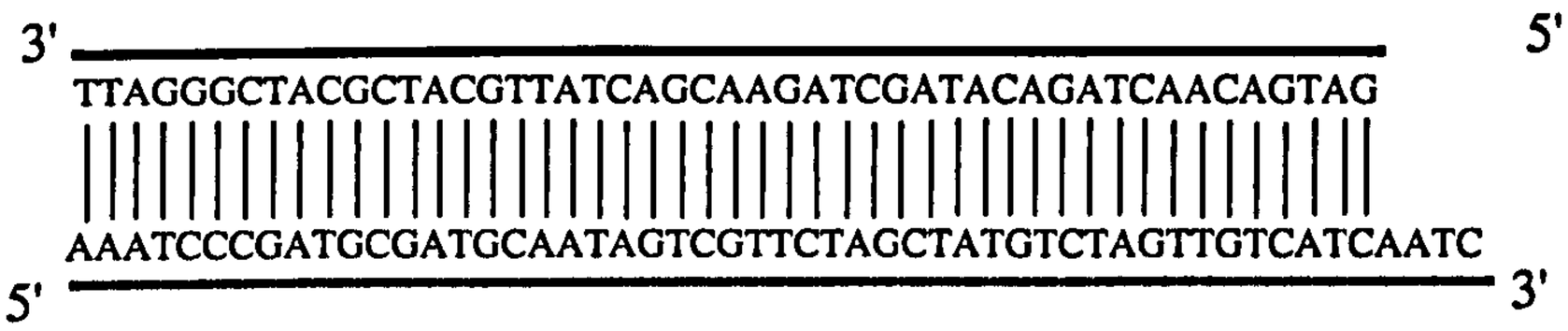
Following replication, the primers are removed





Gap filling can only occur by extension from 3' ends (◄—)





3' overhangs are left on both new molecules. Telomere shortening, then, occurs when the chromosome is replicated during cell division (diagram adapted from Blackburn and Greider, 1995).

The erosion of telomeres and eventual loss of genes, with advancing cell division, provides a model for replicative senescence. Increased rate of Telomere shortening has been shown to occur in a range of progeroid syndromes - conditions which cause premature ageing (Kipling and Faragher, 1997). Nevertheless, the role of telomere dynamics during the ageing process is not clear. Telomere length varies between chromosomes within a single nucleus (Henderson *et al*, 1996), in addition to variation in mean length between tissues. For example, the cells lining human arteries have been shown to have an increased rate of telomere loss with age, when compared to those of veins which sustain less haemodynamic stress and, presumably, have a lower turnover during life (Chang and Harley, 1995). The importance of telomere attrition to ageing would, therefore, depend on the rate of mitosis of primary cells in a tissue and possibly upon the number of chromosomes within the cells of that tissue which have become critically shortened.

Telomerase

Telomere loss can be counteracted by the enzyme telomerase. Telomerase contains an RNA template for the telomeric sequence and maintains telomere length in a range of tissues. Human telomerase is expressed in germline cells (Frenck, *et al.*,1998), hematopoetic stem cells, the basal layer of the skin, basal crypt cells of the small intestine, lymphoid cells and thymocytes. The activation of the gene for human telomerase (hTERT) has also been shown to produce immortal cells in culture. Telomerase negative cells reached crisis (critical telomere length) at around 4kb whereas hTERT cells maintained telomeres at 9kb long. (Bodnar *et al.*, 1998; Counter, *et al*, 1998; Vaziri and Benchimol, 1998). Telomerase is largely down-regulated in somatic tissues, possibly as a mechanism

to inhibit uncontrolled cell division and tumour formation. The relationship between telomerase and cancer has been extensively investigated in recent years (see deLange and Jacks, 1999 for review). Several types of cancer have been shown to be associated with high telomerase activity (Kim *et al.*, 1994).

Telomerase activity is high in mouse tissues, and some strains have extremely long telomeres, up to 150kb. Mice from a germline lacking telomerase were found to exhibit telomere shortening, which in turn caused end-to-end chromosome fusions and tumour formation (Blasco *et al.*, 1997). Rudolph *et al.* (1999) found that the telomerase negative strain showed accelerated senescence and symptoms of ageing, such as impaired wound healing. It is possible that mice avoid telomere shortening, in tissues with high turnover, by maintaining a high level of telomerase activity at the expense of risking uncontrolled cell division. This may be a feasible strategy given that mice are short-lived and the mortality risk associated with cancer is low when compared to environmental threats to survival.

Avian telomeres

Most research on telomeres has been carried out using human or rodent subjects. Avian telomeres have been studied by Venkatesan and Price (1998), who demonstrated that telomere length decreases with age in cultured chicken fibrobasts (*Gallus gallus domesticus*), which show little telomerase expression. However, telomerase was found to be active *in vivo* in a variety of tissues from embryos and chicks.

2.2 Methods

Telomere Restriction Fragment (TRF) measurement has been extensively used in investigations of human and mouse cellular senescence. An equivalent measurement of telomeric DNA abundance can also be made using fluorescence *in situ* hybridisation (FISH), which involves hybridisation of telomeric DNA to a fluorescent probe, within intact cells, and quantification by flow cytometry. A comparison by Hultdin *et al.* (1998) found a highly significant correlation between the mean telomeric fragment length found using the former method and the quantitative fluorescence value.

The application of a protocol designed for mammalian telomere analysis to bird DNA requires modification of standard techniques. Avian TRF measurements have been made by Venkatesan and Price (1998) from cultured chicken cells, although not without difficulty.

DNA extraction: phenol/chloroform method

The analysis of telomere fragment length requires large quantities of high quality DNA. Phenol/chloroform extraction was used to purify genomic DNA for this purpose. Mixing the digested samples with phenol (an organic solvent) serves to remove proteins to the organic phase, whilst the DNA is partitioned in the aqueous buffer. The two phases can then be separated by centrifugation (phenol being denser) and the upper aqueous layer retained for further purification. Phenol also denatures protein, assisting the removal of protein from DNA. Further treatment of the DNA with a mixture of phenol and chloroform improves the efficiency of protein denaturation and phase separation. In addition chloroform

removes lipid (Wallace 1987) whilst final purification with chloroform removes any traces of phenol from the DNA preparation (Sambrook *et al.* 1989).

1) Approximately 10µl blood was mixed with 300µl of SET, 30µl of 10%SDS and 2.5 units/ml of Proteinase K. (12.5µl of 10mg/ml proteinase K in this case) in a 2.5 ml eppendorf. Samples were digested overnight in an orbital incubator at 55°C, then allowed to reach room temperature.
2) 300µl phenol was added and the samples placed in a rotary mixer 15 minutes. The tubes were centrifuged at 18 000 × *g* for 10 minutes and the aqueous (upper) layer was then removed, with a pipette, to a clean tube.
3) 150µl of phenol and 150µl $CHCl_3$ were added and mixed again for 15 minutes then centrifuged and the aqueous layer removed, as before.
4) Step 3) was repeated, but using $CHCl_3$ and centrifuged for 5 minutes.
5) DNA was precipitated by adding 2 volumes of absolute alcohol and 0.1 volumes of sodium acetate (3M pH5.2). For a 400µl sample, 40ul of 3M NaOAc (pH 5.2) and 800µl of 100% EtOH were added. Samples were mixed by inversion and placed at -20°C for 1 hr (Optional). The tubes were centrifuged for 10 minutes and the EtOH poured off.
6) The DNA pellet was washed 80% EtOH and dried by leaving for 15-30 minutes at room temperature.
7) Each DNA sample was resuspended in 50µl TE in an orbital incubator at 55°C overnight. Samples were stored at -20°C.

Restriction enzyme digestion and agarose gel electrophoresis

Restriction enzyme digestion of genomic DNA (effectively of entire chromosomes) that leaves the telomere intact, is made possible by selection of enzymes that cut genomic DNA frequently but that will not cut strands within the repeated telomeric sequence. This should leave behind large telomeric fragments with a short, adjacent, sub-telomeric portion that leads to the nearest cutting site in the chromosome from the telomere.

Restriction digestion was carried out using 2 units of *Hinf1* (Promega) and 2 units of *Alu1* (Promega) (Venkatesan and Price, 1998) per 40µl sample, in reaction buffer with 1% BSA, overnight at 37°C. A 5µl aliquot of each sample with 10 × Orange G loading dye was loaded into a 10cm 0.8% agarose gel (stained with ethidium bromide). A 4µl aliquot of 1kb DNA ladder (Promega) was included in each row of lanes, as a scale, and the gel was run for 20 - 30 minutes at 100V in 1 × TBE buffer. The gel was then placed on a transilluminator and photographed, in order to check that the genomic DNA had been successfully extracted and adequately restricted. 30µl of each sample with 10 × Orange G loading dye was then loaded into a 15cm 0.8% agarose gel (stained with ethidium bromide) in 1 × TBE buffer and run for 16 hours at 30V. A lane containing 5µl λ *EcoR I* marker (Promega) with 6 × loading dye (supplied with marker), was included in the gel to provide scale of six DNA fragments ranging from 3.5 - 21.2 kb in length.

During electrophoresis (Figure 2.3), the large telomeric fragments are retained in the gel and should travel at distances that are proportional to the $\log_{10}$ of the number of base pairs (Helling *et al.*, 1974). Following electrophoresis, the gel was placed on a transilluminator and photographed.

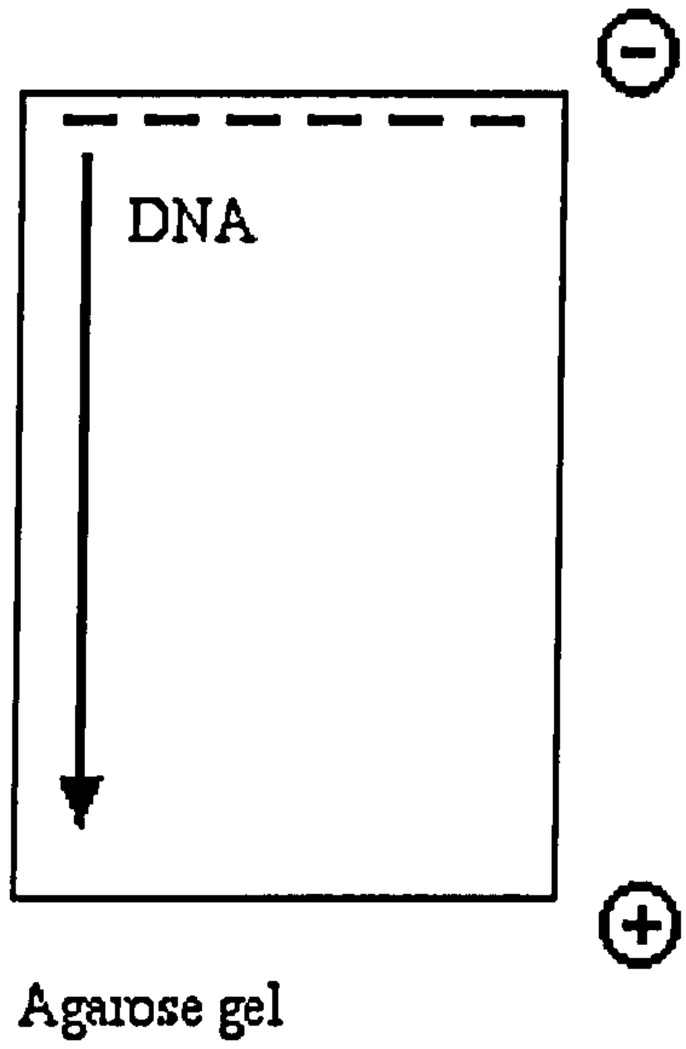


Figure2.3: DNA samples are loaded into wells at the top of the gel, which is placed in an electrolyte buffer. The negatively charged fragments migrate through the gel towards the positive electrode. The distance moved is logarithmically proportional to the DNA fragment length

Southern hybridisation

The gel was denatured (to separate the DNA into single strands) and fragments transferred to a nylon membrane by Southern blotting (Figure 2.4) using methods described by Sambrook *et al.* (1989). This transfer does not alter the relative position of fragments produced by electrophoresis.

Southern blot

1) Depurination (15 minutes) 0.2M HCl (rinse in dH_2O).

2) Denaturation (2x 30 minutes) 0.4N NaOH, 1M NaCl.

3) Neutralisation (2x 30minutes) 0.5M TrisCl, 1M NaCl pH 7.2.

4) Ran in 10 x SSC buffer overnight (see figure2.4).

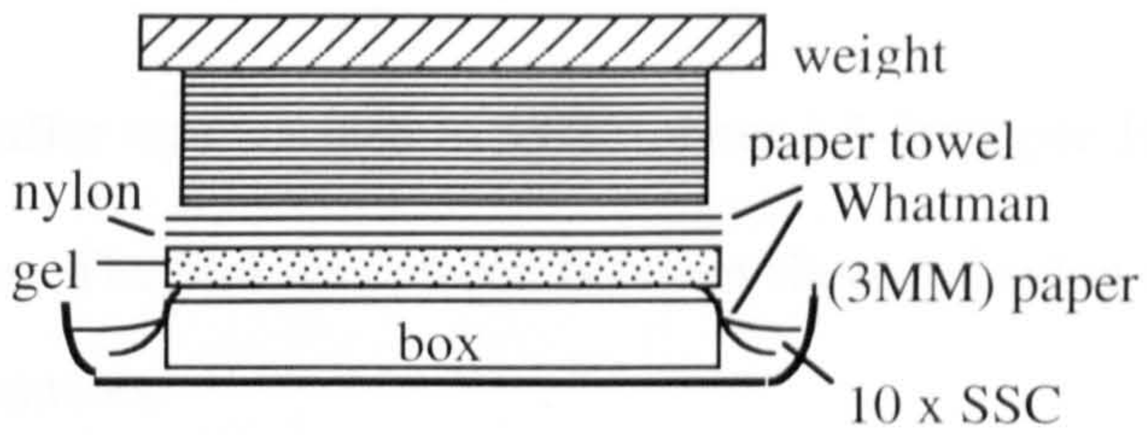


Figure 2.4: Arrangement of gel, filters and nylon (Hybond) for Southern blotting.

Hybridisation to a radio-labelled probe was then necessary to determine mean fragment length. A single stranded analogue of telomeric DNA [$(TCCCAA)_3TCC$] was end-labelled with $\gamma^{33}P$ ATP, using the enzyme T_4 Polynucleotide Kinase (T_4 PNK) (Promega).

End-labelling a $(TCCCAA)_3TCC$ probe

$(TCCCAA)_3TCC$ (100 ng/µl)	1
T_4 PNK buffer	1
$\gamma^{33}P$ ATP (20µCi)	2
(specific activity 3000 Ci/mmol)	
T_4 PNK (10 units/µl)	1
Sterile water	5
Total	10µl

The above mixture was incubated at 37°C, for 1 hour, then heated to 70°C for 10 minutes, to kill the enzyme.

Hybridisation

1) Hybridisation buffer was warmed to 59°C (about 1.5-3mls per 10cm^2 gel) in a hybridisation bottle. The blot was placed between sheets of nylon mesh and pre-hybridised for 20 minutes.

2) The buffer was replaced and a small amount of buffer added to the probe. The probe was added to the bottle and hybridised overnight at 59°C.

3) Washing was carried out in 0.25x SSC at room temperature for 30 minutes then 0.25x SSC for 2 x 1 hour at 42°C.

Optimal hybridisation conditions were chosen according to the length and composition of the probe. Hybridisation temperature (T_m –5) was derived from the following equation:

$$T_m = (A+T) \times 2 + (G+C) \times 4 \ ^\circ C$$

A stringent washing procedure ensured that the binding of the probe was limited to telomeric sequences. The use of a low molarity washing buffer (0.25 × SSC), at moderately high temperature (42°C), prevents non-specific hybridisation. The blot was exposed to film to produce an autoradiograph of the hybridised telomeric fragments.

Initial experiments using Lesser black-backed gull (*Larus fuscus*) blood samples, for which known age adult and chicks were available, produced faint telomeric smears. The appearance of these suggested that the genomic DNA samples were not properly restricted. The yield of DNA produced by phenol/chloroform extraction from around 30-50µl of blood was also rather low, with around 2µg per sample being required for initial restriction and subsequent

Southern analysis. It was also suspected that DNA extracted from these refrigerated samples was degraded.

In order to obtain larger amounts of undamaged DNA for developing the measurement protocol, liver, heart, kidney, testes, brain and lung tissues were sampled from freshly culled Zebra finches (*Taeniopygia guttata*). The radioisotope used to label the telomeric DNA was altered from ^{33}P to ^{32}P, which greatly increased the hybridisation signal from the resulting smears. These new results provided autoradiographs showing a fairly large proportion of restricted genomic DNA remaining in the wells of the agarose gel following electrophoresis. This problem may have arisen due to the presence of residual protein in the sample, to which DNA will remain bound. In order to limit this, further extractions were made using purification columns (Qiagen Tissue Extraction Kit). This did not seem to alter the appearance of the smear, suggesting that additional or alternative explanations need be sought. One prospect is that the DNA fragments are simply too large to overcome the 60kb mobilisation limit (Sambrook *et. al.*, 1989) for conventional agarose gel electrophoresis.

Non-denaturing hybridisation

Venkatesan and Price (1998) found similar results (using Southern analysis for chicken telomere length measurement) to those I have reported and suggest that the appearance is due to telomeric sequences being present at internal sites within the chromosome. This could, in theory, prevent adequate restriction and leave non-telomeric DNA within the analysis. They suggest that chicken chromosomes do seem to have interstitial telomeric sequences, which are visible during fluorescence *in situ* hybridisation. They also showed that the telomeric

smears were resistant to pre-treatment of the DNA with *Bal31* nuclease. This exonucleic enzyme removes nucleotides from the both ends of double-stranded DNA molecules, in a stepwise manner, which would result in terminally derived telomeric DNA becoming progressively shorter. Meyne *et al.*, (1990) also suggest the presence of satellite telomeric sequences, outwith chromosomal termini, in a variety of bird species.

In order to overcome these features Venkatesan and Price (1998) used a G-overhang assay. This technique does not denature the DNA so that the telomeric probe exclusively targets the single-stranded guanine-rich overhang, found at the end of telomeres. The remainder of the telomere and any interstitial telomeric tracts are left double-stranded, in a dried non-denatured gel, and cannot hybridise to the probe. By increasing the selectivity of hybridisation in this way, Venkatesan and Price were able to show that telomere length becomes shorter with increasing number of cell divisions of chicken skin cells in culture.

I adopted this method (including further advice from the authors) and attempted to carry out G-overhang assays on DNA extracted from Zebra finch tissues. DNA restriction and electrophoresis was carried out as before. The λ *EcoR I* marker was end-labelled with γ^{32}P ATP (1µl of 0.5µg/µl stock was end-labelled, as described earlier for the telomeric probe, and diluted to 10ng/µl with TE and 6 × loading buffer). Between 10 and 20µl of the labelled marker (depending on decay of activity during storage) was included in a lane of the gel during electrophoresis. Instead of Southern blotting, following electrophoresis, the gel was soaked 2x SSC for 30 minutes, a double layer of Whatman 3MM paper was immersed in 2x SSC and placed under the gel on a gel drier, covered with cling film. The gel was dried at room temperature for 1 hour or until evenly flat;

then at 60°C for 1 hour or until the paper was dry. Hybridisation and autoradiography was carried out as before, except that the hybridisation temperature was lowered to 37°C.

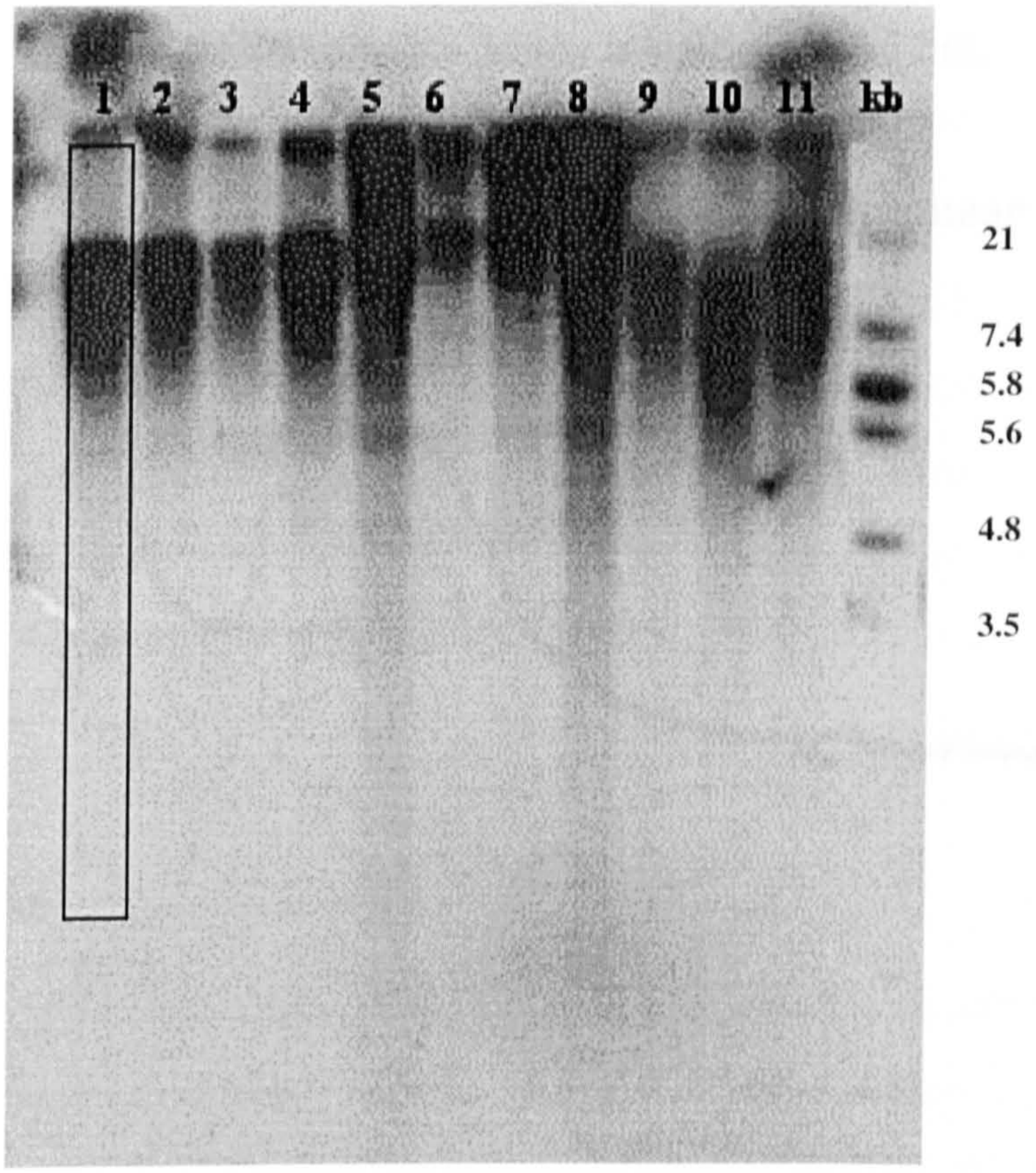


Figure 2.5: Autoradiograph showing telomeric restriction fragments, from Zebra finch DNA, hybridised in a non-denatured dried gel to a γ^{32}P labelled telomeric probe. Each lane contains telomeric restriction fragments from one sample of genomic DNA (lane 1: testes DNA; lanes 2 - 6: DNA from blood samples from 5 individuals; lanes 7-11: DNA from liver samples from 5 individuals). The far right lane contains the labelled λ *EcoR I* marker with fragment lengths (kb) shown on the right.

Densitometry

Mean telomere length for each genomic sample can be measured against the end-labelled λ *EcoR I* marker, which consists of DNA fragments of known length (see Figure 2.5). The autoradiograph was scanned to produce a digital image. A density profile was plotted and mean telomere length defined as the length on the x-axis where density is highest (Figure 2.6).

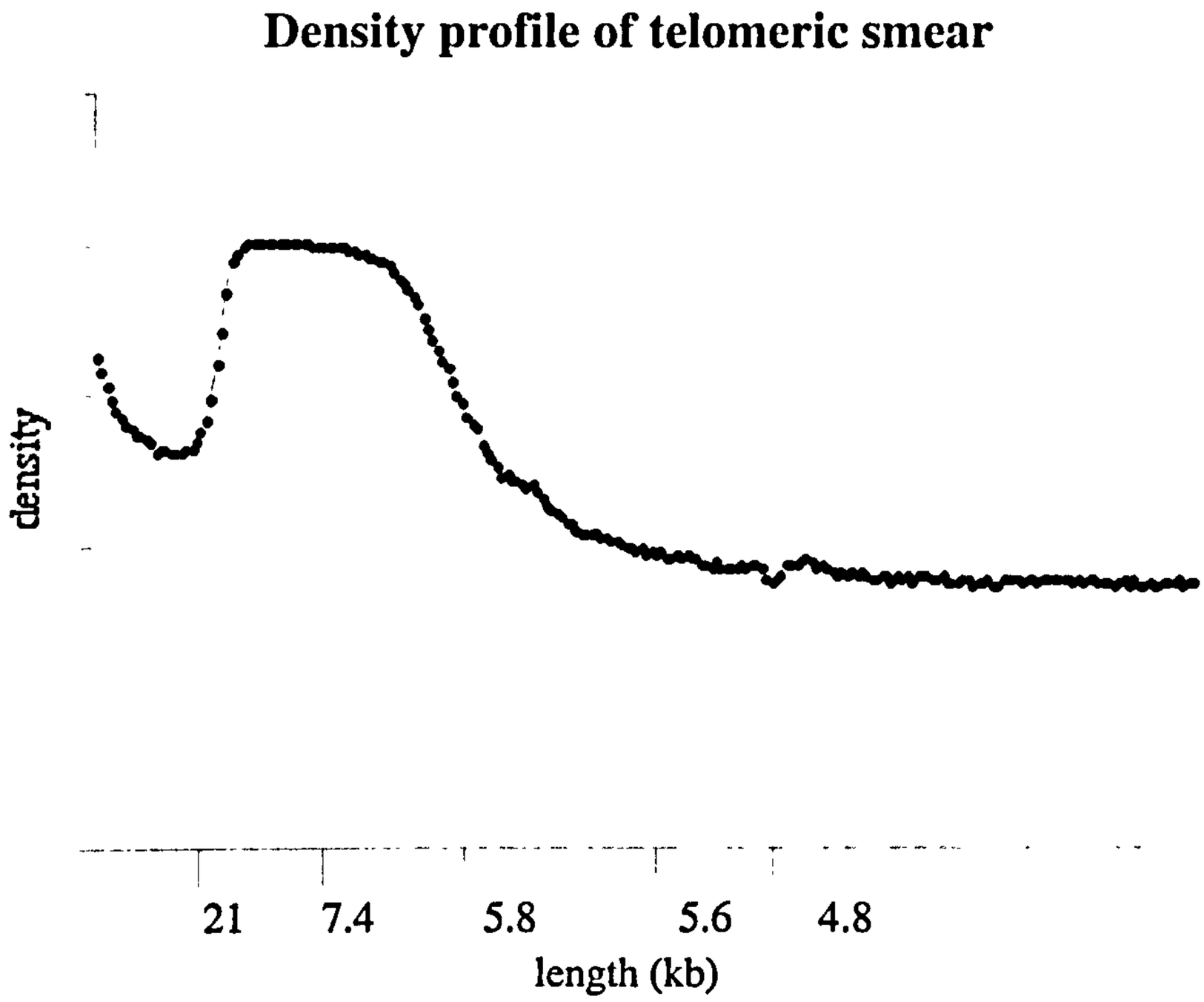


Figure 2.6: density profile of a telomeric smear (lane 1, Figure. 2.5) as generated from the scanned autoradiograph by NIH image software (NIH, V1.61, available at <http://rsb.info.nih.gov/nih-image/>).

The telomeric restriction fragments resulting from the G-overhang assay, continued to be obscured by DNA retained in and near the wells of the gel (see Figure 2.5). Assuming that this DNA is exclusively telomeric, a remaining fault could be that some of the telomeres are sticking together through interactions

between the guanine residues on the single stranded overhangs (Zahler *et al.*, 1991; Hud *et al.*, 1999; Parkinson *et al.*, 2002). This phenomenon is possibly aggravated by exposure to salt solutions and freeze/thaw cycles (Carolyn Price, pers. com.).

These potential difficulties were minimised and repeated sample measurements attempted using DNA extracts from Zebra finch liver. However, variations in the concentration of DNA between smears, within a gel, meant that producing a suitable image for densitometry and analysis was confounded by differences in autoradiograph signal intensity between individual samples. Variations in exposure time to optimise autoradiography of different lanes were insufficient to gather repeatable measurements. Smears of highly concentrated DNA were often over-exposed during autoradiography, so that the peak density of fragments was obscured (Figures 2.5 and 2.6). For many fainter smears, the background fogging of the film was too high to detect the peak density.

DNA quantification

A reliable method of standardising the DNA quantity was required, as well as ensuring that the DNA was of sufficient quality across samples, so that comparable telomere measurements might be made. The following methods of determining DNA concentration were explored:

1) Ethidium bromide staining: DNA intercalates with ethidium bromide and produces an increase in fluorescence of the dye, under UV. Ethidium bromide is commonly added to agarose gels and allows estimation of quantity and position of DNA (Sharp *et al.*, 1973). However, quantification of DNA in a gel requires internal DNA standards of known concentration to be run simultaneously. A high-

resolution photograph taken under UV light can then be taken to compare intensity of fluorescence between the samples and standard.

2) Spectrophotometry: DNA in solution absorbs UV light maximally at 260nm. The optical density (OD) at this wavelength can be measured in a spectrophotometer, with an OD of 1 corresponding to DNA concentration of 50µg/ml (Sambrook *et al.*, 1989).

3) Fluorometry: Hoechst 33258 dye binds to grooves in the DNA molecule to produce a highly fluorescent complex. Resulting fluorescence from a dilute solution of DNA can measured at an excitation wavelength of 360nm and an emission wavelength of 460nm (VersaFluor Fluorometer System, Biorad).

Of the above methods, fluorometry proved most effective for detecting differences in concentration between relatively small amounts of DNA. Ethidium bromide gel photographs were scanned to compare fluorescence intensity across a DNA dilution series using NIH-image software. However, the resolution was insufficient to provide more than a fair estimate of DNA quantity. A 5-50µl aliquot of DNA, diluted to 1ml for spectrophotometry, produced too low a signal to detect the small changes in optical density that occurred between samples, even though these variations in concentration produced significant changes with respect to autoradiography.

Problems continued in obtaining measurable telomeric smears from Zebra finch tissues, as a consequence of DNA retention in the wells. In addition, the hybridisation procedure with dried gels produced erratic results. Often, the background signal from the dried gel obscured any telomeric smears present. It is

possible that the radio-labelled probe or unincorporated γ^{32}P ATP is more easily absorbed into a gel than by the nylon membrane used in Southern Blotting. I attempted to purify the radio-labelled probe, by removal of unincorporated labelled nucleotides using MicroSpin™ G-25 Columns (Amersham-Pharmacia), with little success.

Refinements were also made to minimise background noise on autoradiographs. The addition of degraded Salmon sperm DNA to the hybridisation mixture serves to block non-complimentary sequences from the telomere probe. The viscosity of the hybridisation solution was also increased by the addition of Denhardts solution (Sigma-Aldrich). This should have increased the efficiency of the binding reaction.

The possibility remained that Zebra finches have extremely long telomeres, for example certain species of mice have telomeres which are up to 150kb long and cannot be measured using conventional electrophoresis (Starling *et al.*, 1990). However, the smears that I obtained from telomeric restriction fragment analysis of Zebra finch liver DNA, that seemed measurable, appear to have a mean length of approximately 10 kb (Figures 2.5 and 2.6). The chicken telomeres, measured by Venkatesan and Price (1998) have a similar size range to that of humans (10-15kb).

2.3 Discussion

Protocol

Progress towards solving problems with telomere measurement was slow. Potential solutions have taken a long time to test, the protocol taking between five and six days from start to finish. The failure to produce repeatable characteristic telomeric smears on the resulting autoradiographs cannot be attributed to a particular step in the protocol.

In summary, DNA samples may be damaged before or during preparation; however, this should have been avoided in experiments using fresh tissues. Protein contamination may also be ruled out through the use of purification columns. It remains possible that isolation of telomeric fragments might be prevented by the presence of internal telomeric sequences (although using the G-overhang assay should avoid this) and the telomere fragments, once produced, might stick together. To show that only the G-overhang is hybridised using the non-denaturing protocol, exonuclease control experiments could be carried out. However, the formation of stable telomere fragment aggregations in solution is difficult to prove.

Hybridisation of the telomere probe to G-overhangs was also not always successful, possibly because much less single stranded DNA is available to the probe than would occur with a denatured gel.

Correlation of blood cell telomere length with age

The possibility of using telomere length from blood samples of birds as a biomarker of senescence would be dependent upon the level of telomerase activity in the bone marrow. There is evidence to suggest that telomerase is active in

chicken embryos and to a lesser extent in two-year-old birds (Venkatesan and Price 1998). Telomerase detection involves preservation of fresh tissue at -70°C, so that the enzyme remains active, then subsequent preparation of a cell extract. This can be assayed using a PCR (polymerase chain reaction) based protocol, during which any active telomerase will extend artificial telomeric DNA fragments of predetermined length. Whether telomerase is active in juvenile and mature birds of other species remains to be seen. A telomerase repeat amplification protocol was attempted with extract from a zebra finch liver and no telomerase was detected (TRAPeze telomerase detection kit, Intergen). However, this may have been due to enzyme denaturation during the sample preparation.

Haematopoetic cells divide at regular intervals, to renew red and white blood cells, throughout the life-span of an individual. In humans, it has been suggested that telomerase activity would be necessary to provide sufficient replicative capacity and that stem cells do not undergo senescence (Iscove, 1997). Nevertheless, telomere shortening has been shown to occur in peripheral blood cells (Vaziri *et al*, 1994; Weng *et al.*, 1995 Iwama and Toyama 1998; Rufer *et al.*1998). The notion of limited replicative capacity in haematopoeitic cells is also supported by Lansdorp *et al.* (1997). It is calculated that one stem cell need only undergo 55 divisions in order to supply the entire 4×10^{16} cells required for lifetime turnover. Also, the study of bone marrow transplants has shown that the amount of telomere shortening in the haematopoetic cells of a recipient is inversely correlated with the number of donor cells infused. The rate of telomere decline, in the bone marrow, has been found to vary with age and is most rapid early in life (Notaro, 1997). Telomerase activity is relatively low in adult stem cells (Frenck *et al.*, 1998). Therefore, high telomerase activity and lack of replicative senescence in bone marrow need not be predicted. However, the Hayflick limit has recently

been disputed by Cristofalo *et al.* (1998) who found no connection between cell proliferative potential and human donor age.

Alternative mechanisms of telomere shortening may be of greater importance than that produced during cell division. Makarov *et al.* (1997) suggests a C-strand degradation mechanism to operate, resulting in long G-overhangs. Chronic to mild hyperoxia is also shown to accelerate shortening (von Zgliniki *et al.*, 1995), indicating that oxidative stress may play an important role. Single strand breaks have been found to be the major cause of telomere shortening by von Zgliniki *et al.* (2000) whilst Hamilton *et al.* (2001) show that oxidative damage to DNA occurs, with advancing age, in rats.

Given that telomere shortening occurs independently of cell division, it may be doubtful that telomere length is a reliable indicator of cellular senescence. The additional factors, mentioned above, may shorten telomeres irrespective of the age of the individual. Replicative senescence itself may also vary according to tissue type and growth period. It is possible that, even if they could be easily measured, avian telomeres might not give a good correlation of length with age.

Difficulties with the protocol, used in the present study, led to the abandonment of experimental attempts to measure avian telomeres. However, Haussmann and Vleck (2002) have recently published a method for measuring telomeres from zebra finch blood samples. The method used is similar to the non-denatured gel hybridisation protocol that I used. Differences include the use of restriction enzymes *HaeIII, HinfI,* and *MspI,* and the gel was dried for only 30 minutes at room temperature. From a sample of 27 birds, ranging in age from 4 to more than 18 months old, the authors were able to correlate telomere length with age of the individual. Mean telomere length ranged from approximately 8.7kb in the youngest individuals (< 4 months) to 7.9 kb in old birds (>18 months). This

length variation seems narrow; however, the ages of the zebra finches sampled ranged over only half of the species natural life span. The telomere length values are similar to my estimated value of 10kb from zebra finch tissues (Figures, 2.5 and 2.6) -although this was not measured precisely (due to problems with autoradiography exposure) and cannot be seen as accurate because repeatable measurements were not made.

The measurement of telomeres might be better applied as an indicator of the residual longevity of an organism e.g. Life-span in rats is related to maternal nutrition, with undernourished mothers producing offspring with a lower life expectancy (Desai and Hales, 1997). It is possible that catch-up growth, occurring under these circumstances, results in accelerated telomere shortening and early senescence.

Another interesting avenue of further research might be based on Rhome's (1981) description of variation in the Hayflick limit, across mammalian species. Birds provide a selection of species, with large differences in expected life-span. Comparative study of telomere dynamics in birds would predict that short-lived groups, such as small passerines, would show higher rates of attrition than long-lived species. Alternatively, telomerase activity may vary, such that short-lived birds have active telomerase (analogous to the situation in mice) whilst longer-lived species might suppress telomerase, in a similar way to humans.

The potential avenues for telomere research in ecological or evolutionary studies are interesting. Nevertheless, in order to be used as an indicator of ageing in birds, telomere restriction fragment length analysis, would seem to require fairly large, high quality genomic DNA samples. The protocol is also fairly time-consuming and costly - requiring the use of radioactive substances and specialised equipment, so that it may not be of extensive practical use to field biology studies.

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Chapter 3

Variation of Offspring Sex Ratio in the Herring Gull, *Larus argentatus*: The Effects of Season and Hatching Sequence.

3.1 Introduction

Sex allocation theory, according to Trivers and Willard (1973), predicts that where species show extensive parental investment, the sex and number of offspring produced will be influenced by the phenotypic quality of the mother. Natural selection should favour a biased sex ratio when there is a difference between the cost and benefit of producing males and females, such that a female in poor condition is likely to overproduce the cheaper sex so that offspring quality and survival is maximised.

Experimental induction of extended clutch size in Lesser black-backed gulls, *Larus fuscus* (Nager *et al.*, 1999) have shown that reproducing females, under nutritional stress, lay an increasing number of female eggs. This is thought to be an adaptation to increase reproductive success. Male gull chicks, being skeletally larger but not heavier at hatching, carry less reserves upon hatching and suffer reduced survival (Griffiths, 1992).

It remains to be seen whether a sex ratio biasing mechanism operates widely in wild bird populations in response to maternal quality. This might be expected for synchronously breeding species where there are limits upon time and resources available for reproduction.

Offspring sex ratio was examined in a population of Herring gulls, *Larus argentatus*, on Walney Island, Cumbria throughout a single breeding season. Blood sampling of chicks immediately after hatching and sexing by DNA analysis allowed the primary sex ratio of broods to be established (without confounding effects of post-hatch mortality). Egg size and laying date are good indicators of maternal quality in gulls (Parsons, 1970; Perrins, 1970; Nisbet and Drury, 1972). Individuals that are in better condition lay heavier eggs and breed earlier so that, as the breeding season progresses, a larger number of female offspring would be

expected to be recorded. Also, the chicks from the last laid eggs of each clutch (the smallest in a clutch of three) are expected to have a greater likelihood of being female than larger siblings are.

Sex allocation

Fisher (1958) states that frequency-dependent selection stabilises the sex ratio near unity, assuming a linear net relationship between reproductive investment and return. Charnov (1982) suggests that changes in investment may not be proportional to changes in returns; if marginal returns (in terms of fecundity) vary with the sex of the offspring then investment in males and females may not be equal. Frank (1990) gives a comprehensive review of sex allocation theory.

Avian sex ratio variation

Male birds are Homogametic, with two sex chromosomes (ZZ), whilst females are heterogametic (WZ), producing eggs with Z or W sex chromosomes, so in theory could control the offspring sex. Mechanisms of vertebrate sex ratio manipulation have not yet been demonstrated. Female birds could potentially alter the primary sex ratio (sex ratio of eggs laid) at any point between the production of W or Z-chromosome bearing gametes and the shell formation of fertilised eggs (Krackow, 1999). Variation in the primary sex ratio can be measured at hatching provided that all eggs in the clutch can be sexed, avoiding any possible bias in embryonic mortality. Differential post-hatching mortality may also occur leading to variation in the secondary sex ratio at fledging.

Primary sex ratio manipulation has been shown to occur with hatching order within clutches of eggs in polygynous House wrens, *Troglodytes aedon*,

(Albrecht, 2000). The last hatched chick, which receives less parental care, is more likely to be a female than a male (the latter sex facing greater disadvantage, in terms of mate competition, if last hatched). The reverse is found in Bald eagle (*Haliaeetus leucocephalus*) clutches where the larger, more expensive, female offspring are more often first hatched (Bortolotti, 1986). A similar trend is found in Zebra finches (*Taeniopygia guttata*) where female fecundity benefits are relatively greater when fledging weight is high. Female eggs are therefore more likely to be laid earlier in the clutch (Kilner, 1998).

Hatch order effects, under experimental manipulation, have also been recently demonstrated in a closely related species to gulls, the Great skua (*Catharacta skua*). Using similar methods of egg-removal and induction of extended laying to Nager *et al* 's (1999) gull study, Kalmbach *et al.* (2001) measured the primary sex ratio of extended skua clutches. Sexual size dimorphism in the skua contrasts that of gulls, with females being the larger and more vulnerable sex. Later hatched chicks, of nutritionally stressed mothers, were more likely to be male (Kalmbach *et al.*, 2001) reinforcing the hypothesis that differences in body mass between the sexes result in selection pressure for sex ratio manipulation.

It has also been suggested that the female may bias the sex ratio produced in response to paternal quality. Burley (1986) manipulated the attractiveness of Zebra finch mates using coloured leg bands. Red-banded males, that were shown to be most attractive, fathered male-biased clutches. However, this variation became established post-hatching and a parental role in sex-biased mortality is inferred (via the possibility of selective feeding or eviction from the nest box), rather than a skewed primary (ovulatory) sex ratio. However, the primary sex ratio does seem to be manipulated in response to paternal attractiveness in

Collared flycatchers (*Ficedula albicollis*). Male-biased broods were produced when males with a large forehead patch, a character that attracts females, fathered them (Ellegren *et al.*1996). Further study of this species includes the determination of sex of extra-pair offspring. It might be expected that females seeking additional mates of good quality would, accordingly, invest the genes in male offspring. However, the sex of extra-pair chicks was found to be randomly determined (Sheldon and Ellegren, 1996).

Polygynous males of the Great reed warbler *Acarocephalus arundinaceus,* (Westerdahl *et al.*, 1997; Nishumi, 1998) provide parental care to the offspring of primary females. Secondary females receive less paternal investment. Sons are larger and more expensive to raise and so the sex ratio in primary nests is often skewed towards males.

A striking sex ratio bias is found with territory quality in Seychelles warblers *Acarocephalus seychellensis* (Komdeur *et al.*, 1997). Pairs nesting in good territories overproduce daughters that remain with the parents to assist future breeding attempts. The offspring of parents at poor quality sites produce more sons that disperse from the natal territory, thus avoiding competition for food. The Green woodhoopoe (*Phoeniculus purpureus*) is also a co-operatively breeding species. Again, the female offspring are altruistic and are also smaller and less costly to rear. Females are therefore more likely to be produced by pairs with small groups (Ligon and Ligon, 1990).

Seasonal adjustment of sex ratio is suggested to occur in the Common grackle, *Quiscalus quiscula* (Howe, 1977). The chicks are sexually size dimorphic, with males being larger and suffering from increased mortality. Early in the season, when conditions are poorer, greater proportions of the offspring produced are female. The opposite situation occurs in raptors, where females tend

to be the larger sex. Peregrine falcons that nest early (*Falco peregrinus*) are more successful and produce a female-biased brood. Earlier eggs in the clutch are also more likely to be female (Olsen and Cockburn, 1991).

Laying date may affect the reproductive status of the offspring the following season. The probability of female recruitment in the Spotless starling, *Sturnus unicolour,* is increased with early maturation and a shift in the sex ratio from daughters to sons is found as the season advances (Cordero *et al.*, 2001). Daan *et al.*, (1996) produced a simulation model based on the premise that genders whose maturation time is reduced by early birth date should be produced first. This is supported by empirical data from five raptor species.

To summarise, offspring sex ratio has been shown to vary within clutches, in response to parental quality, paternal investment and resource abundance. There are also examples of seasonal sex biases. Although these examples may appear strategic, in terms of increased offspring survival or future reproductive success, interpretation of sex ratio variation as adaptive relies on the assumption that active parental manipulation is taking place.

Analyses of bird species, that exhibit sexual dimorphism of body mass, has shown that the sex ratio often becomes biased between hatching and fledging due to differential survival of chicks. The larger sex is assumed to be more susceptible to starvation. Higher male mortality occurs in Rooks, *Corvus frugilegis,* (Slagsvold *et al.*, 1986) and Lesser black-backed gulls, *Larus fuscus,* (Griffiths, 1992) – both species with males larger than females. Sexual dimorphism of body mass is reversed in the Blue footed booby, *Sula nebouxii,* and female offspring suffer greater mortality, a risk that is increased for last hatched chicks (Torres and Drummond, 1997).

Nevertheless, many studies fail to find an expected offspring gender bias. Examples include Koenig and Dickinson's (1996) extensive study of Western bluebirds (*Sialia mexicana*), the measurement of paternal quality and offspring sex ratio in the Barn swallow, *Hirundo rustica*, (Saino *et al.*, 1999) and the investigation of differences within clutches and across the breeding season of the European sparrowhawk, *Accipiter nisus* (Newton and Marquiss, 1979). Trends in sex ratio variation found in the Great tit (*Parus major*) were inconsistent between years (Radford and Blakey, 2000) whilst Hartley *et al.* (1999) found no effect of timing of breeding, year, polygyny, brood size or female size upon the offspring sex of Corn buntings, *Miliaria calandra*. In addition, a recent study by Leech *et al.* (2001) found no effect of parental quality or extra pair paternity upon offspring sex ratio in a population of Blue tits, *Parus caeruleus*. The latter analysis examined 1483 chicks in 154 broods and the authors suggest that offspring sex determination, at the level of the individual egg, may not be easily controlled.

The following study aims to show whether or not Herring gulls carry out adaptive sex ratio manipulation, under natural conditions. A seasonal trend is expected, whereby poorer quality parents that nest later in the breeding season are likely to overproduce the cheaper sex (females). The effect of laying/ hatching order on sex ratio will also be studied, under the hypothesis that chicks hatched from the third (smallest) egg of the clutch will be more likely to be female.

3.2 Methods

Blood was sampled from Herring gull chicks, during the breeding season, from the 23rd of May until the 11th of June (2000) at Walney Island Nature Reserve, Cumbria, UK. The breeding colony was mainly composed of breeding Lesser black-backed gulls and Herring gull nests were identified amongst these by the presence of at least one brooding parent. Nests containing three eggs (the most common clutch size) were selected for sampling when the chicks were beginning to hatch. These nests were assumed to belong to parents who were incubating their first clutch of eggs of the breeding season. It was also assumed that each clutch of three represented A, B and C eggs laid upon consecutive days, without replacement, and that the hatching sequence would match this laying sequence.

Blood collection took place outwith the public access hours of the reserve and the nests used in the study were often situated near footpaths. This restriction was necessary, in part, to avoid disturbing areas that were being used for other studies. Certain areas were also avoided if nesting Eider ducks, *Somateria mollissima* were present. Many Herring gull nests were sampled from colony patches where Herring gull nests were clustered outwith larger areas of Lesser-black-backed gull predominance. These factors may have resulted in non-random sampling of clutches with a possible bias towards nests that were most conspicuous and those subjected to higher levels of disturbance by human visitors. In addition, at the early and late extremes of the hatching period, the encounter rate with nests that contained hatching chicks was low and the sampling area became slightly more wide-ranging.

Blood was collected from chicks with dry feathers within 12 hours of hatching. A sample was obtained by puncturing the leg vein with a sterile needle and approximately 0.1ml of blood was collected from each chick using a glass

capillary tube. Blood samples were dispensed into prepared 1.5 ml plastic tubes containing 1ml pure ethanol and stored at -20°C within four hours of collection.

Following blood collection from the first (A) hatchling, nests were discreetly marked so that the remaining chicks in a clutch could be sampled later. The second egg (B) generally hatched within one day of the first (A) and the third egg (C) two days after the first. A and B chicks could not always be distinguished from each other, whilst the identity of the later hatching, smaller, C-chick was more obvious. Thus, for the majority of samples a conservative assignment of "A/B" or "C" was made for each chick within a nest.

It is presumed that all of the chicks sampled from a clutch, at hatching, shared the same parents. This assumption is based upon evidence that the frequency and success of extra-pair copulation in gulls is relatively low. A taxonomic review of cuckoldry in birds by Møller and Birkhead (1993) list the frequency of extra-pair copulation in the Lesser black-backed gull (Brown, 1967) and Herring gull (Fitch and Shugart, 1984) as zero. This is in common with most colonial nesting seabirds, which exhibit a high degree of monogamy. In addition, Gilbert *et al.* (1998) found that although extra-pair copulation occurred in Western gulls (*Larus occidentalis*), genetic fingerprinting of broods revealed no extra-pair paternity.

DNA extraction - Chelex protocol

PCR based sex determination requires only a small amount of genomic DNA, of which a fraction is subsequently amplified. For this purpose, extraction of DNA from bird blood was carried out using Chelex100 resin (Biorad). This polymer resin contains chelating groups that sequester metal ions, however, its exact role in DNA purification is not entirely clear. Chelex suspension is incubated with homogenised tissue, at a high temperature, which is postulated to release DNA from cells. It is assumed that Chelex has a protective role in removing metal ions that would otherwise cause DNA damage, at high temperature, and inhibit subsequent PCR (Walsh *et al.*, 1991).

1) A small piece of (coagulated) blood, around $1mm^3$, was mixed in a 2.5ml eppendorf with 200µl, 5%, Chelex 100 resin (Biorad, UK) and placed on an orbital incubator for 20 minutes at 55 °C.

2) Samples were vortexed for a further 10 seconds then the lids of the tubes were pierced and incubated at 90 °C for 10 minutes.

3) Samples were vortexed for 10 seconds and centrifuged for 3 minutes at 18 000 × *g*. The supernatant was removed with a pipette into collection tubes and stored at -20 °C until use.

Sex determination by PCR

Reactions were carried out in a total volume of 10µl under the conditions listed overleaf. The small volumes listed were measured more accurately (and to save time) by multiplying all quantities (excluding DNA) by the number of DNA

samples used in the PCR (e.g. 96) and preparing a "mastermix". A 9µl aliquot of this mixture was then be added to each 1µl DNA sample.

primers P2/P8 (0.1 mg/ml)	0.8 µl
dNTP (10mM)	0.8 µl
10 × PCR reaction buffer (Promega)	1.0 µl
$MgCl_2$ (25mM)	1.0 µl
dH_2O	3.725 µl
Taq (5 units/µl, Promega)	0.075 µl
DNA	1.0 µl

The reaction conditions were: 2 minutes at 94°C, 30 cycles of 1 minute at 46°C, 1 minute, 30 seconds at 72°C and 45 seconds at 94°C followed by 1 minute at 46°C and five minutes at 72°C. Primers were used as described in Griffiths *et al.*, (1998):

P8 (5'-CTCCCAAGGATGAGRAAYTG-3')

P2 (5'-TCTGCATCGCTAAATCCTTT-3')

A negative control was included from each batch of extracted DNA (containing no sample, to check for contamination). In each PCR experiment, a positive control of known sex male and female DNA was included as well as a PCR negative control containing no DNA sample. PCR products were loaded with 10 × Orange G loading dye into 10cm 3% agarose gels (stained with ethidium bromide) with a 4µ1 aliquot of 1kb DNA ladder (Promega) included in each row

of lanes, as a scale. Gels were run in 0.5 × TBE buffer at 120V for 20-30 minutes (Figure 3.1).

The primers used amplify the CHD (chromobox-helicase-DNA-binding) gene from avian W and Z chromosomes. The CHD genes of birds show sequence similarity to the CHD 1 gene in mice. However, unlike mice, birds possess two sex-linked homologues of the gene, CHD-W and CHD-Z (Griffiths and Korn, 1997). CHD proteins are thought to modify chromosome structure, thus controlling access of transcriptional apparatus to the DNA template (Woodage *et al.*, 1997). The CHD gene is, therefore, highly conserved and can be used to sex a wide range of bird species (Griffiths *et al.*, 1998). In most cases, including that of the Herring gull, the PCR product from the CHD-W gene is larger than that of the CHD-Z gene. Thus, DNA from females (WZ) will yield two differently sized PCR products whilst that from males (ZZ) will produce a single product.

A photograph of an agarose gel, used to analyse the products of a Herring gull sexing PCR is shown in Figure 3.1. The lower band, seen in both the male and female PCR products, at around 360 bases long, is a section of the CHD - Z gene. This band is present in both sexes and acts an internal control, its presence indicating that the PCR reaction has been successful. The upper band of around 400 base pairs in length, is a fragment of the CHD-W gene. This product is only obtained from females and so acts as a sexing marker.

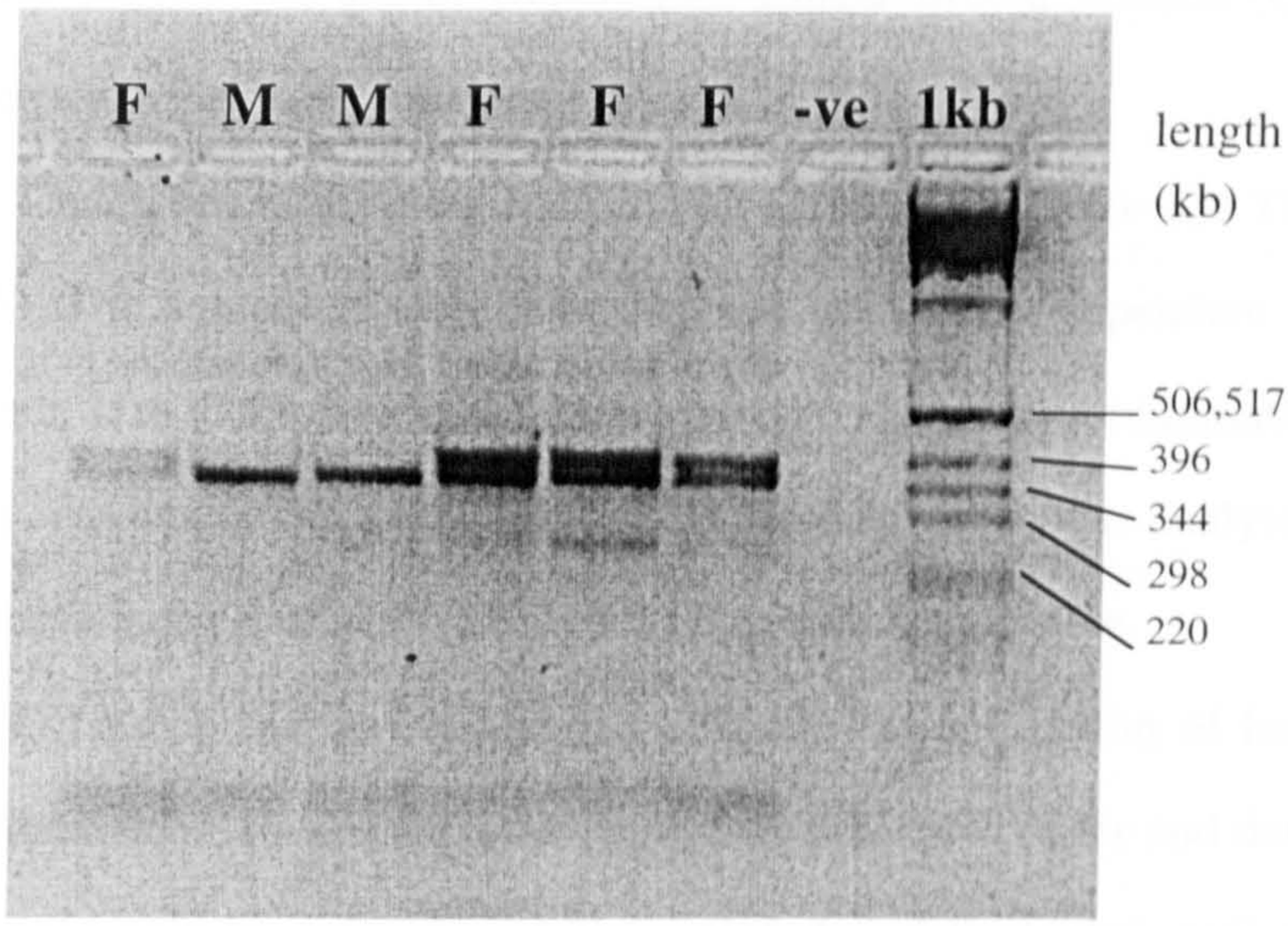

Figure 3.1: photograph of an agarose gel with PCR products from two male (M) and four female (F) Herring gulls. The right hand lanes contain the PCR negative control and a 1kb DNA ladder.

Statistical analysis

The number of male and female offspring was found for 174 complete 3-egg clutches. Clutch sex ratios were expressed as the proportion of males in each clutch and the mean hatching date for chicks within each clutch was calculated, to the nearest day. Since the proportion of males hatched lies between 0 and 1, Generalised Linear Modelling (GLM) was used to examine the binomially distributed data. However, standard GLM includes one error term, whilst the nested structure of brood data contains two sources of random variation - that within and between clutches. The sex of each individual chick does not represent an independent observation, as chicks within a brood may be interconnected by maternal identity. Thus, treating the sex of each chick as a single datum results in pseudoreplication (Hurlebert, 1984).

Krackow and Tkadlec (2001) addressed the problem of clustered brood data and suggest the use of Generalised Linear Mixed Models (GLMMs) that include random effects of maternal identity in the analysis.

Clutch sex ratios were analysed by GLMM (Krackow and Tkadlec, 2001) in SAS. The number of male offspring was used as the dependent variable with the clutch size (3 in each case) entered as the binomial denominator. Maternal identity was entered as a random factor. Clutch sex ratio was analysed in relation to the mean hatch date of each brood, as a continuous variable.

Offspring sex was analysed by GLMM as a function of hatching order individual hatch date and the interaction between hatch order and date. Hatching order could only be defined as A/B (pooled A and B chicks) or C as the laying order of the first two eggs could not be inferred from hatching order (often synchronous). Five clutches were excluded from the analysis of hatching order where the identity of the C chick had also been ambiguous. Maternal identity was entered as a random factor. Non-significant terms were removed from the model in a step-wise fashion, starting with non-significant interactions.

The data was also used to examine the allocation of sex within clutches. The number of males and females in each clutch is expected to follow a binomial distribution. For this analysis, a goodness-of-fit test was used to compare the number of three egg clutches falling into the different categories (3 male; 2 males + 1 female; 2 females + 1 male; or 3 females) to the number of broods expected in each category if sex allocation occurs by chance.

3.3 Results

There was a significant effect of mean hatch date upon clutch sex ratio ($F_{1,172} = 3.91$; $p = 0.0497$) when date was entered as a continuous variable. This seems to be a result of an increase in the number of males hatching towards the end of the breeding season (Figure 3.2).

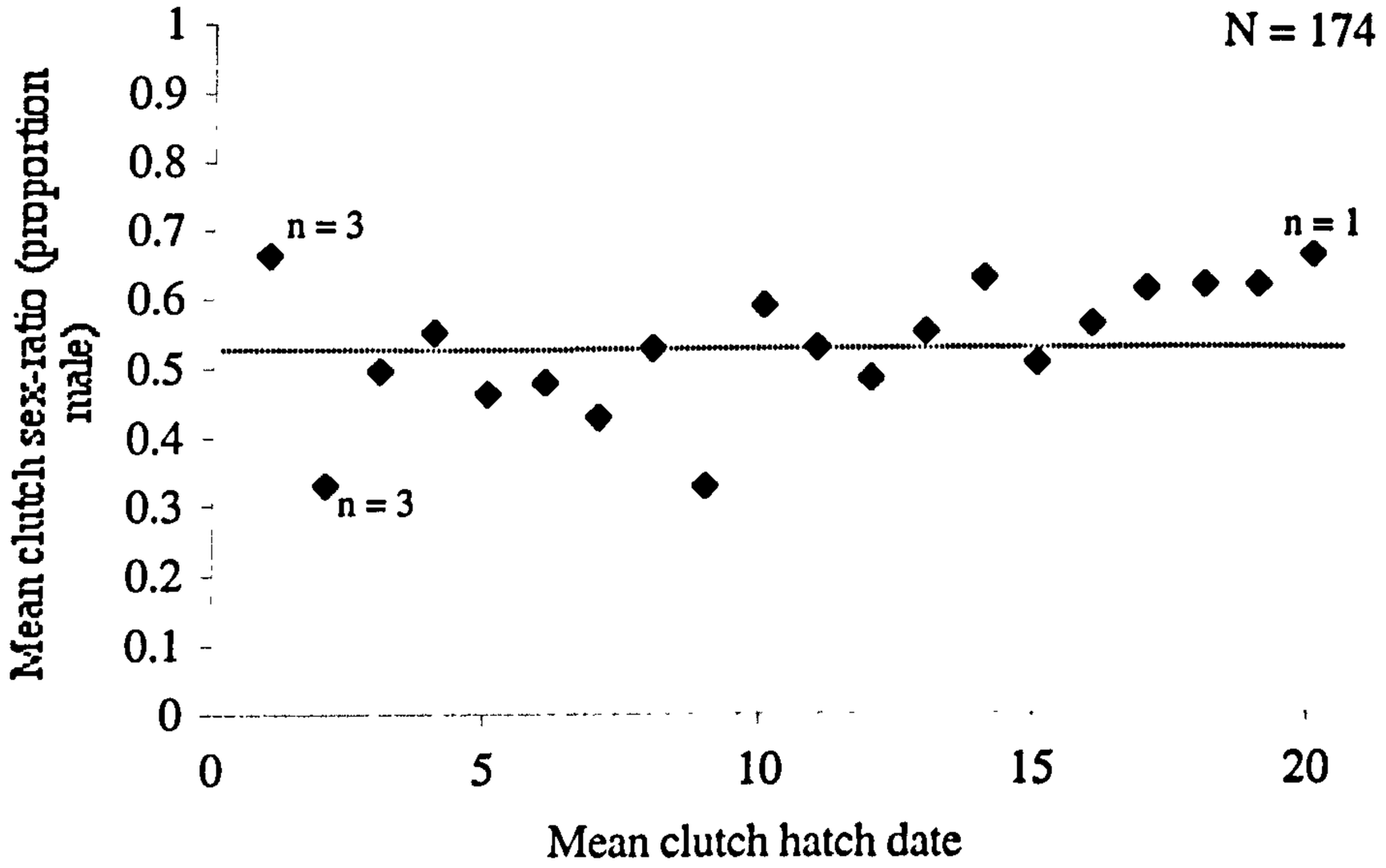

Figure 3.2: Mean Herring gull clutch sex ratio variation with mean clutch hatch date (23rd May to 11th June numbered as days 1 to 20). The dotted line indicates the mean, overall, sex ratio (0.52). Mean daily clutch sex ratios have been calculated from 174 complete clutches. The number of clutches (sample size) for each day varies ($\bar{x} = 8.65$; *s.d.* = 4.43; max. = 20; min. = 1). Mean clutch sex ratios calculated from a sample size of: $n < 5$ are annotated.

When offspring sex was analysed at the individual level as a function of hatching order and date, there was no significant interaction between sex and hatching order (Figure 3.3). Hatching order was removed from the model, leaving a significant effect of date upon the offspring sex ($F_{1,180} = 5.03$ $p = 0.0262$).

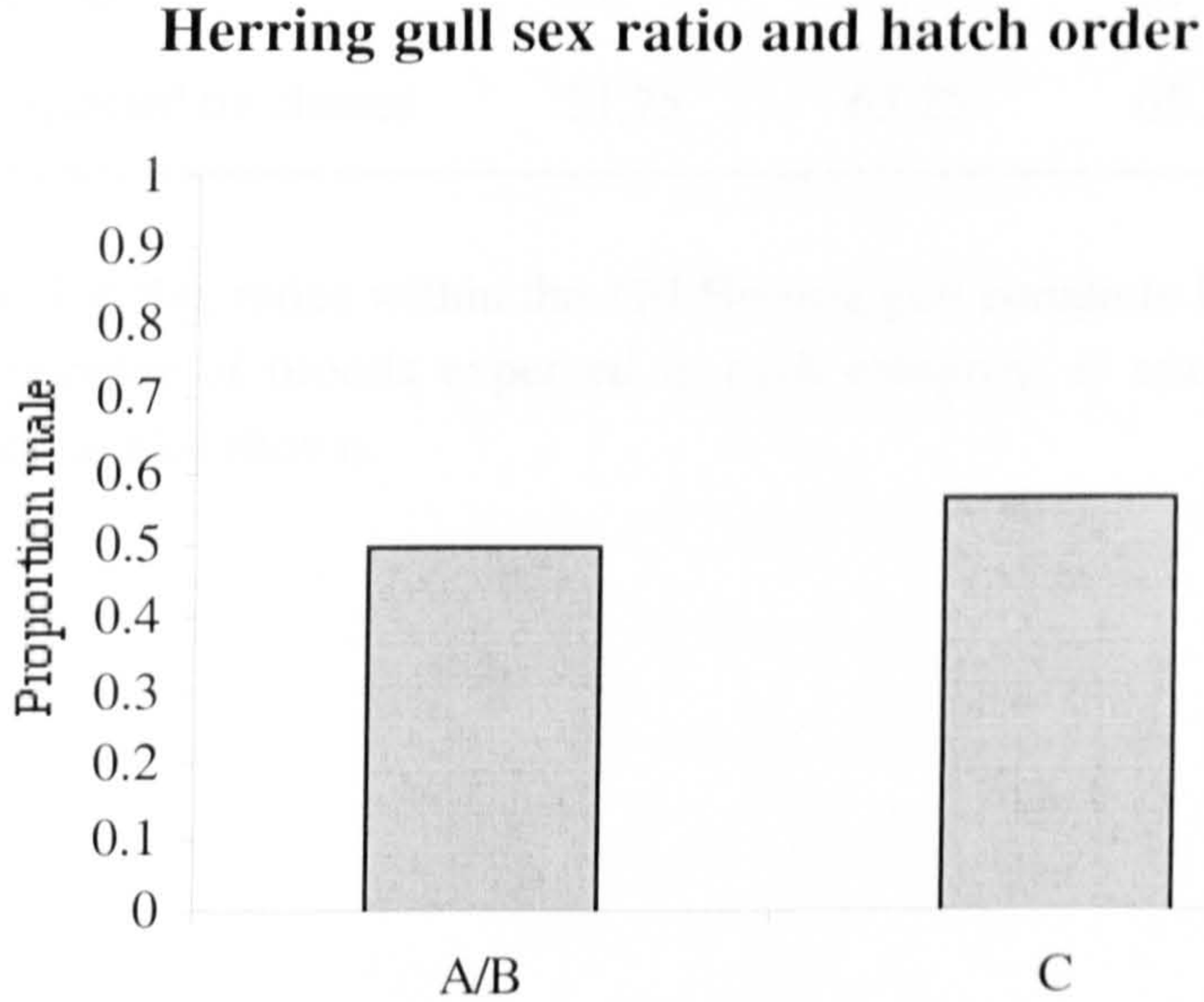

Figure 3.3: Proportion of males hatched from A or B eggs (first or second in laying sequence) and C eggs (third laid). Proportions are calculated from chicks (n = 507) that could be assigned a hatching order from complete three-egg clutches (n=169).

The number of clutches containing different possible sex ratio combinations (Table 3.4) was compared to that expected in each category, should allocation occur by chance, using a chi-squared goodness-of-fit test. The actual numbers of broods in each category did not differ significantly from those

expected, under random allocation of sex within broods ($x^2 = 5.37$; d.f. = 3; $0.2 > p > 0.1$).

Allocation of sex within Herring gull broods

	3 males	2 males + 1 female	2 females + 1 male	3 females
Actual no.	31	57	67	19
No. expected by chance	21.75	65.25	65.25	21.75

Table 3.4: Sex ratios within the 174 Herring gull complete broods of three chicks. The number of broods expected in each category, if sex allocation occurs by chance, is also shown.

3.4 Discussion

Seasonal variation of offspring sex ratio in the Herring gull

The finding of the present study, that an increase in male Herring gull chicks hatching occurs towards the end of the season, contradicts the predicted seasonal change in sex ratio. Hatching and fledging success, egg size and maternal age in gulls are presumed to decrease seasonally, so that those individuals nesting later are expected to overproduce the cheaper sex (females).

Nisbet and Drury (1972) found that early-hatched Herring gull chicks showed lower post-fledging mortality than those hatching later in season. Brown (1967) and Parsons (1975a) found similar trends, although both studies showed an additional increase in mortality of very early clutches. In contrast, a study by Harris (1969) found that the breeding success of a colony of Herring gulls increased towards the end of the season. On the balance of this information, it seems possible that producing males at the beginning of the breeding season could also be counterproductive.

Parsons (1975a) experimentally extended the laying season, in Herring gulls by egg-removal. Chicks hatching from the repeat clutches showed a similar level of success, at the peak of delayed laying to those peaking earlier, in the control groups. He, therefore, suggests that hatching success is correlated with nest synchronisation, irrespective of laying date. Should this be the case, then overproduction of males would seem most likely to occur during the middle of the season. The data from Walney does not support this idea. However, the degree of nest synchronisation at the colony, during the latter study, was not known.

Chick survival also is related to egg size. Parsons (1970) found a positive relationship between egg size and the weight and lipid content of Herring gull chicks at hatching which, in turn, correlated negatively with post-hatching

mortality. Parsons (1972) shows that egg size, in the Herring gull, decreases through the breeding season. A seasonal shortening in the incubation period was also apparent and it is suggested that the decreased incubation time relates to egg size. This agrees with observations of the same species by MacRoberts and MacRoberts (1972), who attribute a seasonal decline in incubation time to changes in adult incubation behaviour (decreased attentiveness towards the end of the season) and an increase in temperature during the summer. Ideally, egg size, incubation period and chick survival should have been recorded, in the present study, and may have provided insight in to the unexpected variation in sex ratio observed.

Davis (1975) investigated the relationship between clutch volume, female age and laying date in Herring gulls. Clutch volume was estimated from egg length and breadth measurements, using calculations described by Harris (1964). The study by Davis found that females advanced their laying date with age, up to nine years, and that clutch volume showed a similar increase with age, up to seven or eight years, before declining. During three years of data collection, only one season showed a decline in clutch volume with laying date and another a trend towards increased survival with egg size and hatching weight. In two of the years of study, later breeders were least successful. The author suggests that changes between years may be due to differences in the age structure of the population, and that the correlation between egg-size and chick survival is a result of an excess of young breeders, that are less experienced in foraging and parental care, laying small eggs. Information about maternal age, for the clutches sampled on Walney, would also have been beneficial to the analysis of sex ratio. However, sampling data, from a wide age range of individuals requires extensive longitudinal study.

Nevertheless, it might be possible, in future, to utilise telomere length, as a correlate of ageing in birds (see Chapter 2).

A study of Glaucous winged gulls, *Larus glaucescens*, by Hunt and Hunt (1976) also showed inconsistent effects of hatching date upon breeding success. In a year of low food availability, chicks hatching early and on large territories were more likely to survive to fledging. Two years later, when food availability at the colony was higher, there was no effect of timing of breeding and territory size upon chick mortality. The authors devised models that predicted early hatching to be most beneficial when intra-specific cannibalism (driven by low food availability) is the predominant cause of chick mortality. Chicks hatching during the middle of season are favoured when predation outweighs the risk of interference by neighbours. It is possible that, in the colony on Walney, food availability was high, and/or the risk of chick loss to cannibalism was lowest at the end of the season so that late nesting parents were at a breeding advantage that allowed the overproduction of males.

With regard to the hatching sex ratio in gulls, a significant seasonal bias in hatching sex ratio has not been observed, until now. Sayce and Hunt (1987) found no seasonal effect upon the number of males and females hatching at a colony of Western gulls. The sample size of their study is large (739 chicks); However, the analysis of the data is not controlled for the clustering of individuals within broods and the effect of maternal identity. Sayce and Hunt (1987) also report that male mortality, between hatching and fledging, seemed to be greater but the bias found was not significant. It is suggested that post-fledging differences in mortality account for a skew towards females found in the adult population. In addition, Griffiths (1992) found no seasonal trend in the allocation of sex within broods of the Lesser black-backed gull, *Larus fuscus*. Although egg volume declined

seasonally, there was no difference in egg volume or its seasonal decline between the sexes. Again, males showed an increase in post-hatching mortality, this time significant.

The experimental study by Nager *et al.* (1999) provides strong evidence that sex ratio manipulation can occur in gull populations. Females were induced to lay additional eggs by removal, a treatment that decreases female condition and thereby her ability to produce high quality eggs, so that male offspring become less viable to produce. Survival to fledging was reduced in males, but not females, that came from less well-provisioned eggs. With increasing number of eggs laid, egg mass and chick survival decreased and the sex ratio at hatching became significantly female-biased, reaching 75% at the last chicks (in a clutch of twelve to thirteen).

Male gull chicks appear more expensive to produce, in terms of food requirements and survival risk, and it seems that sex ratio manipulation by gulls is possible. Nevertheless, studies of seasonal breeding success of gulls give inconsistent results and we do not know what the seasonal survival prospects of male and female Herring gull chicks were, during the period of study on Walney. Dr Nanette Verboven (University of Glasgow) collected survival data from breeding Lesser black-backed gulls, during the same season, on Walney. Although her study involved experimental treatment, the observations made on control chicks (that had been cross-fostered but were otherwise unmanipulated) may be relevant. There was no seasonal effect upon survival, from hatching to fledging, of control Lesser black-backed gull chicks and no difference in survival between the sexes. Herring gulls are closely related to Lesser black-backed gulls, so it could be assumed that laying date was not an important component of offspring survival or differential mortality during the breeding season studied.

However, the sample sizes of Lesser black-backed gulls are rather low (17 males and 20 females) and the analysis was not controlled for laying order and nest of origin.

In summary, the sex ratio data for Herring gulls collected here, contradict the expectation that males become less likely to survive with advancing laying date and the subsequent prediction that poor quality parents, laying later, are more likely to overproduce females. Thus, the assumptions that parental quality and the likelihood of male survival decrease with laying date could be flawed. Obvious improvements could be made to the interpretation of the results, if measurements of parental quality, such as mean clutch mass and data on chick survival had been collected.

It would also have been interesting to examine the effect of egg size upon offspring survival and the possible correlates of egg size, considered to reflect maternal quality, such as age and breeding experience, female mass and body condition (see references in Risch and Rohwer, 2000). It should be considered that males also invest significant time and energy in reproduction, sacrificing energy reserves stored as fat before and during the laying period through courtship feeding, mate guarding and territorial behaviour (Hario *et al.*, 1991). Thus, male condition may also have a significant effect upon offspring quality and survival.

Unfortunately, additional information was not collected, owing to time constraints, although data from Lesser black-backed gulls collected at the same period did not reveal any seasonal effect upon offspring sex and survival. Variation, in seasonal effects upon gull breeding success is suggested to relate to food availability (Hunt and Hunt, 1976) and population age structure (Davis, 1975) and it is possible that these factors contributed to unexpected trend observed during the present study.

Hatch order and sex ratio in the Herring gull

The Herring gull is typical of many gull species in that the third egg (C) of a three egg clutch is smaller than the first two (A and B). Nager *et al.* (2000) demonstrate the effects of egg sequence and quality in gulls upon offspring survival. By using extended laying sequences and cross-fostering in Lesser black-backed gulls the potentially confounding effects of parental quality and decreased parental condition (induced by additional laying) were removed. The hatching sex ratio was biased towards females with increasing position in an extended laying sequence and there was an accompanying decline in chick survival . The later eggs in extended clutches were not significantly different in fresh mass from normal C eggs but differed in composition - containing relatively less lipid and more water. The hypothesis that a reproductive trade-off occurs between egg number and quality was supported, with clutch size being constrained by the ability of parents to produce good quality offspring in terms of chick survival. Under natural conditions, it is expected that C chicks are less likely to be male and also that all male clutches should be avoided by poor quality gull parents.

Parsons (1970) showed that although the hatching success of Herring gull eggs in a clutch of three did not vary, there was a significantly greater decrease in post-hatching survival of last hatched chicks, than would be predicted from egg volume effects alone. In an egg transfer experiment, Parsons (1975b) interchanged A and C eggs that were beginning to hatch, between nests. C-chicks that hatched first in a clutch showed increased survival compared to C-chicks hatching in their normal position. Nevertheless, the survival of C-chicks hatching first was still lower than that of its siblings and A-chicks moved to third in a hatching sequence survived better than a C-chick in the same position. Therefore, some of the differential mortality can still be attributed to egg volume effects.

It seems clear that C-chicks have an increased mortality rate, which would be exacerbated in male chicks that carry fewer reserves on hatching and have a higher growth rate. However, there was no evidence in the present study to suggest that C-chicks were more likely to be female. These findings agree with those from Herring gulls (Ryder and Termat, 1987) and Western gulls (Sayce and Hunt, 1987). Neither study found an effect of hatching order upon sex ratio at hatching, nor did Griffiths (1992), studying Lesser black-backed gull sex ratios, although there was an increase in post-hatching mortality with hatch-order. In a later study of Lesser black-backed gulls, Bradbury and Griffiths (1999) created experimental synchronous and asynchronous broods by grouping chicks that were beginning to hatch in clutches of three, of equal egg mass, at the same stage of hatching or clutches of three that simulated normal, asynchronously hatching broods. The allocation of sex with hatching order did not differ from random. Also, in the synchronous broods there was no difference in post-hatching survival time between the sexes. However, in asynchronous broods last-hatched females survived longer than males whilst of the chicks hatching earlier in the sequence, males were longer lived. In this case, it seems that chick sex ratio is affected by hatching asynchrony, but at the level of differential post-hatching mortality between male and female chicks in different hatching positions.

Few studies show any effect of clutch sequence upon gull hatching sex ratio. Ryder (1983) describes a bias in Ring-billed gulls, *Larus delawarensis,* towards males in the first hatched eggs of clutches of three. However, the trend is seen only in two of the three seasons studied. A further publication by Meathrel and Ryder (1987) includes sex ratio data from the same colony, for an additional two years. There was no effect of sequence upon hatching sex ratio in either season, indicating that the previously observed trend is inconsistent. In addition,

the sample sizes are smaller (approximately 30 clutches per year) than that of the current Herring gull study and there is no control for the effect of clutch/female identity in the statistical analysis (Krackow and Tkadlec, 2001).

The numbers of broods with different sex ratio combinations, at hatching in the Herring gull colony on Walney, did not differ from those expected by chance. This agrees with a similar comparison by Griffiths (1992) in Lesser black-backed gulls. There is no evidence to suggest that, in gulls, certain combinations of males and females in a 3-egg clutch are more likely to occur than others.

Given evidence that last-hatched gull chicks (the smallest of a clutch of three) are less likely to survive and that increased mortality amongst third-hatched males seems to occur, the prediction that males are less likely to be produced from C-eggs seems reasonable. The study of Herring gulls on Walney found no difference in sex ratio between A or B eggs and C eggs, in accordance with most other findings in gulls. It is possible that any effect of differential mortality of male C-chicks, in the Herring gull population, was not strong enough to produce a significant benefit from sex ratio manipulation. Unfortunately, A and B eggs, in the current analysis, were often not distinguished from each other, preventing a more detailed comparison. Laying sequence was inferred from hatching order, so that only the later hatching, smaller C-chicks were easily recognisable.

Further study

It would be interesting to carry out further study of hatching sex ratio and subsequent offspring survival at the Herring gull colony on Walney, in relation to additional factors such as parental condition, age and breeding experience. The possibility remains that the clutches sampled at hatching, during the present study, may have included replacement eggs or second clutches. Ideally, nesting pairs for

which laying date and the outcome of any breeding attempts made (earlier in the season) was known should have been used. Studying pairs during laying would also have allowed A and B chicks (as well as C chicks) to be marked and separated in the analysis of sex ratio and egg sequence.

Other variables contributing to gull reproductive success include territory size (Hunt and Hunt, 1976) and nest cover (Brown 1967). Potentially confounding variables could either be controlled for or examined as covariates of seasonal or hatch-order effects, in relation to hatching sex ratio. However, the relative costs and benefits of brood sex ratio manipulation are also likely to be influenced by stochastic variables such as weather conditions, food availability and levels of predation and disease. Any patterns in sex ratio variation should, preferably, be studied across a number of breeding seasons.

The landfill site on Walney, adjacent to the study colony, was closed during the winter following the present study. Although the relative importance of landfills and anthropgenic food supplies to gull breeding colonies is somewhat unclear (Belant, *et al.*, 1998), it would be worth using the opportunity to compare the sex ratio of young produced by Herring gulls, at the same colony, before and after the removal of a convenient food source.

Mechanisms of sex ratio adjustment

Although sex ratio manipulation does seem to occur in birds, the skews reported by many studies are subtle. Considering a significant skew in offspring sex ratio to be an example of adaptive manipulation is dubious, when little information is available about the costs or physiological mechanisms involved.

Krackow (1999) discusses the potential mechanisms of avian sex ratio manipulation. These include segregation distortion, selective ovicide and the

possible effects of maternal testosterone. Because female birds are heterogametic (producing oocytes containing Z or W sex chromosomes), the opportunity to adjust the primary sex ratio is, conceivably, available to the female at any time between the production of gametes at meiosis and the calcification of fertilised eggs prior to laying.

Krackow (1999) doubts that there is a suitable mechanism through which distortion of segregation of the sex chromosomes, during meiosis, could occur whilst Williams (1979) points out that gametes should be selected to oppose parental attempts at control of the offspring sex ratio. Reiss (1987) explores the conflict between parent and gamete over control of the offspring sex ratio by genetic modelling. He demonstrates that gametic autosomes would be selected to promote sex ratio distortion, only when profitability of producing an individual of one sex exceeds the other by at least a factor of three.

Given the millions of oocytes available in the female's ovary, Oddie (1998) suggests that oocytes might be differentially provisioned, according to sex, thus manipulating the hierarchy of gamete maturity and likelihood of ovulation. Krackow (1999), who refers to the parent-gamete conflict (Reiss 1987) that predicts selection for oocytes to avoid sex-discrimination, deems suppression of ovulation improbable. The possibility that females arrest the development of follicles of the wrong sex is also discounted, owing to the rarity of observation of atretic follicles in birds (references in Krackow, 1999).

The remaining window for maternal control of offspring primary sex ratio lies between ovulation and shell formation. In the chicken, fertilised eggs are calcified between 4 and 5 hours after ovulation and during this period, re-absorption of the egg may occur, appearing to do so in 5 to 40% of ova (Sturkie, 1986). Selective re-absorption of fertilised eggs is likely to be costly. However,

female gulls are able to lay one or two replacement eggs, depending upon protein reserves (Houston *et al.*, 1983) so could, potentially, sacrifice eggs of the wrong sex.

None of these suggested mechanisms of sex ratio distortion are supported by evidence of how the female recognises the sex of gametes or embryos to be sacrificed. Nevertheless, recognition of sex need not be necessary if the condition of the female (that results in sex ratio manipulation being profitable) were to trigger a physiological change that affects male and female gametes or embryos differentially. In the laboratory rat, offspring sex ratios are distorted by natural variation in the mother's oestrogen level and by treatment of the female with the adreno-cortico-trophic hormone or gonadotrophin (references in Reiss, 1987).

Recent interest has surrounded the discovery that yolk testosterone levels vary during the laying sequence (Schwabl, 1993) and with offspring sex (Petrie *et al.*, 2001) of the eggs of some bird species. The testosterone in eggs is suggested to be provisioned by the mother (Schwabl *et al.* 1997). However, whether or not this hormone could control sex determination, during sex-chromosome segregation - as suggested by Petrie *et al.*, (2001) seems controversial (Cunningham and Russell, 2001). Krackow (1999) discusses how sensitivity to testosterone levels might be dependent upon embryonic gender. In female birds, the Z-chromosome is non-dosage compensated (Jablonka and Lamb, 1988) so that heterogametic female embryos (ZW) would be expected to under-produce Z-chromosome products relative to male embryos (that have two Z-chromosomes). If Z-chromosome gene products were involved in counteracting possible adverse affects of testosterone upon embryonic development, then female embryos might suffer increased mortality. In the same way, W-specific gene products could potentially confer differential protection upon developing females.

The data of Komdeur *et al.*, (2002) suggest that pre-ovulatory control of sex ratio is the most likely mechanism in the Seychelles warbler. Birds that were translocated to good quality territories were more likely to produce females that, unlike males, serve to assist future breeding attempts. Many of these pairs were able to lay a second egg, which was also significantly female-biased. More importantly, the second laid (sex-biased) eggs were laid within 24 hours of the first - a time scale that greatly diminishes the possibility that ovulation and subsequent re-absorption of unprofitable male eggs could have occurred. The authors propose that a sex-biased release of gametes might only evolve in species such as the Seychelles warbler, where the difference in benefits gained from the production of sons or daughters is exceptionally high.

The most plausible mechanism for avian sex ratio manipulation, in other cases, would seem to be selective re-absorption of fertilised eggs, in the oviduct, before calcification. As yet, there is no evidence to show that female birds use this mechanism, during laying. Primary sex ratio manipulation is, presumably, physiologically costly, given that selection pressures have not led to strong variation in the hatching sex ratio in many of the bird species studied.

Adaptive sex ratio manipulation: a matter of opinion?

The occurrence of facultative offspring sex ratio manipulation in vertebrates remains a topic of debate. There is likely to be a publication bias against results that show no, potentially adaptive, sex ratio manipulation - so that those published could be viewed as those showing statistical coincidence.

Brown and Silk (2002) present a meta-analysis of the relationship between maternal condition (derived from social rank) and offspring sex ratios in primate groups. Although there did not appear to be a publication bias between significant

and non-significant results in the literature investigated, effect sizes found in various studies were shown to vary with sample size. When sample sizes were small, large deviations in sex ratio were observed whilst the effects of maternal quality tended towards zero for larger sample sizes. In addition the mean variation in sex ratio observed between high and low-ranking females was zero, suggesting that the observed effects are a product of stochastic variation.

A survey of studies in ungulates, in which good quality females are predicted to overproduce males, also illustrates inconsistencies between results (Hewison and Galliard, 1999). Several studies are listed as producing significant or near-significant negative relationships between female quality and the proportion of male offspring. These findings have particular relevance to the model of differential sex allocation proposed by Trivers and Willard (1973) who based their predictions upon a polygynous ungulate, the caribou *Rangefer tarandus*.

With reference to sex ratio studies in birds, West and Sheldon (2002) analysed the results of 11 studies testing the hypothesis that sex ratio is adjusted in response to mate attractiveness and five studies predicting offspring sex ratio manipulation in co-operatively breeding species. These studies were shown to be consistent in their findings of predicted sex ratio skews. The authors argue the importance of environmental predictability in the evolution of sex ratio manipulation - selection pressure for adjustment will only lead to adaptation, if the conditions that lead to the differential reproductive value of the offspring are predictable.

To conclude, the results of the present study do not support the predicted offspring sex-ratio variation, with season or hatching order in the Herring gull. The unexpected trend (that of an overproduction of sons, at the end of the breeding

season) is difficult to explain without further information regarding seasonal offspring survival rates or more direct measures of parental quality. The only convincing demonstration of adaptive sex ratio manipulation, at hatching, in gulls is that by Nager *et al* (1999) which used egg-removal to lower female quality to the extent where female offspring were overproduced. It seems possible that, under natural conditions, that the difference in the relative reproductive value of male and female offspring often cannot be predicted or is insufficient to drive costly mechanisms of sex ratio control.

Chapter 4

Differences in Offspring Sex Ratio in the Three-spined Stickleback (*Gasterosteus aculeatus*): The Relationship with Paternal Attractiveness

4.1 Introduction

The three-spined stickleback

The three-spined stickleback, *Gasterosteus aculeatus*, is a small sexually dimorphic fish. The species is common in fresh water and brackish ecosystems throughout northern temperate regions. Stickleback have long been the subject of extensive study by ethologists (Warington, 1855; Tinbergen, 1948) and behavioural ecologists (Huntingford, 1984; Krebs and Davies, 1993) and more recent interest encompasses research in the fields of ecotoxicology (Armin *et al.*, 2000; Bervoets *et al.*, 2001) and genetics (Griffiths *et al.*, 2000; Peichel *et al.*, 2001).

Stickleback are relatively easy to breed and maintain in large numbers in aquaria, under semi-natural conditions. A new molecular tool can be used to reveal the sex of the stickleback, as early as newly hatched fry (Griffiths *et al.*, 2000) and this chapter describes two studies examining the gender of stickleback offspring. The first study examines the primary sex ratio of fry in relation to paternal quality and was carried out in collaboration with Dr Iain Barber at the Institute of Biological Sciences, University of Wales, Aberystwyth, Ceredigon, UK. The second study involves the determination of sex from fertilised eggs, at various stages of embryonic development and was conducted at the University of Glasgow.

Section I: The Relationship of Offspring Sex Ratio with Paternal Attractiveness

4.2 Introduction

Male stickleback exhibit strong, red, nuptial colouration, which is variable (McLennan and McPhail, 1989a) and appears to be heritable (Bakker, 1993). All other factors being equal, females generally prefer redder males as mates (Milinski and Bakker, 1990; Bakker,1993; Bakker and Mundwiler, 1995 but see Bakker and Milinski, 1991; Milinski and Bakker, 1992, Braithwaite and Barber, 2000) therefore, red breeding colouration can be considered to have evolved via sexual selection (Andersson, 1994).

The intensity of red colouration has been found by a number of authors to increase with body condition or behavioural vigour (McLennan and McPhail, 1989b; Frischknecht, 1993; Bakker *et al.*, 1999). Thus, male red nuptial colouration may be described as a condition-dependant sexually selected trait. However, not all studies find a consistent trend. Barber *et al.* (2000a), found that the intensity of male nuptial colouration correlated positively with body condition when individuals were measured following capture in the wild but not after a period of maintenance in the laboratory. A positive correlation was found by Bakker and Mundwiler (1995), but only at one of two field study sites whilst Fitzgerald *et al.*, (1994) report no association of colour score with body condition.

In parallel with humans, female stickleback are homogametic (their sex being determined by a homologous pair of sex chromosomes, XX). Male stickleback are heterogametic (possessing X and Y sex chromosomes) and the gender of the offspring is determined during fertilisation of the female's

homogametic eggs by X or Y sex chromosome bearing sperm (Griffiths *et al.*, 2000). The male stickleback invests significant time and energy in parental care and might recoup reproductive costs by producing offspring that have maximum opportunity for future reproductive success. The production of a biased sex ratio in response to parental quality is discussed in Chapter 3. If we accept that attractiveness is heritable, a male that is sexually attractive might be expected to skew the offspring sex ratio towards male offspring. Male progeny, inheriting attractiveness, would achieve relatively greater reproductive success than female siblings, due to an increased advantage in mate competition. Poor quality males are predicted to benefit more from over-producing females that are more likely to secure mating opportunities.

Reproductive biology of the three-spined stickleback

A comprehensive account of stickleback biology is given by Wootton (1976) and courtship behaviour by Rowland (1994). The breeding season occurs in spring and early summer months, varying with latitude. At the onset of reproductive activity, the male stickleback defends a nesting territory and begins nest construction. During this phase, males develop nuptial colouration - the iris of the eye turns from grey to iridescent blue whilst the throat and underside develop red pigmentation. Females remain cryptic, although, when gravid, the distended belly becomes silvery and dark stripes may develop dorsally. The nest is composed of a mat of sand and plant material, through which a tunnel is excavated, and is secured by the excretion of spiggin, a glycoprotein glue (Jakobsson *et al*, 1999), produced in the male kidneys.

Following nest completion, the male begins to court gravid females. The courtship display develops as a stereotypical series of stages that progress as the

male becomes more receptive. The initial response of a nesting male to a female is to attack and bite the intruder, further to which the male performs a "zigzag" dance. The male will then attempt to lead the female to the nest, followed by creeping through the tunnel, into which the female may then enter and spawn.

Should a female choose to spawn, her readiness is indicated by the adoption of a "head-up" posture, which displays the distended belly. Following fertilisation of a clutch, the male enters a parental phase, during which the nest is defended and the eggs oxygenated by fanning of the pectoral fins. Egg development lasts approximately 11 days, although this may vary with temperature (Wootton, 1976). The hatched fry may be guarded for a short period whilst in the vicinity of the nest prior to dispersal.

Sexual selection

The nuptial colouration of the male stickleback is an example of a sexually selected character. Fisher (1999) describes how female preference for a trait becomes genetically coupled to female preference for that trait by conferring a reproductive advantage on her male offspring. Models of Fisherian sexual selection propose that at first the male trait may improve male survival and when coupled to the female preference becomes a runaway evolutionary process, only counteracted by negative natural selection e.g. increased risk of predation or metabolic costs of maintaining colouration.

The trade-off between natural and sexual selection is illustrated in the guppy, *Poecilia reticulata*. In a study of this species by Nicoletto and Kodric-Brown (1999), regional variation was found in the brightness of colouration. In headwater habitats with low predation risk, male guppies are more conspicuous and have higher display rates.

It has also been suggested that mate-choice criteria may evolve from biases that already exist in the sensory system of a particular organism. A study of guppies by Rodd, *et al.* (2002) investigated whether female preference for orange colouration in the male was based on a general attraction to orange objects. It was shown that both males and females were significantly more attracted to orange coloured discs than to other colours, indicating a possible non-sexual origin of the preference. The authors propose that the bias may have evolved as a result of a preference for foraging upon orange coloured fruit. Female stickleback have been shown to exhibit a sensory bias (Ryan, 1990) towards red colouration during the breeding season, with an increased optomotor response at the red end of the spectrum (Cronly-Dillon and Sharma, 1968). However, this physiological change is not found in male stickleback and there is, as yet, no evidence to suggest that female preference for red is evolutionarily derived from a corresponding food colour detection bias.

Kirkpatrick and Ryan (1991) and Andersson (1994) summarise the mechanisms of sexual preference evolution. Selection for male sexual characteristics may result from direct advantages to the female (not considered by the Fisherian model). For example, search costs might decline with increased conspicuousness. Other direct benefits may include increased fecundity as a result of choosing a particular male; in the case of the stickleback, this might involve variations in the quality of paternal care. Paternal condition, was manipulated by Stanley (1983; in Wootton, 1994) by altering the rations fed to captive reproductive males. Paternal investment, in terms of nest fanning activity was reduced in males on low-rations, although this did not affect the hatching success of fertilised clutches. McKinnon (1996) correlated red colouration of males, in the parental phase, with the intensity of nest defence. Redder males may provide

better protection to offspring against conspecifics, as redness plays an important role in male-male interactions (Rowland, 1983; Kraak *et al.* 1999).

Selection for female preference may also act indirectly, if male condition-dependant traits affect offspring viability. An initial survival advantage of the trait is not required, as pleiotropy or genetic drift might give rise to a preference for ornamentation (Andersson, 1986). Good genes models suggest that females choose males on the basis of apparent genetic quality, as was first suggested by Williams (1966). The selected trait should be correlated with male fitness components such as growth rate, predator avoidance, disease resistance and competitive ability. Unlike Fishers hypothesis, the fitness of both male and female offspring should be correlated to the father's fitness (Kodric-Brown and Brown, 1984).

Milinski and Bakker (1990) have shown that female stickleback avoided males infested by white-spot fungus, which were recognised by their less intense colouration. Female choice may, therefore, be selecting for parasite resistance genes. Choosing healthy males may also directly benefit the female as she is less likely to become infected, with directly transmitted parasites, during mating. Barber, *et al.*, (2000a) were able to demonstrate the genetic effects of paternal quality upon offspring disease resistance by comparing the maternal half-siblings of bright and dull-coloured males. Clutches derived from individual females were divided between two males using *in vitro* fertilisation and incubated artificially (Barber and Arnott, 2000), thus controlling for maternal quality and any direct effects of paternal care. The offspring of brighter males were more resistant to a controlled exposure to the parasite *Schistocephalus solidus*, (Cestoda: Pseudophyllidea) and had higher white blood cell counts if infected, suggesting that red colouration is an indicator of genetic quality.

Additional studies of parasitic infection and male red colouration show the relationship to be less straightforward. Of the five parasite species found in a population of breeding males by Folstad *et al.* (1994), only three were associated with red colouration. The incidence of two parasite species correlated positively with male colouration and the authors suggest that the redness of infected males functions to broadcast to females the degree of exposure to parasites as well as genetic resistance. Fish infected with *S. solidus* in the study by Folstad *et al.* (1994) showed decreased red colouration. However, Tierney *et al.* (1996) found no effect of *S. solidus* on breeding colouration and Fitzgerald *et al.*, (1994) found no correlation between male redness and infection with skin-encysted trematodes.

Nuptial colouration in the stickleback is based upon carotenoid pigments (Brush, 1965; Czeczuga, 1980) and the intensity of colouration increases with carotenoid colouration (Wedekind and Jakobsen 1998; Barber *et al.*, 2000b). Carotenoid-dependant sexual signals may indicate resistance to oxidative stress (von Schantz *et al.*,1999; Lozano, 2001). The immune and detoxification systems of animals generate reactive metabolites and free radicals that in turn may be scavenged by carotenoids. The availability of pigment for display may therefore indicate a healthy immune system (Lozano, 1994). Carotenoid colouration may also display superior foraging ability, with the extent or intensity of colouration reflecting the ability of the individual to accumulate carotenoid containing nutrients from natural sources (Endler, 1980). Frischknecht (1993) carried out feeding experiments upon male sticklebacks to investigate the costs of extraction of pigment from food and deposition into chromophores. Although there was no direct correlation between food availability and red colouration, individuals whose condition increased during the experiment developed a more intense red colouration.

Reproductive costs and benefits

Reproduction in the stickleback is costly for both sexes. A clutch of eggs costs the female around 14% of her post-spawning body weight, 200% of a daily maintenance ration (Wootton *et al.*, 1995). Free-living females spawn, on average, 3.4 times within a season, and the inter-spawning interval can be as little as three days (Mori, 1993). In addition, a gravid female has impaired swimming ability and increased conspicuousness so is at greater risk of predation (Wootton *et al.*, 1995). Reproductive males expend a similar proportion of their daily energy budget to that of females, during breeding. Besides nest building, defence and courtship, the eleven day fanning cycle to keep the developing eggs oxygenated requires 200-300 J/g (body mass)/day (Wootton *et al.*, 1995).

Males that display costly secondary sexual traits during breeding might be assumed to face intra-sexual competition for mates (Darwin, 1871; Bateman, 1948; Trivers, 1972). Bateman (1948) attributes variability in male reproductive success to the relatively low cost of sperm production, so that a male's investment in reproduction is limited by his ability to acquire mates and fertilise eggs. In contrast, production of eggs by females, is generally more costly, thus constraining the potential reproductive rate.

Trivers (1972) extends the definition of parental investment to include the cost of any investment that increases offspring survival. Parental investment in the stickleback, therefore, should include the cost of paternal care as well as that of gamete production. Also, the cost of sperm production should not be ignored. Spermatogenesis in the male stickleback is inhibited during breeding and each mating uses around 5% of the male's sperm store, with a significant reduction in males that have bred several times (Zbinden *et al.*, 2001).

Clutton-Brock and Parker (1992) propose that it is the potential reproductive rate of males and females, that primarily determines which sex competes for mates (the operational sex ratio). The male stickleback can increase his reproductive rate at less cost than the female, by incubating more than one clutch at a time, so that females become the limiting sex. Wootton *et al.* (1995) give a simulation model, that predicts the operational sex ratio of breeding sticklebacks, based upon the length of periods of receptivity and unreceptivity in both sexes. The model suggests that the operational sex ratio is male-biased, although factors such as food availability and sex-biased mortality are likely to vary the intensity of intra-sexual competition. Thus, it may be assumed that, under most circumstances, males face more competition for mates than females.

It is hypothesised that redder males might produce more male offspring during a single breeding attempt to maximise transmission of genes for male signalling. The male might achieve offspring sex ratio distortion by altering the ratio of X or Y chromosome-bearing sperm used in fertilisation (primary sex ratio), or by selective cannibalism of the clutch during incubation (adjusting the secondary sex ratio). The latter strategy would only be effective if the cost to reproductive success incurred by eating viable eggs is outweighed by the future benefit of maintaining body condition for future reproductive effort.

The aim of the present study is to investigate the possibility that the heterogametic male might bias the sex ratio of a clutch of homogametic eggs, at fertilisation. The sex ratio of offspring, at hatching, will be measured in clutches sired by males of differing quality. Given that previous studies of the relationships between the intensity of nuptial colouration and male attractiveness and reproductive success have shown inconsistency, female preference for male redness shall also be investigated. Mate choice trials will be used to measure

female response to courting males, from a wild caught population, in the laboratory. Eggs fertilised by males (whose colouration and attractiveness to females has been recorded) will be incubated, artificially, between fertilisation and hatching, in order to measure the primary sex ratio of the offspring. It is predicted that the redder, more attractive males should skew the clutch sex ratio towards more profitable male offspring.

4.3 Methods

Husbandry

In order to test the idea that males control offspring sex ratio, wild fish were captured from Llyn Frongoch, Ceredigion, Wales, before and during breeding (April-July, 2000). Fish were housed in aquaria and experiments carried out at the Institute of Biological Sciences, Edward Llwyd Building, The University of Wales, Aberystwyth, Ceredigion, UK.

Males were placed in individual 16 litre nesting tanks with a 15cm plastic plant, for cover, and aeration was provided by a biofoam airlift filter. An area of sand was placed upon the gravel substrate, near the plant and two hundred lengths of black nylon thread (8cm) were provided as nesting material. Tanks were separated by grey opaque dividers to prevent male interaction. Males were fed twice daily on chironomid larvae. Females were held in shoals in large (160 litre) tanks and fed *ad libitum* on chironomid larvae. All fish were subject to a 16h light: 8h dark regime, at 19°C. Seawater (approximately 20% of the water volume) was added to all tanks to inhibit white-spot infection (*Ichtyoptheirius multifillis*), which is endemic in the native population.

Males were presented with a gravid female by placing her inside a glass jar in the nesting tank for 20 minutes, twice daily, to encourage nesting. Nest building behaviour and the males reaction to the female was monitored. Once the nest was complete and the male had reached the stage of courtship where creeping through the nest occurred, he was used in mate choice trials with gravid females.

Mate choice trials

Males were classified according to intensity of red nuptial colouration by eye using a (0-10) redness index from a colour chart (see Appendix II) Pairs consisting of a bright (colour score: 0-5) and dull (colour score 6-10) male were used for each trial. Each pair of males was presented to two females, consecutively, and a total of 9 pairs of males were used in mate choice trials. Since few receptive males were available for use in mate choice trial at any given time, the pairs of males were not size matched. Only gravid females that showed a characteristic "head-up" response when presented to a courting male, indicating readiness to spawn, were used in the trials.

The experimental females were acclimatised to the choice tank (Figure 4.1) for around ten minutes before removing the divider between the female and the nesting tanks containing the courting males. Female choice was measured for 10 minutes by recording her orientation with respect to each male (area A or B in the female tank) every 20 seconds (see tank set-up Figure 4.1,). The female was observed without disturbance by fixing a mirror above the tank at a 45° angle, facing the observer.

Time spent orientated towards each pair of males was recorded for two gravid females. When a female did not enter the zone adjacent to both of the males, the preference result was discarded as the female might be focussing her attention on the first and only male she has seen rather than making a mate comparison.

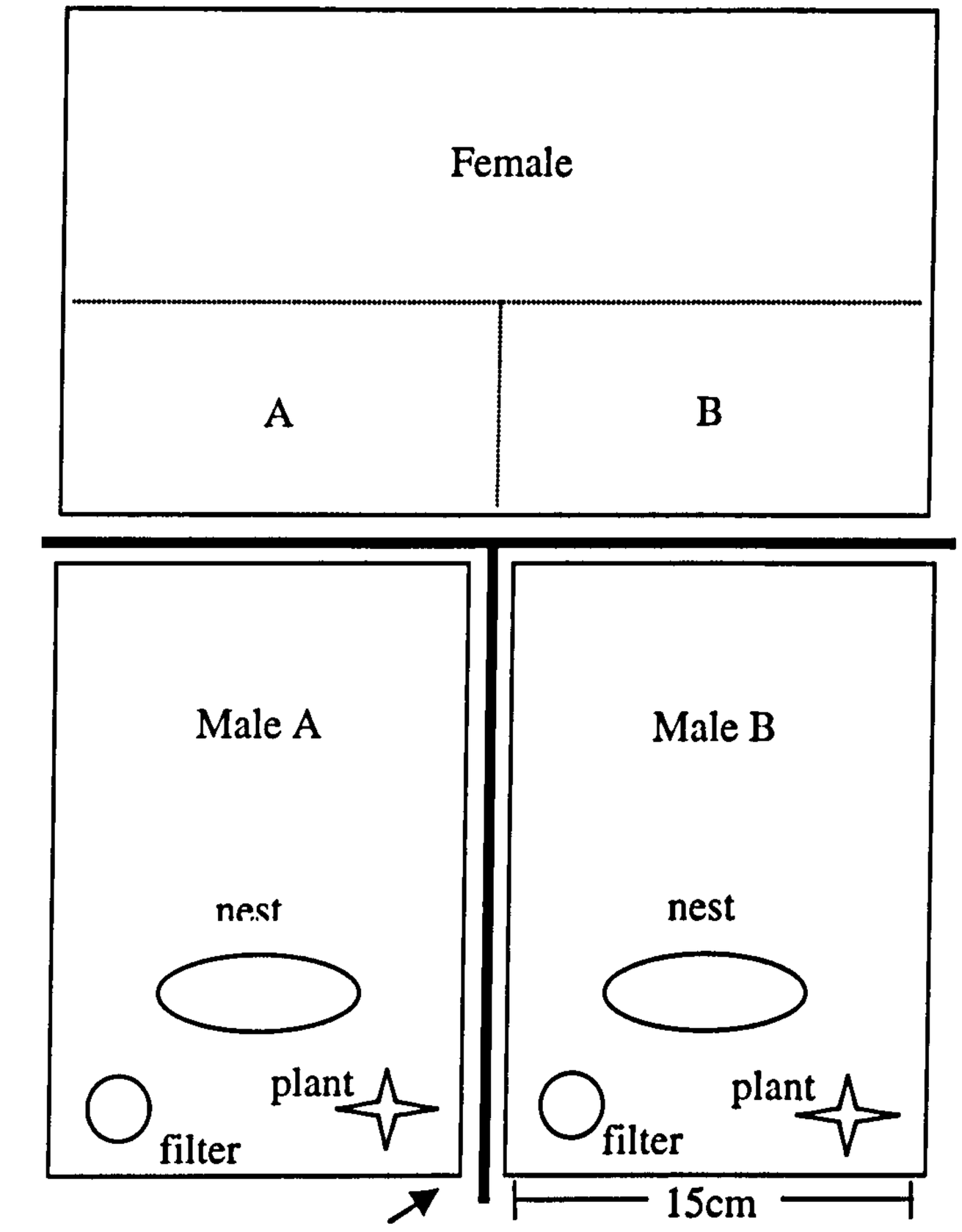

Figure 4.1: For mate choice trials, two nesting tanks containing males are positioned next to a larger tank containing a gravid female. Time spent by the female in areas A and B, facing each male is recorded.

Split clutch *in vitro* fertilisation

Following the trial, each male was killed humanely (with an overdose of anaesthetic). Male length was recorded by measuring the distance from the tip of the snout to the fullest extent of the tail, when fanned out, to the nearest 0.5 mm. The testes were removed and sperm released from both into a watch glass by

finely chopping the tissue. Eggs were stripped from each female, of the pair used in the trial, and the clutches split in half and fertilised separately using sperm from different males (Barber and Arnott, 2000). After twenty minutes the fertilised eggs were transferred from the watch glass to an incubating tank. The eggs were incubated, in specially designed incubators (Barber and Arnott, 2000) at 19°C for 11 days or until hatching. The fry were then collected and stored in 100% ethanol for sexing analysis.

By dividing the sperm from each male between clutches, using a split-clutch *in vitro* fertilisation (IVF) protocol (Barber and Arnott, 2000), it is possible to compare separately the male and female effects upon offspring sex ratio (Figure4.2).

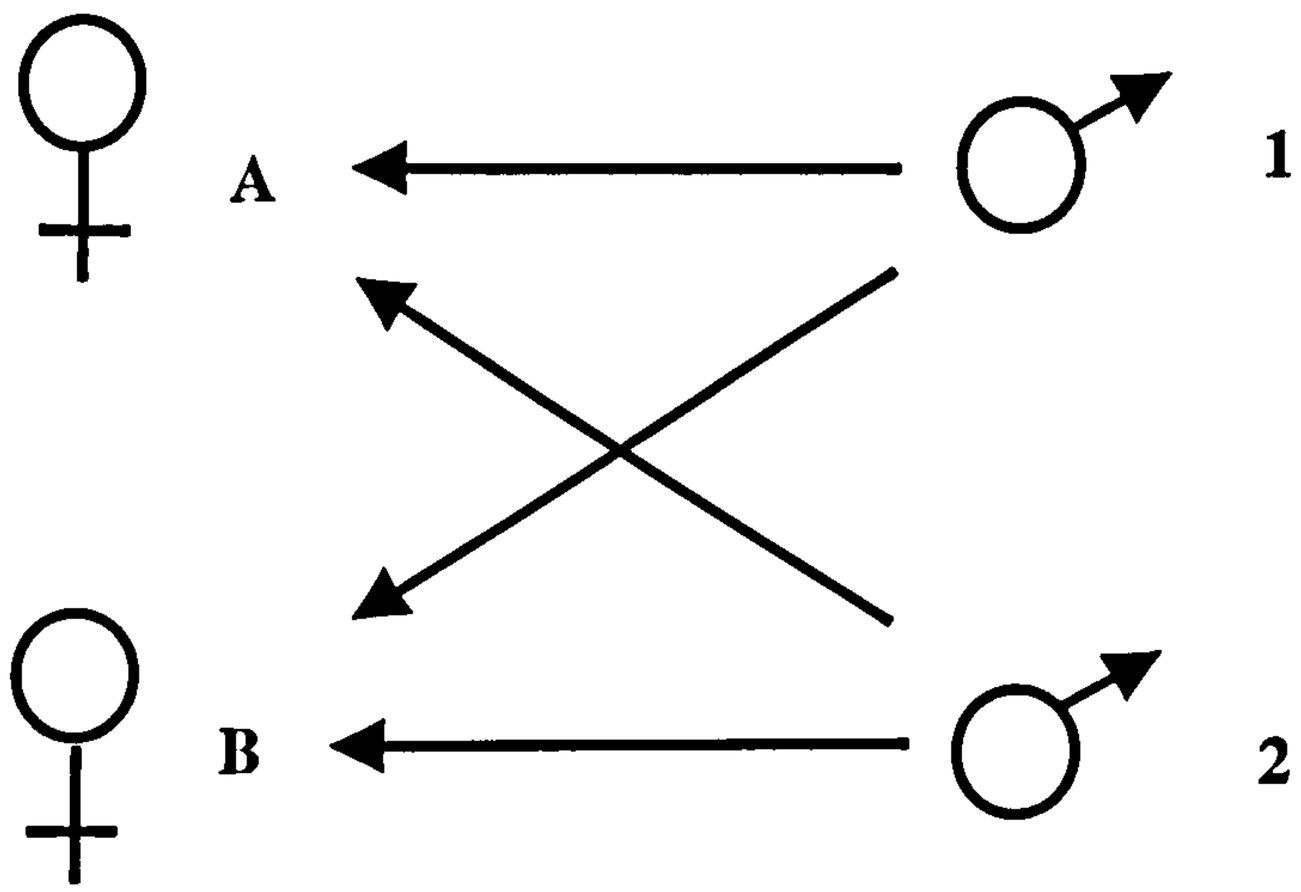

Figure 4.2: Split clutch *in vitro* fertilisation – each male of a pair (1 and 2) is used to fertilise two females (A and B), to control for maternal affects upon the offspring.

Each fry was homogenised in a 2.5 ml eppendorf, containing 200µl of ethanol, using clean forceps and scissors, washed in 0.5 M HCl. 5µl of each

sample was then mixed with 200µl 5% Chelex and extracted as described in Chapter 2.

Sex determination by PCR

Sex determination by PCR of sex-linked markers was carried out, at Glasgow University, as described in the introduction. Reactions were carried out in 10µl under the following conditions. A "mastermix" of reactants for each batch of DNA samples was prepared as described in Chapter 3:

Primers Ga1F/Ga1R (0.1 mg/ml)	0.8 µl
dNTP (10mM)	0.8 µl
10 × PCR reaction buffer (Promega)	1.0 µl
$MgCl_2$ (25mM)	1.0 µl
dH_2O	3.725 µl
Taq (5 units/µl, Promega)	0.075 µl
DNA	1.0 µl

Thermal cycling began with 2 minutes at 94°C then 30 cycles of 1 minute at 46°C followed by 1 minute at 72°C and 45 seconds at 94°C, 1 min at 46°C and five minutes at 72°C. Primers were used following design by Griffiths *et.al.*, (2000) and the primer sequences are shown overleaf.

Ga1F (5'-CTTCTTTCCTCTCACCATACTCA-3')

Ga1R (5'- AGATGACGGTTGATAAACAG-3')

Each batch of samples used in DNA extraction contained an extraction negative (containing no fry sample) which was also included in the PCR to check for extraction contamination. Control samples of DNA from male and female stickleback and a PCR negative, containing no DNA, were also included in each PCR. Thus, it was possible to check that the PCR was successful from the known male and female control samples and that no contamination occurred during PCR set-up using the PCR negative control. PCR products were loaded, with 10 × Orange G loading buffer, into 10cm 3% agarose gels (stained ethidium bromide), with a 4µl aliquot of 1kb DNA ladder (Promega) included in each row of lanes, as a scale. Gels were run in 0.5 × TBE buffer, at 120V for 20-30 minutes, then placed on transilluminator and photographed (Figure 4.3).

The primers used amplify an Y-linked marker, around 371bp long (Figure 4.3, C) that identifies males (XY). However, a control product is necessary that is present in both males and females (XX) to distinguish female DNA samples from an unsuccessful PCR. This could be done with a second set of primers but, conveniently, Ga1F and Ga1R also produce a second band of around 600bp in both sexes (Figure 4.3, B). The uppermost band visible (Figure 4.3, A) is an additional fragment of DNA, that has been amplified by the PCR reaction, and is not sex-specific.

Stickleback sexing gel

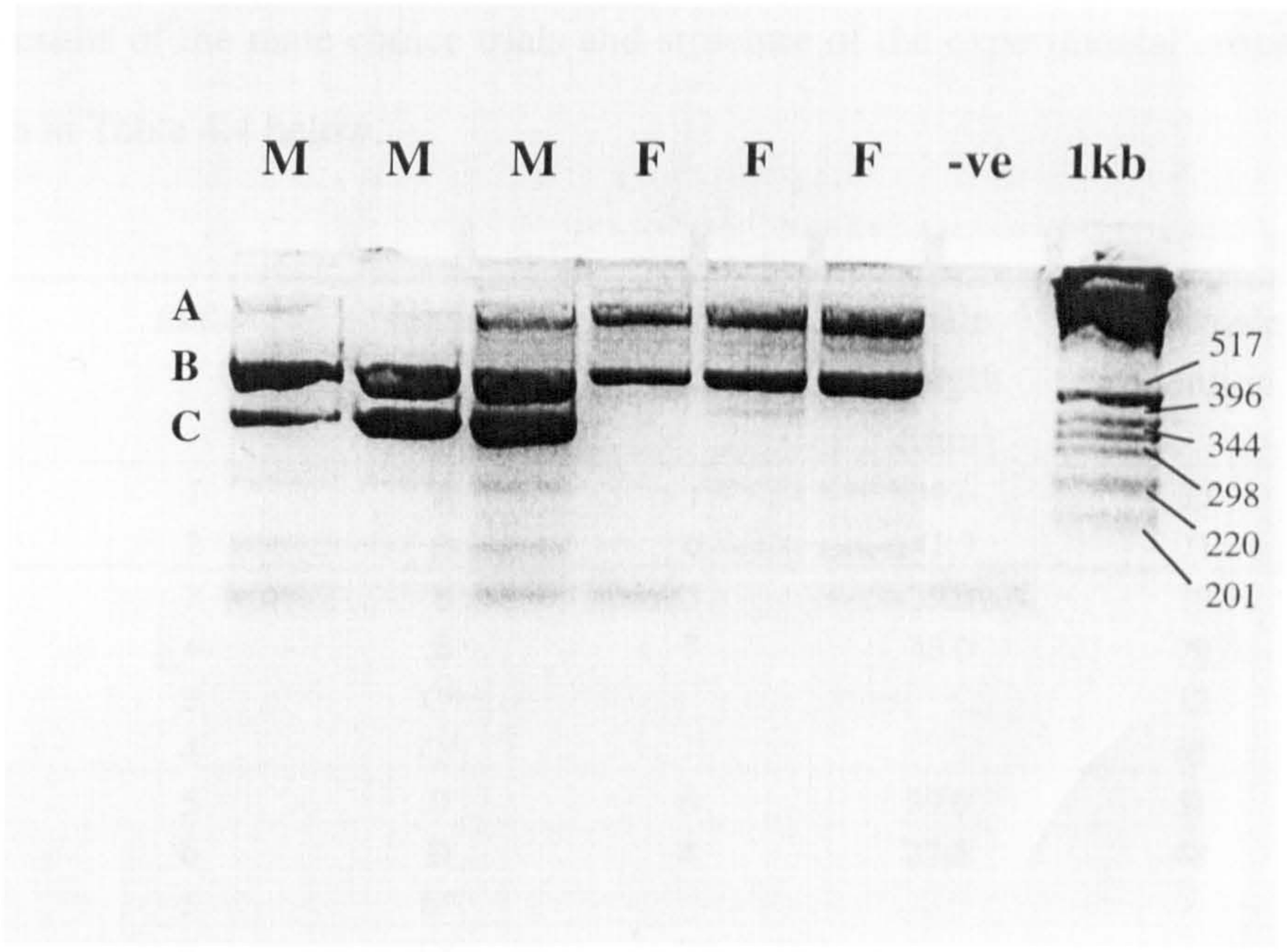

Figure 4.3: photograph of an agarose gel with PCR products from 3 male (M) and 3 female (F) stickleback. The uppermost band (A) is an additional DNA fragment, that has been amplified, and is not sex-specific. The middle band (B) is a DNA fragment amplified from both sexes that acts as a control, whilst the lower band (C) is a Y-linked sex-specific DNA fragment. The right hand lanes contain the PCR negative control and a 1 kb DNA ladder.

4.4 Results

Mate choice and IVF

Results of the mate choice trials and structure of the experimental crosses are shown in Table 4.4 below.

clutch	male	female	male colour score	male length (mm)	% female attention
1A	1	A	1	39.0	25
2A	2	A	6	41.0	71
3B	3	B	5	39.0	19
4B	4	B	7	43.0	70
-	3	C*			12
-	4	C*			65
5D	5	D	6	39.0	51
6D	6	D	4	37.5	48
-	5	E*			-
-	6	E*			-
7F	7	F	8	42.0	58
8F	8	F	0	42.0	13
-	7	G*			70
-	8	G*			19
9H	9	H	7	39.5	6
10H	10	H	3	40.5	93
9I	9	I			32
10I	10	I			6
11J	11	J	6	39.0	3
12J	12	J	4	34.0	93
11K	11	K			87
12K	12	K			10
13L	13	L	7	38.0	71
14L	14	L	0	35.5	23
13M	13	M			45
14M	14	M			54
15N	15	N	6	37.0	48
16N	16	N	3	44.5	6
15O	15	O			-
16O	16	O			-

Table 4.4, continued overleaf

clutch	male	female	male colour score	male length (mm)	% female attention
17P	17	P	4	43.5	54
18P	18	P	7	39.5	38
17Q	17	Q			10
18Q	18	Q			90
19R	19	R	1	49.5	-
20R	20	R	7	37.0	-
19S	19	S			-
20S	20	S			-
21U	21	U	5	43.0	-
22U	22	U	0	44.0	-
23V	23	V	7	35.0	-
24V	24	V	0	44.0	-
25W	25	W	4	35.0	-
26W	26	W	4	40.0	-

Table 4.4: Results of mate choice trials for each male and the structure of experimental clutches. Each row shows the parents crossed to fertilise each clutch. Females marked (*) failed to release eggs. Entries in the % female choice column marked (-) indicate that the female did not visit both males during the trial (females E and O) or that trials were not carried out (females R-W). Each section (of 4 rows) contains the pairs of males and females used in a mate choice trial and half of each female's eggs were fertilised using half of each male's sperm. Sections (of 2 rows) containing a single female (females A, U, V, and W) and pair of males show crosses where only one gravid female was available for fertilisation. Male length (to nearest 0.5 mm) and colour score is shown for each male, along with the percentage of attention, given by the corresponding female to that male, during the trial.

A total of 26 males were used to fertilise split clutches from 22 females. Some of the gravid females used in mate choice trials failed to release eggs when gentle pressure was applied to the distended belly, or only a single female was available for fertilisation. Thus, not all fertilisations followed the split-clutch

pattern shown in figure 4.2. Instead, some pairs of males were used to fertilise a split clutch of eggs from a single female.

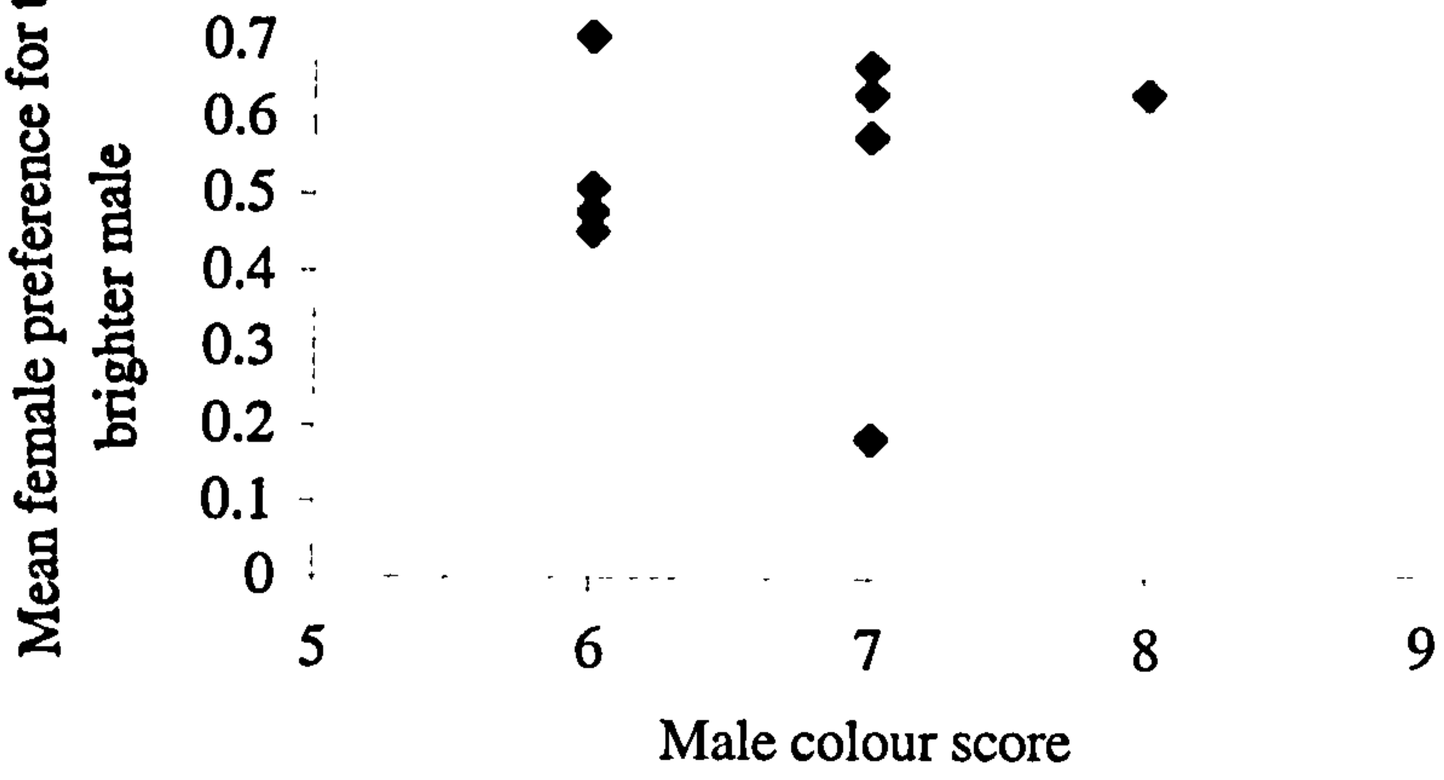

Figure 4.5: Correlation of male colour score (0 - least red colouration, 10 - most red colouration) and female attention. Each co-ordinate represents the mean proportion of time spent by two females in the vicinity of the brighter male, during the mate choice trial (n=9).

Female preference data, expressed as proportions, was arcsine transformed (Sokal and Rohlf, 1995) for subsequent analysis. Spearman's rank correlation (Statview 4.5) was used to compare the proportion of time spent by each of the two females in the vicinity of each male, to check the repeatability of mate preference. There was no significant correlation between females in the proportion of time spent oriented to individual males ($r_s = -0.36$; $p = 0.23$; $n = 9$).

The relationship between male colour score (ranked from 0-10 with 10 being the brightest) and mean proportion of female attention, directed to the

correlation: $r_s = 0.35$; $p = 0.31$; $n = 9$). Male length may also affect female choice (Kraak *et al.*, 1999), Spearman's rank correlation between male length and mean proportion of female attention, directed at the longer male, during the mate choice trial was also not significant ($r_s = -0.37$; $p = 0.32$; $n = 9$).

Because the two comparisons of length and colour score with female preference are not independent, the probability of a type 1 error (incorrectly rejecting the null hypotheses - that there are no effects of either male colour or length upon female attention) is increased. The type 1 error of the statistic of significance ($\alpha = 0.05$) can be lowered so that the probability of making a type 1 error at all in the series of tests does not exceed α. The Dunn - Sidák method was used to calculate a value of α' for each comparison, so resulting in a conservative test when the individual significance tests are not independent (Sokal and Rohlf, 1995). For two tests, that are not independent α' for each test is 0.025.

Hatching success, male colouration and clutch sex ratio

Unfortunately, approximately two thirds of the fertilised clutches were completely lost, due to fungal infection. Table 4.6 shows the parents and sex ratio of the clutches that survived to hatching.

In order to control for possible female effects upon sex ratio, a comparison was made within split clutches (A, B, H and K). Males were classified as bright (colour score 0-5) or dull (colour score 6-10). There were no significant differences in the proportion of male offspring that hatched between the bright and dull groups ($\chi^2 = 3.456$, $0.5 \leq p \leq 0.3$, 3 *d.f.*). The overall sex ratio of hatched fry (calculated from total numbers of males: $n = 197$, and females: $n = 132$, from all clutches) was 60% male. The number of males was significantly greater than that

expected (equal numbers of males and females), $\chi^2 = 6.09$, $p \leq 0.02$, 1 *d.f.*, with Yates correction.

clutch	colour (bright/ dull)	number of fry sexed	% hatched	% male
1A	dull	27	93	57
2A	bright	29	93	52
3B	dull	26	86	73
4B	bright	45	90	65
5E	bright	23	77	50
9H	bright	20	91	70
10H	dull	28	97	66
11K	bright	33	90	63
12K	dull	10	32	50
19S	dull	9	n/r	44
21U	dull	17	n/r	81
25W	dull	22	n/r	54
26W	dull	30	n/r	50

Table 4.6: Hatching success and the sex ratio of experimental clutches. Males have been classified as "dull" (colour score: 0 - 5) or "bright" (colour score: 6 -10). Clutch names indicate male (numbers) and female (letters) parents (see table 4.4). "n/r" denotes that hatching success was not recorded.

The overall male-bias found in the sex ratio may have been due to differential embryo mortality during incubation. If this were the case, it would be expected that the skew in sex ratio would relate to the number of eggs that were lost from the clutch and not sexed. If increased female mortality occurred, resulting in a male bias in the remaining clutch, then clutches with reduced mortality should be less male-biased.

Figure 4.7 shows the relationship between clutch sex ratio and the proportion of eggs that survived to hatching. There was no significant correlation

between clutch sex ratio and clutch mortality (arcsine transformed proportions, Spearman's rank correlation, $r_s = 0.34$; $p = 0.329$; $n = 9$).

Clutch sex ratio and hatching success

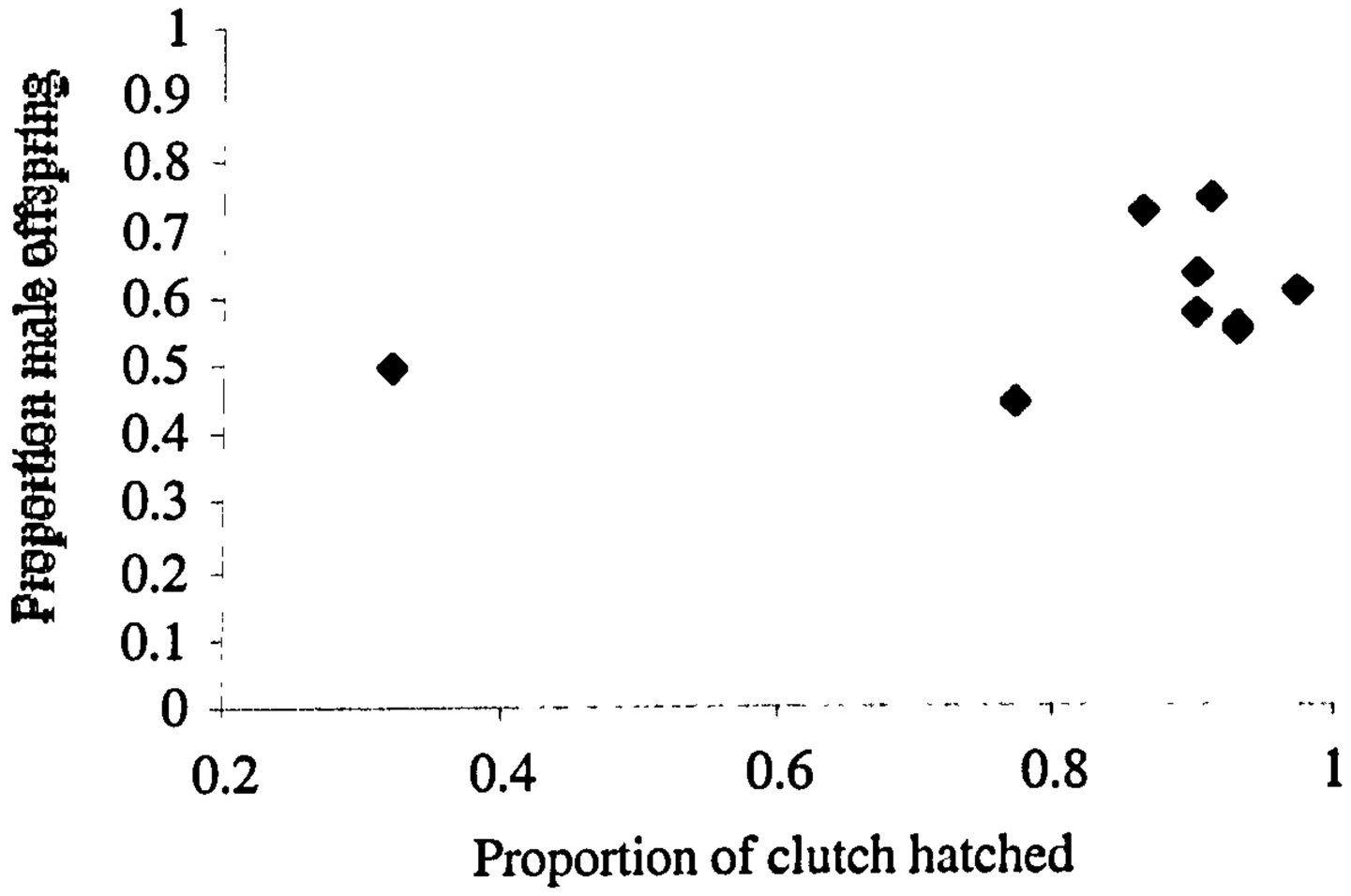

Figure 4.7: The relationship between mortality during incubation and clutch sex ratio. The hatching success of each clutch is plotted against the proportion of male fry, sexed at hatching.

4.5 Discussion

Female preference for redder males

The proportion of time spent by females in the vicinity of courting males did not vary significantly with male colour score, although the number of trials carried out to verify this was rather small. In total, data from 16 females was used as a measure of preference between nine pairs of males. However, studies that have found a positive association of male brightness with female preference have used similar numbers of subjects.

Millinski and Bakker (1990) used 13 females in mate choice trials with 15 pairs of males. Different combinations of three females were used with each pair of males, and the average duration of female "head-up" response correlated significantly with male brightness. Also, Bakker and Milinski (1991) used a total of nine males to generate combinations of three (bright, medium and dull) that were compared, sequentially, by 28 females. Again, females gave significantly more "head-up" response time to brighter males. The lack of association between female preference and male colouration, found in the present study, might be due to the use of time spent in the vicinity of the courting male, rather than duration of head-up responses, as a measure of mate preference. Also, there were large differences in time spent with the brighter male, between the two females that were used, sequentially, to measure preference between each pair of males. In addition, Rowland (1982) suggests that there are inter-population differences in the extent of female preference for redder males.

Alternative indicators of male quality exist. In the wild, females have been found to choose males upon the basis of territory size, nest concealment, level of aggression and timing of nesting (Goldschmidt and Bakker, 1990). Females also prefer to spawn in nests that already contain eggs (Goldschmidt *et al.*, 1993).

Also, cues such as male body size and condition, courtship behaviour and blue eye colour are important (Kraak *et al.*, 1999). However, body size, in our study did not seem to affect female choice. Kunzler and Bakker (2000) suggest that pectoral fin size affects male ability to oxygenate eggs and may be used by females as an indicator of male quality. In the latter study, the area of pectoral fin was experimentally manipulated, with reduced fin size leading to extended offspring development time. It is, therefore, suggested that pectoral fin size may affect female choice.

Male trait expression may alter according to the presence of predators or neighbouring dominant males. Male-male interactions cause inferior males to reduce colouration and expend less energy in courtship (Candolin, 1999a). This was avoided, during the present study of male colouration and offspring sex ratio, by placing opaque dividers between nesting tanks.

Candolin (2000) also studied changes in male colouration across successive breeding cycles. Large males increased their colouration across cycles, whilst smaller males, that were unable to complete as many cycles as large males, did not. At the penultimate cycle, bright males cannibalised some of the eggs, thus reducing the reproductive success of that clutch. Consequently, females choosing bright males at the end of season might reduce the survival of her offspring. In contrast, Bakker and Mundwiler (1995) found that intensity of male colouration decreased across the breeding season. In order to control for these possible effects in our sex ratio study, all males should ideally have been caught from the wild prior to nesting so that each experimental individual is used during their first breeding opportunity.

Female choice may also be affected by the extent to which males differ in colouration. Braithwaite and Barber (2000) highlight inconsistencies in female

choice during mate choice trials. It is shown that female mate choice correlates with nuptial colouration, only when the males to be discriminated between vary greatly in colour. I examined the effect of the difference between male pairs used in the present trials. The mean duration of female attention, given to the brighter male, did not increase significantly with the difference in colour score between the pair of males (Spearman's rank correlation: $r_s = 0.28$; $p = 0.43$; $n = 9$). A similar regression, of the difference in length between males, in a pair, and mean female preference for the longer male was also non-significant (Spearman's rank correlation: $r_s = -0.51$; $p = 0.18$; $n = 8$)

In addition, during mate choice trials that present males to females sequentially, there may be an effect of previous encounters. Bakker and Millinski (1991) address this topic. When a sequence of males was presented to a female, her response was affected by the attractiveness of both the present and the previously encountered male.

The mate choice trials carried out in the above sex ratio study do not support the hypothesis that male colour score is good indicator of female attention. Also, the time spent in the vicinity of a given male does not necessarily reflect the probability that a female would spawn with that male. However, female attention, measured as time spent oriented towards a male is used as an indicator of female preference by several authors including Milinski and Bakker (1992) and Braithwaite and Barber (2000).

Many other studies use the duration of female "head-up" posturing in the vicinity of a courting male as a measure of female preference (Milinski and Bakker, 1990; Goldschmidt *et al.*, 1993; Bakker and Mundwiler, 1995; Candolin, 1999a). McLennan and McPhail (1990) show that a female's "head-up" responsiveness correlates positively with likelihood of her spawning with one of a

pair of males used in mate choice trials. Using a different experimental arrangement, where six potential mates in a large arena surrounded a female, Ward and Fitzgerald (1987) recorded male and female behaviour before and after raising gates to allow female access to the nests. There was no significant correlation between female preference measured in time spent in a male's vicinity or duration of "head-up" posturing with her likelihood of spawning with a given male.

It would be interesting to carry out mate choice trials similar to those in the present study, where females were given the opportunity to choose to spawn with either male. To investigate this involves practical difficulties, in that each pair of males would need to be encouraged to nest in a large tank with removable dividers separating the males from each other and also from a section where females could be placed to carry out mate choice trials.

In our study of male quality and offspring sex ratio, the assignment of a redness colour score was subject to human error. Many studies have followed Frischknecht's (1993) method of measuring the intensity of breeding colouration. The male is photographed ventrally and laterally and a brightness value calculated from a scanned image. A red brightness value is assigned at random, or at several anatomically distinct points, by dividing the redness component by the sum of the red, green and blue component values. Percentage red area may also be calculated (Candolin, 2000) and included in assessment of male quality.

Further improvements to the experiment would include the use of size-matched pairs of males. It might be also worth using a photographic method to assess redness. However, care should be taken during handling and photography as the fish may alter in colour whilst stressed (Candolin, 2000).

Male quality and offspring sex ratio

There was no evidence to suggest that brighter males produced more male offspring. However, that data set for offspring sex ratio was rather small due to loss of many eggs to fungus. Data collection would have been made more efficient if the offspring were sexed as eggs, avoiding loss of sampling due to fungal infection (see section II of this chapter). Sexing the eggs shortly after fertilisation would also control for any effects of differential embryo mortality.

The expectation of adjustment of sex ratio by the male to maximise his offspring reproductive success assumes that nuptial colouration is a heritable trait, that confers a reproductive advantage to offspring. Bakker (1993) carried out mate choice trials and breeding experiments that showed genetic correlation between male colour and female preference for brighter males. Intensity of colouration was also found to be heritable.

Good quality males would gain advantage by over-producing male offspring, only if there were competition for females. Wootton, (1976) found that males in better body condition were brighter and received a higher number of eggs, whilst Bakker and Mundwiler (1995) studied a wild population where the nests of redder males were also found to contain more eggs. In a field study by Kraak *et al.* (1999), reproductive success correlated with throat redness in only one of two separate populations, and only during periods when overall reproductive success was low.

Candolin (2000) studied male reproductive success in the laboratory under high and low food availability. It was shown that increased red colouration correlated with increased hatching success of eggs when energy was constrained but not when males had access to unlimited food. Thus, the breeding conditions in the laboratory may affect interactions between male quality and reproductive success. In addition, Barber *et al.* (2000b) found that when males

were captured in the wild and used in laboratory husbandry, there was an increase in the average intensity of male colouration, with the dullest males gaining most.

In order to investigate the effects of male quality upon reproductive success and offspring sex ratio in the natural competitive environment, field study is required. Male sticklebacks and the contents of their nest could be collected during the breeding season and the eggs sexed. Male colouration could be recorded and female preference for males would be reflected in the number of eggs belonging to each male.

However, female identity and the direct affects of paternal care could affect the sex ratio of eggs collected under these conditions, which are controlled for in our laboratory study through the split-clutch IVF design. Also, "sneaking" males may fertilise clutches of eggs in the nests of other males or males may steal the fertilised eggs of conspecifics (Mori 1995). Genetic evidence for these activities has been supplied by microsatellite analyses of paternity (Rico *et al.*, 1992; Largiader *et al.*, 2001). It should, therefore, be possible to analyse the sex ratio of eggs of differing parental origin by including microsatellite identification of parentage in field investigation.

In addition, differential embryonic mortality may occur before eggs are collected. Nevertheless, if the sample of clutches was large and the eggs examined to determine the stage of embryonic development (Wootton, 1976), the relationship between the clutch age and sex ratio would show whether survival differed between eggs of either sex. Should this be the case, then any sex ratio variability between clutches of the same age could be attributed to parental effects.

Candolin (1999b) examined the correlation of male stickleback redness and body condition in a wild population. Males in good condition had larger red areas than those in intermediate condition, as would be expected, but males in poor

condition were found to increase their signalling intensity. It is suggested that the poor quality males invest more in current reproduction as a terminal effort, made necessary to ensure some reproductive success, given diminishing prospects of survival. Should nuptial colouration be an unreliable indicator of male quality, it would have been desirable, in the present study to use additional measures of male condition. Body condition was quantified by Candolin (1999b) by measuring the male lipid content. Also, the relative liver weight can be calculated, which represents medium term energy reserves (Chellappa *et al.*, 1995). Measurements of immunological status include relative spleen weight (the spleen becomes enlarged during infection or disease) and the proportion of white to red blood cells (Barber *et al.*, 2000b).

Sex ratio manipulation

If differences had been found between males in offspring sex ratio, it would be difficult to obtain evidence as to how this is achieved. Williams (1979) emphasises that the sex ratio at the level of gamete production is restricted to 1:1, in the heterogametic sex, as an automatic result of the segregation of sex chromosomes at meiosis. It is possible that male stickleback could alter the sex ratio of the sperm produced by selective destruction before fertilisation. This might involve differential immunological responses to male and female sperm, mediated through antibody production. However, there is no evidence to support this idea.

An alternative strategy might be selective cannibalism of embryos during incubation. Egg cannibalism in male sticklebacks is suggested to increase future reproductive success, as bodily reserves are maintained for future parental care (Rohwer, 1987). Filial cannibalism should be a viable strategy for the parental

male, only if leaving the nest to feed represents a risk to his offspring. Egg raiding by conspecific males and females is common in sticklebacks, the eggs being more nutritious than other foods (Fitzgerald, 1991). By eating his mate's eggs, the male exploits the female's increased foraging opportunities. However, eating fertilised eggs of the sex that has lower future reproductive value would require the male to be able to discriminate between embryos of each sex. This might be investigated experimentally by leaving half-clutches of eggs to develop in the nests of males and comparing the sex-ratio of hatched offspring with a control group of artificially incubated eggs. Should selective cannibalism be found to occur, a greater challenge would be the detection of plausible cues that the male could use to recognise the sex of developing embryos.

Section II: Determination of Sex from Fertilised Eggs

4.6 Introduction

Following large losses of fertilised stickleback embryos to fungus during incubation, it was decided to investigate the possibility of sampling and sexing eggs at an earlier stage of development. The following breeding season (April - July 2001) males were captured from the wild and encouraged to begin nest building in aquaria, at Glasgow University, as described earlier. Pairs of fish were allowed to mate in the male's nesting tank and the fertilised eggs removed to incubators. Eggs were sampled daily until hatching and the DNA extracted and quantified. Sex determination of each egg by PCR was then attempted.

4.7 Methods

Gravid females were introduced to the nesting tank of a receptive male, allowed to spawn in the nest and then removed. Clutches of fertilised eggs were transferred from the nest to individual incubation tanks. Between 3 and 8 eggs (depending upon initial clutch size) were sampled from each of ten clutches, daily, from fertilisation until hatching and stored in 100% ethanol at -20°C.

DNA was extracted from each egg/fry using the phenol/chloroform method (as in chapter 1, but omitting the initial phenol extraction step) and following precipitation, resuspended in 30µl TE. PCR sex determination was carried out, also as described earlier in this chapter. When the initial PCR of a sample of embryo DNA yielded no product, the PCR was repeated on up to two more occasions. The DNA concentration of each sample was measured using Hoechst 33258 dye (Biorad) that binds to grooves of DNA molecules to produce a highly

fluorescent complex. Resulting fluorescence from a dilute solution of DNA (5µl sample in 1ml Hoechst dye solution) was measured at an excitation wavelength of 360nm and an emission wavelength of 460nm (VersaFluor™ Fluorometer, Biorad). The DNA concentration was calculated from its fluorescence relative to a dilution series of standard calf thymus DNA (Biorad).

4.8 Results

DNA yield

Figure 4.8 shows the mean mass of DNA extracted from individual stickleback embryos. The DNA yield ranges from around 60ng from a 1-day-old embryo to over 1μg from a hatched fry on day 10.

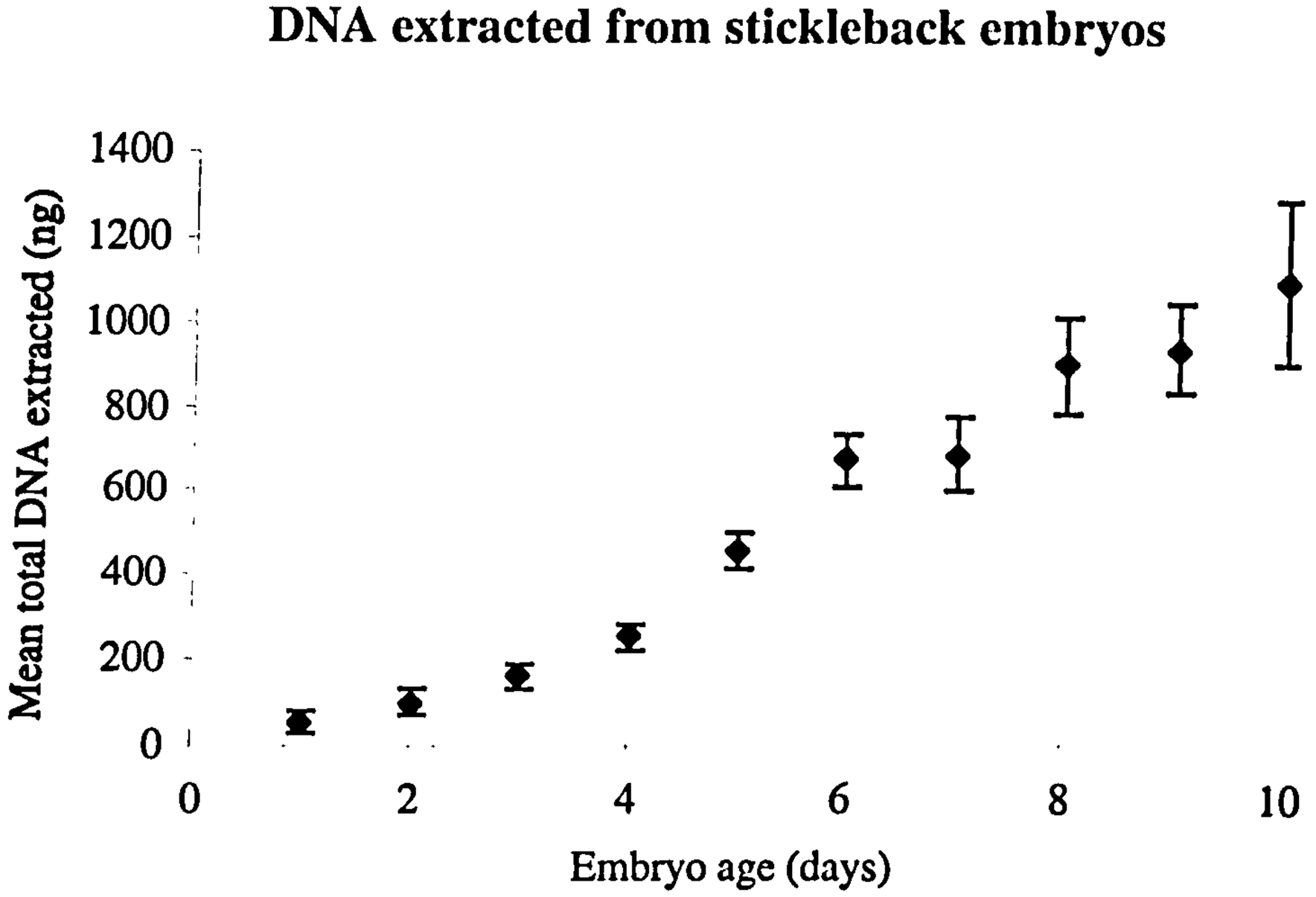

Figure 4.8: The mean of the total DNA extracted from each embryo is shown, along with the standard error. The sample size for each day is around 50 embryos.

PCR efficiency

The percentage of successful PCR reactions (the number of embryos that could be sexed within three PCR attempts, expressed as a proportion of the total number of embryos from each group) for each of the groups is shown in Figure 4.9. Embryo growth is such that 98% of embryos can be successfully sexed by the

third day of incubation. PCR efficiency was lower (80%) for embryos aged between 6 and 8 days.

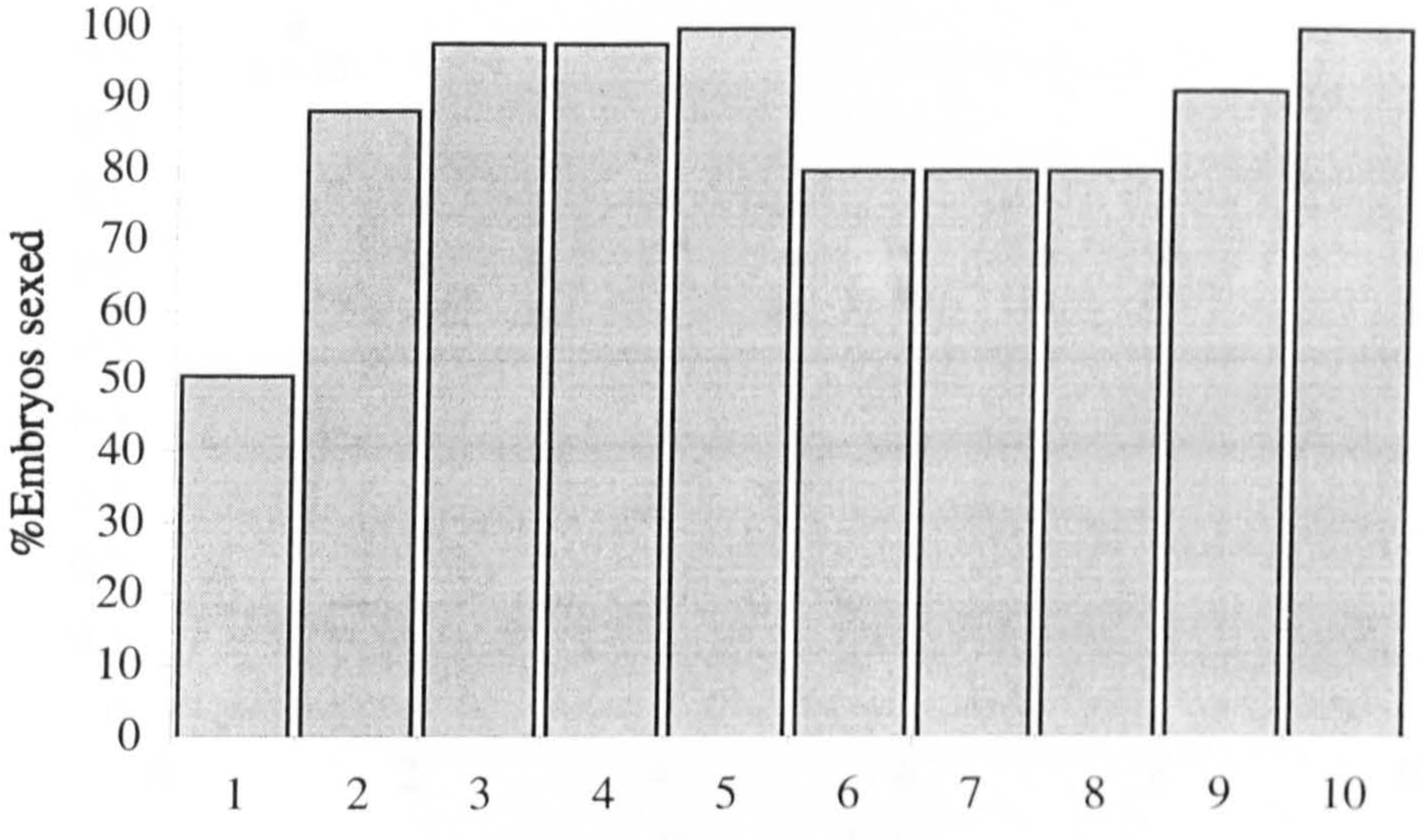

Figure 4.9: The percentage of embryos that could be sexed by PCR, following DNA extraction. PCR was attempted a maximum of 3 times with samples that were unproductive.

Sex ratio

The proportion of male embryos sampled daily is shown in Figure 4.10. A significantly greater proportion of embryos that were sampled shortly after fertilisation (day 1) were male than during the remainder of the sampling period (days 2-10) ($\chi^2 = 4.3$; $p \leq 0.05$; 1 *d.f.* with Yates correction). The overall sex ratio of embryos collected (excluding embryos sampled on day 1) was 64% (males: n = 211; females: n = 120). The number of samples sexed as males (excluding embryos sampled on day 1), was significantly greater than the expectation of equal

numbers of males and females ($\chi^2 = 11.46$; $p \leq 0.001$; 1 *d.f.* with Yates correction).

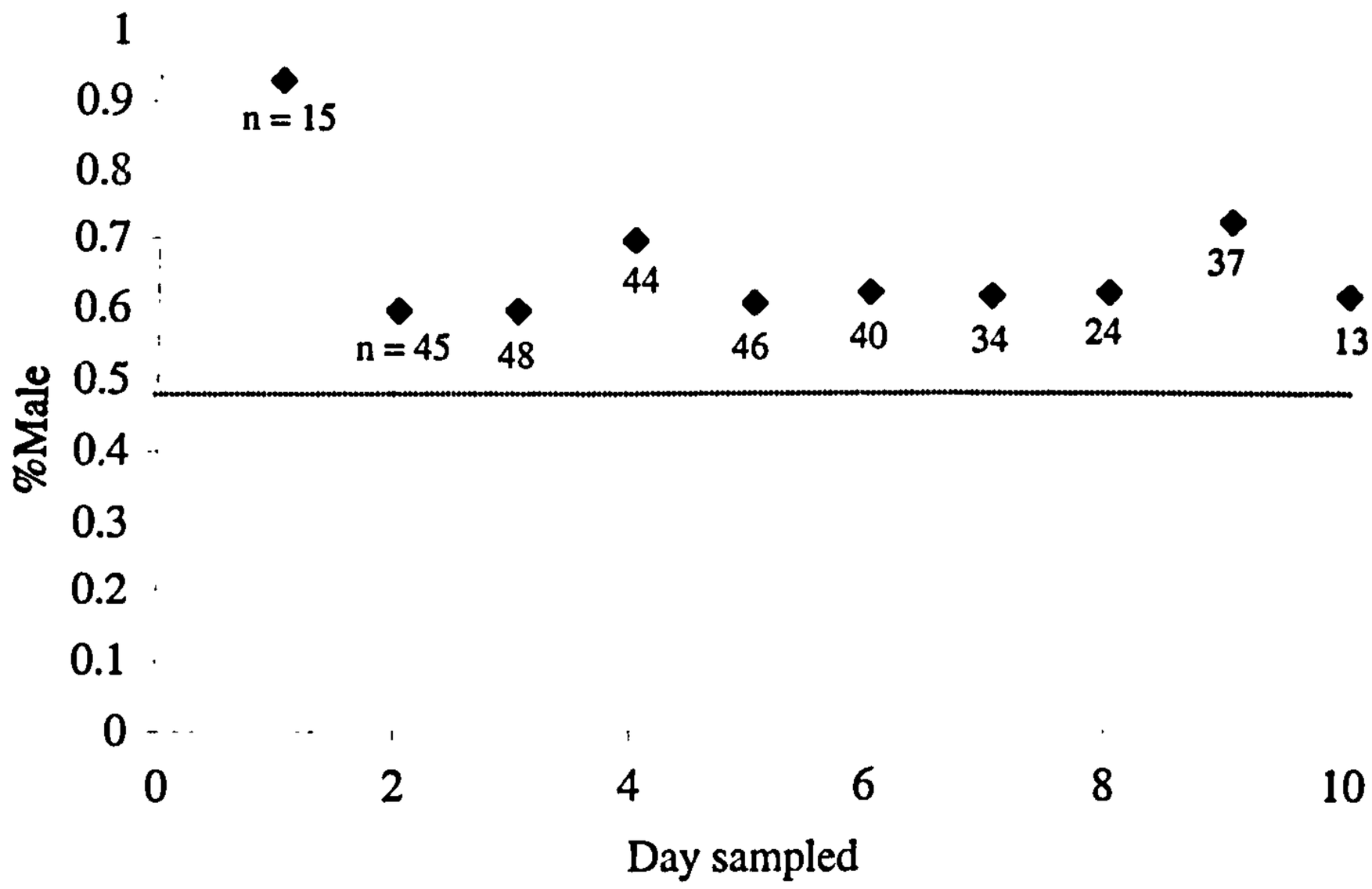

Figure 4.10: The proportion of stickleback embryos sexed as male, sampled during development (days 1-10). The dotted line shows the expected proportion of males (0.5). Each co-ordinate is annotated with the sample size (n), used to the calculate proportions (minimum, n =13; maximum, n = 48).

4.9 Discussion

Sexing stickleback embryos

The results of the embryo sexing experiment show that successful offspring sexing can be carried out when embryos are 3 days old. Since the total mass of DNA extracted doubles between embryos of 3 and 4 days of age, it would be sensible to use four-day-old embryos for sex ratio analysis. In addition, the age at which embryos can be sexed is likely to vary with temperature, as this affects the rate of embryo development (Wootton, 1976). The decrease in success of sexing embryos between 6 and 8 is puzzling, as they seemed to contain adequate DNA when quantified by fluorometry. Each of these DNA samples failed 3 separate PCR reactions; therefore it might be assumed that they were remained contaminated by PCR inhibitors following extraction. Unfortunately, as the entire embryo is used in the DNA extraction procedure, there is no possibility of re-extracting failed samples.

Stickleback sex ratio

It is interesting that the overall hatching sex ratio of stickleback fry in both the embryo sexing (this section) and the previous experiment (section I) was significantly male-biased. This bias could represent the primary sex ratio at fertilisation or be a result of differential embryonic mortality (if females were more likely to die during incubation).

The results of embryo sexing (Figure 4.10) show that there are significantly more embryos sexed as male on day 1 than during the remainder of the development. The high proportion of males (93%) found at fertilisation is possibly due to the presence of Y-chromosome bearing sperm in the samples, that

may have been washed away by the time that embryos were collected from the incubators on following days. The embryos sampled on day 1 were, therefore, excluded from the calculation of pooled embryo sex ratio. The sex ratio seems to remain constant across the subsequent incubation period, and few of the embryos were lost to fungus. It would appear that the sex ratio of fertilised embryos at day three or four of artificial incubation is a good indicator of the sex ratio at hatching, whilst also reflecting the sex ratio at fertilisation.

Little is known about the juvenile or adult sex ratio in wild stickleback populations. The sex ratio of two populations of fish, from the Baltic Sea and Llyn Frongoch, in Wales, was estimated by collection during breeding and found to be female-biased (Aneer, 1973 and Allen, 1980, cited in Wootton, 1984). Wootton (1984) suggests that sampling sex ratio during the breeding season is complicated by the behaviour of territorial males and schooling females, juveniles and unsuccessful males. Differences in the likelihood of catching males and females make it difficult to obtain a representative sample.

Molecular sexing of spine samples from adults captured in Inverleith Pond, Edinburgh was carried out between March and October (Arnold *et al.*, unpublished). The sex ratio of fish caught in early spring was even, with adult males decreasing significantly throughout the breeding season. Dermal cysts of the parasite, *Glugea*, were also counted on each individual and found to be more prevalent in males.

Sex differences in parasitism have been found in another population. Reimchen and Nosil (2001) found that males and females were infested with differing parasites, which could be related to contrasting feeding ecology. Females of the population studied tended to be limnetic whilst males foraged in the benthic zone, leading to differences in consumption of parasite host prey. It is

suggested that males may be more susceptible to parasitism due to increased physiological stress arising from the demands of sexual selection and parental care. Males may also be more susceptible to infestation as a result of elevated testosterone and stress, which may lead to decreased immunocompetence (Folstad and Karter, 1992). Whatever the cause, it would appear that males are more likely to become diseased and have reduced survival during the breeding season. The techniques described here would be very useful for the further examination of sex ratio in natural populations.

Chapter 5

Post-glacial Microsatellite Variation amongst Three-spined Sticklebacks from North East Scotland

5.1 Introduction

Extensive divergence in morphology, behaviour and life history between populations, within a single species, has been observed in a vast array of organisms. Examples include insects (e.g. Bush, 1966), molluscs (Wilding *et al.*, 2002), isopods (Merilaita, 2001) and echinoderms (Vadas *et al.* 2002) as well as amphibians, birds and fish (reviewed by Smith and Skulason, 1996). Many of these intra-specific alternative phenotypes appear adaptive and may be selected for at a genetic level.

Amongst fish, species that have colonised post-glacial lakes provide numerous examples of niche-based divergence (reviewed by Schluter, 1996) whereby polymorphism within species often entails adaptations that exploit different foraging environments within an ecosystem. Evidence for environmentally induced morphological variation has been found in many salmonid and coregonid species, as well as the three-spined stickleback *Gasterosteus aculeatus.*

The Arctic charr, *Salvelinus alpinus* exhibits morphs that, within a single lake, may include up to two benthic feeding forms, a planktivorous and piscivorous morph. Morphs vary in trophic morphology, colouration and behaviour and also show genetic differentiation, displaying different levels of speciation across localities (reviewed by Jonsson and Jonsson, 2001). Whitefish, *Coregonis clupeaformis* exhibit similar phenotypic diversification in relation to differential resource use. Lu and Bernatchez (1999) found that more highly specialised sympatric Whitefish morphs showed reduced levels of gene flow (measured by microsatellite genotyping) between them, compared to sympatric morphs that showed less phenotypic differentiation. Thus, the selective force that

drives divergence in morphology appears to simultaneously induce reproductive isolation. This process, described as ecological speciation by Schluter (1996) involves population divergence-with-gene-flow (Rice and Hostert, 1993) and differs from more widely accepted models of allopatric speciation. The latter mechanism requires geographic isolation of populations, within a species, by physical barriers that prevent gene flow. Reproductive isolation between allopatric populations is assumed to arise via non-ecological mechanisms such as genetic drift, founder effects or the fixation of alternative alleles in response to similar selection pressures (summarised in Mayr, 1963).

The Three-spined stickleback also shows extensive morphological variation between populations, and the evolution of sympatric morphs in this species has received considerable attention (Nagel and Schluter 1998, Taylor and McPhail, 2000). The ancestral marine form of the stickleback has undergone significant ecological divergence, following widespread colonisation of post-glacial freshwater habitats. The species has a wide geographical range within the Northern Hemisphere and is found around the margins of the Atlantic and Pacific Oceans (Bell and Foster, 1994).

The research described in this chapter collaborates with a study of morphological variation between stickleback populations, from the Moray Firth coastal region of Scotland, an area that is geologically well understood. I have carried out microsatellite genotyping of some of the stickleback populations that were sampled. Stickleback were collected for study by Dr Steve Arnott and Dr Iain Barber, who sampled populations from 14 lochs, in order to relate morphology to post-glacial history. Analysis of eight characteristics (numbers of lateral plates, dorsal rays, anal rays, gill rakers; body depth, jaw length, gill raker length and stomach contents) was used to examine divergence. Of particular

interest was the presence of spineless and spined morphs in Loch Ruthven that may represent reproductively separate sympatric populations. These might arise either through sympatric speciation or through a sequential post-glacial invasion. It was decided to investigate the relationship between these samples and the ancestral form (represented by a local marine population, sampled from salt marshes at Carse of Delnies) and to compare these populations with a solitary population from Loch a` Choire, upstream of Loch Ruthven (see Figure 5.1).

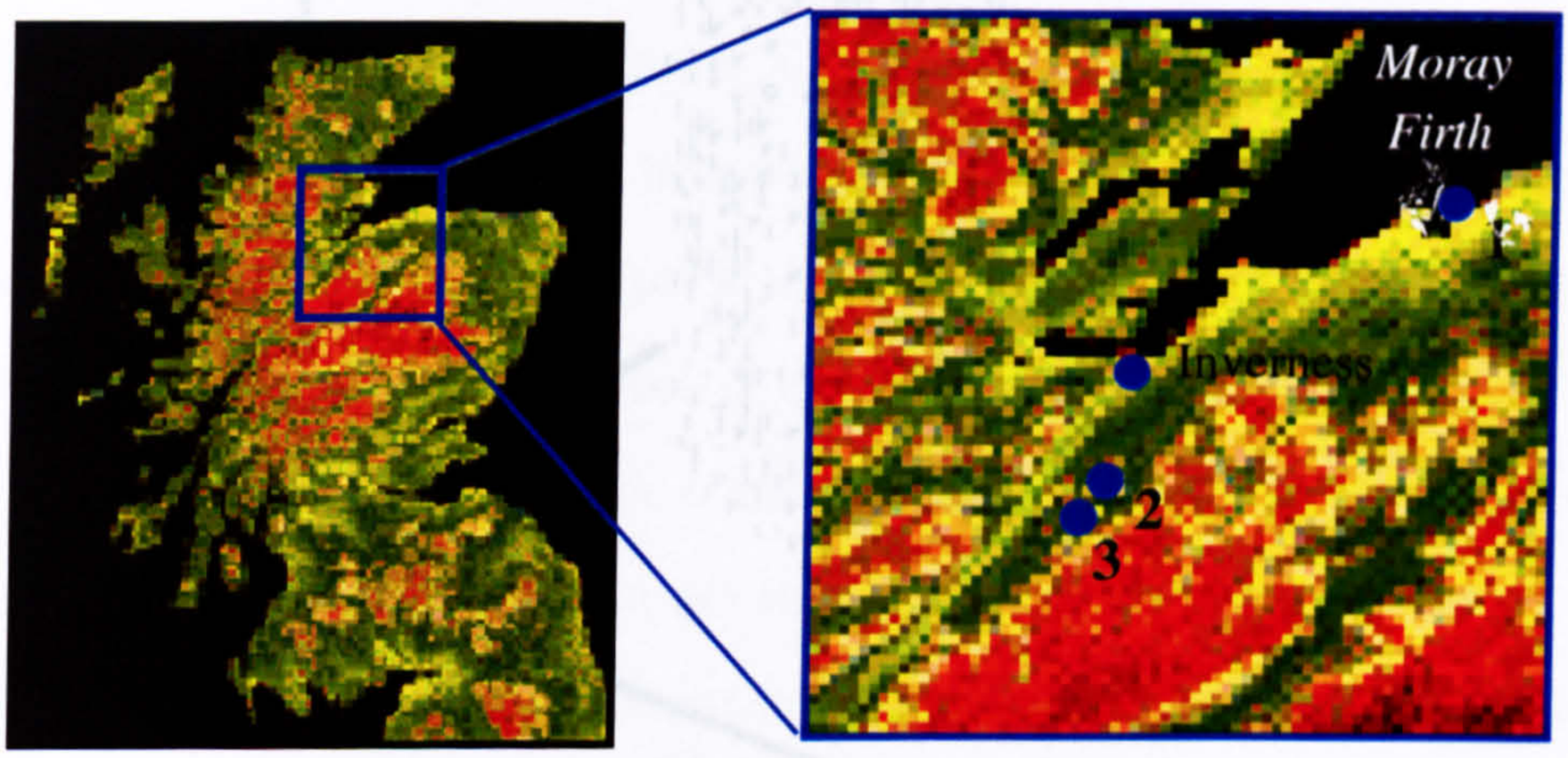

Figure 5.1: Map of Scotland with an expanded view of the Moray Firth coastal region. The locations of stickleback populations that were sampled and used in microsatellite analyses are shown: (1) Carse of Delnies (2) Loch a` Choire (3) Loch Ruthven.

Stickleback speciation

Three-spined sticklebacks present in the marine fossil record, up to 10 million years ago, are very similar to the extant marine form. Outgroups to this species are generally marine, suggesting that *G. aculeatus* is ancestrally marine. From this fairly homogenous marine stock it would seem that populations have

colonised a wide variety of inland habitats. These freshwater groups diverge from the marine ancestral state to form a phylogenetic raceme (Figure 5.2).

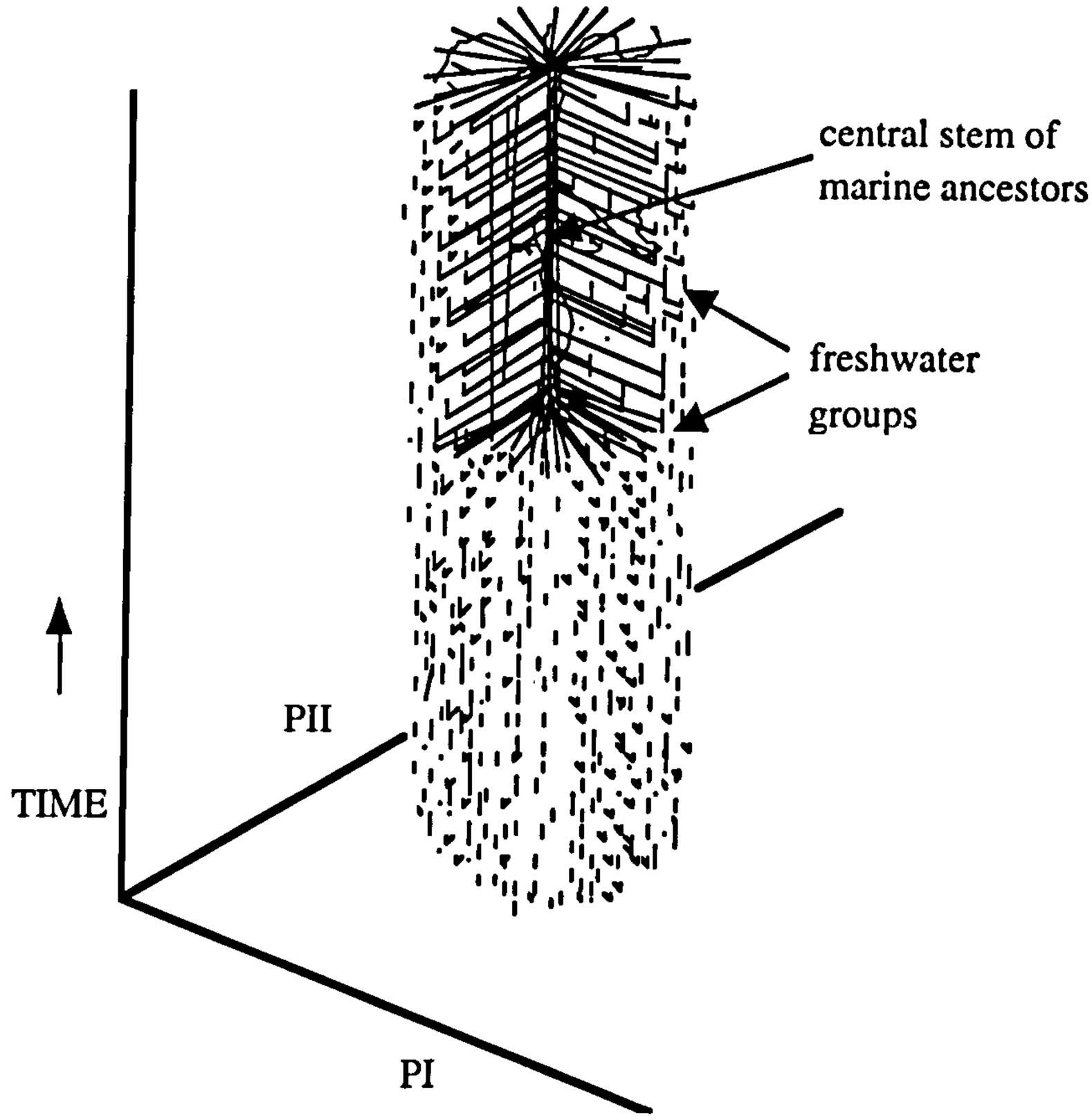

Figure 5.2: The phylogenetic raceme of the *Gasterosteus aculeatus* species complex. Diversification is shown in a hypothetical two-dimensional phenotypic space (abscissas: PI, PII) with an upper section cut away to show the internal structure (from Bell and Foster, 1994).

The pattern of evolution of the stickleback differs from the conventional phylogenetic tree in that there is a central axis (analogous to the central axis of an inflorescence from which many flowers project) consisting of a phenotypically and temporally stable lineage (the marine form). Short-lived specialised isolates (freshwater groups) diverge rapidly and frequently over time from marine

populations, following invasion of heterogeneous freshwater habitats, which are often subject to geographical isolation (Bell and Foster, 1994).

Stickleback populations around the North West of British Columbia are particularly well studied, not least due to the occurrence of sympatric species pairs in six locations. McPhail (1984) describes the pairs present in small lakes on islands in the Strait of Georgia region. He examined benthic and limnetic morphs from Enos Lake for meristic counts (e.g. gill raker number), morphometrics and allozyme variation as well as laboratory-reared offspring from both parental forms and their reciprocal F_1 and F_2 hybrids. Consistent differences were found in gill raker number and body shape in both wild and laboratory reared morphs, whilst hybrids were intermediate in traits. The two forms also differed significantly in allele frequency at two allozyme loci.

These benthic and limnetic morphs show parallel evolution in each lake (Taylor *et al.*, 1997). Limnetics are found in open water (except during the breeding season, when both forms nest in the littoral zone) and are slender bodied and smaller with a narrower gape than benthics. The gill rakers are longer and more numerous, in the limnetic population, so that plankton are efficiently sieved from the water. Limnetic fish are therefore similar to the planktivorous marine population (Taylor and McPhail, 1999). Benthic fish are deeper bodied with a wide gape for feeding upon littoral invertebrates.

Initial analysis of mtDNA suggested that significant genetic differences between the morphs had arisen independently in four lakes and that two of the pairs appeared to be monophyletic, indicating sympatric divergence. In the remaining two lakes the limnetic form was more closely related to the putative marine ancestor, supporting the hypothesis that the plankton-feeding morphs are derived from a secondary post-glacial invasion (Taylor and McPhail, 1999).

Further genetic analysis at six microsatellite loci has shown that these freshwater populations have lower allelic diversity than the marine stock, as would be predicted for populations founded independently from a limited sample (Taylor and McPhail, 2000). The microsatellite analysis also suggests that none of the species pairs are monophyletic. The sympatric divergence of two species pairs suggested by Taylor and McPhail's (1999) mtDNA study seemed less likely following phylogenetic analysis that included the microsatellite data. Instead, the limnetic morphs appeared more closely related to their marine ancestor than the benthics, giving support to the allopatric scenario of a double invasion (Taylor and McPhail, 2000). Nevertheless, ecological selection in sympatry may still contribute to the evolution and maintenance of polymorphism between the pairs.

A low level of hybridisation occurs between wild benthic and limnetic populations (around 1%, McPhail, 1992). Hybrids are fertile but, being intermediate in form, have a foraging disadvantage, with reduced feeding efficiency in both prey habitats (Schluter, 1993). During the breeding season, the two morphs are often found nesting in close proximity in the littoral zone of the lakes (Foster *et al.*, 1998). In order to test whether prezygotic isolation is reinforced in sympatry, Rundle and Schluter (1998) conducted a series of laboratory experiments comparing mating preferences of benthic females with morphologically similar females from solitary (allopatric) populations. Mate choice trials showed that the benthic females discriminated between benthic and limnetic males whereas allopatric females did not. In addition, limnetic males were found to show a departure from the ancestral preference for large females, instead choosing smaller mates. Body size differs markedly between the forms and was found to act as a basis for divergent mate selection between morphs by

Nagel and Schluter (1998). Reproductive isolation may also be facilitated by the evolution of differences in male courtship behaviour (Nagel and Schluter, 1998).

Assortative mating between stickleback morphs may provide an example of reproductive character displacement - whereby low hybrid fitness reinforces premating isolation (Rice and Hostert, 1993). Environmental reduction of hybrid fitness has been tested experimentally in sticklebacks (Vamosi *et al.*, 2000). Artificial ponds and lake enclosures were stocked with laboratory-reared F_1 hybrids and parental species and subsequent growth and survival monitored over several months. Relative survival of hybrids was lower than expected and their growth rate was slightly lower than that of benthics. Limnetics were slower growing, being smaller as adults.

The morphological and behavioural differences that have arisen through stickleback microevolution have been shown to have a heritable genetic basis, and persist for at least two generations when bred in the laboratory (McPhail, 1984). The genes controlling morphological variation of the stickleback have been mapped to chromosomal regions by Peichel *et al.* (2001). Genomic library screening and sequencing identified 227 microsatellite markers that were used to type the offspring of a limnetic/benthic cross. Linkage analysis found quantitative trait loci that accounted for two thirds of the variation in the number of small gill rakers. Other characters such as spine length and lateral plate number were also linked to different loci. Some traits mapped to similar regions of the chromosome indicating the importance of genetic linkage or pleiotropy in the covariation of characters.

Microsatellites

Microsatellites are repeated sequences of DNA, with a repeat unit of less than six base pairs. They are randomly distributed throughout non-coding regions of eukaryotic genomes; with CA repeats being the most common motif in vertebrates (see Hancock, 1999, for review). Unlike allozymes, that show little variation, microsatellites are highly polymorphic. They have a relatively high mutation rate, are easily scored and are therefore extensively used as markers for measuring gene flow and population subdivision (Sunnucks, 2000).

Several functional roles of microsatellites have been proposed (Kashi and Soller, 1999 review). Their properties as regulatory sequences have been investigated by Hamada *et al.* (1984) who inserted a TG repeat array into expression vector plasmids, which were then transfected to cells in culture. The expression of plasmid genes was subsequently enhanced by the microsatellite. Insertion of the Z-DNA repeat $d(TG)_{30}$ is thought to induce a change in strand conformation during homologous pairing of chromosomes that promotes recombination (Wahls *et al.*, 1990). Pardue *et al.* (1987) noted a non-random distribution of certain repeat sequences in *Drosophila* chromosomes. A particularly high density of $(dC\text{-}dA)_n.(dG\text{-}dT)_n$ stretches was revealed on the X chromosome by *in situ* hybridisation. The distribution appears to be evolutionarily conserved between *Drosophila* species and is correlated to dosage compensation (whereby transcription from the single X chromosome of the male is higher than in females, who have two copies of X).

Tri-nucleotide repeat expansions at selected loci are implicated in human disease. Five classes of trinucleotide-repeat diseases have been identified, in conditions such as myotonic dystrophy, Fragile X syndrome and Freidrich's ataxia

(reviewed by Rubensztein, 1999). However, for the purpose of population genetic studies, microsatellites are assumed to be selectively neutral, as loci are selected for comparison at random (Jarne and Lagoda, 1996).

Mutation

Microsatellite mutation is thought to arise by strand slippage during DNA replication. Repetitive sequences in the nascent (newly copied) strand may reanneal out of phase to the template, giving rise to looped-out bases in either strand, shortening or lengthening the product (Hancock, 1999). Schlötterer and Tautz (1992) analysed slippage synthesis of DNA *in vitro*. Simple sequence primers were used in PCR to synthesise varied repetitive di- and tri-nucleotide repeats, indicating that slippage occurs frequently and at a high rate. Unequal chromatid exchange and genetic recombination may also change microsatellite repeat number (Shriver *et al.*, 1993). Estimated rates of microsatellite mutation vary between species (reviewed by Hancock, 1999; Jarne and Lagoda, 1996). Analysis of human pedigrees by Weber and Wong (1993) found a mutation rate of 1.2×10^{-3} per locus per gamete per generation, in microsatellite repeat number.

Microsatellite evolution has been the subject of much theoretical work with three main models of mutation having been proposed. The infinite allele model (Kimura and Crow, 1964) predicts that each mutation creates a new allele at a rate, u. Identical alleles share a common ancestor and novel mutations cannot result in a new allele of the same sequence as those existing previously. The K allele model assumes that the number of possible alleles is K, with a probability (K-1) that the allele will mutate to any other allelic state (Crow and Kimura, 1970). Under the stepwise mutation model (Kimura and Ohta, 1978), each mutation

lengthens or shortens the microsatellite array by one repeat unit. This means that alleles are more closely related when they are similar in size, but that they may also mutate towards allelic lengths already present in the population, that are not identical by descent (reviewed by Estoup and Cornuet, 1999; Balloux and Lugon-Moulin, 2002).

Models of microsatellite evolution may also be rendered inaccurate by bias in the direction of length mutations. Weber and Wong (1993) found that mutations most commonly involved an increase, rather than a decrease in microsatellite length. In addition, there may be constraints upon maximum and minimum allele sizes that result in reduced heterozygosity, relative to that expected under the step-wise mutation model (Neilsen and Pasboll, 1999). Range constraints may vary between loci, along with mutation rate, the effect being most extreme when comparing divergent taxa. Pollock *et al.* (1998) describe procedures for estimating these parameters to improve phylogenetic reconstruction using microsatellite data.

In populations that have undergone a recent bottleneck, most of the existing microsatellite mutations are likely to have arisen from single recent mutations (Estoup and Cornuet, 1999). This reasoning was applied to a study of Honey bee (*Apis mellifera*) sub-populations to compare mutation models. Simulation studies of two loci rejected the stepwise mutation model in favour of the infinite allele model (Estoup *et al.*, 1995). However, the distribution of allele sizes in Weber and Wong's (1993) pedigree study lends support to the single step model. In addition, Shriver *et al.* (1993) compared probability distributions of number of alleles, modes, range in allele size and heterozygosity for a range of mutation rates to expectations derived from computer simulations. Microsatellite

loci were most similar to simulated results under the stepwise mutation model and showed deviations from the infinite allele model.

More recently, a two-phase model was proposed by DiRienzo *et al.* (1994). This model predicts most mutational changes to be of single base pairs with a lower occurrence of larger mutations. The observed variance of the allelic distributions of a sample of ten loci, from a human Sardinian population, was compared with theoretical predictions of the single step mutation model and the two-phase model. For 8 out of 10 loci examined, the single step was rejected in favour of the two-phase model. Nevertheless, it is clear that no single mutation model yet described can accurately describe the evolution of all microsatellite loci.

Measuring population differentiation

The most commonly used statistics for the estimation of population structure from microsatellite alleles are F_{ST} and R_{ST}. Wright's (1951) F_{ST} assumes that the loci follow the infinite alleles mutation model, and can be defined as the correlation between two alleles sampled randomly from sub-populations relative to alleles chosen at random from the total population. The F_{ST} value is 0 when sub-populations have identical allele frequencies and reaches 1 when the sub-populations share no similarities.

Given evidence that most mutations occur as single steps, Slatkin (1995) devised the R_{ST} statistic, appropriate to the step-wise mutation model. Unlike F_{ST}, which is calculated from the variances in allele frequencies, R_{ST} is derived from the variance in allele sizes. Using computer simulation, Slatkin (1995) found that estimates of F_{ST} showed too much genetic similarity between populations, especially following long periods of divergence. Nevertheless, tests based on R_{ST}

can be less powerful, due to its high associated variance (Balloux and Lugon-Moulin, 2002). Comparisons of F_{ST} and R_{ST} under different simulated conditions were made by Balloux and Goudet (2002), who found that R_{ST} gives a better reflection of population structure under low-levels of gene flow, whilst F_{ST} gives better estimates for less highly structured populations with greater levels of gene flow.

5.2 Methods

Fish were collected from lochs in the Moray Firth coastal region of Scotland during the breeding season (May-June) using seine nets and traps. This avoids biased sampling due to trophic selectivity, as the fish nest in littoral zones. Between 40-80 fish were sampled from each site and frozen at -20°C. Following morphological analysis, a small amount of tissue was dissected from the gill opercula for DNA extraction.

DNA extraction: salt method

1) Each tissue sample (around $1cm^3$) was finely chopped in a 2.5ml eppendorf, containing 250µl Digestion solution, using small dissection scissors. The scissors were washed in 0.5M HCl and distilled water between samples to prevent cross-contamination.
2) 5µl (10mg/ml) Proteinase K (Promega) was mixed into each sample using a vortex mixer and digestion was carried out overnight in and orbital incubator at 55°C.
3) 250µl 4M ammonium acetate was added and the samples left at room temperature for 15 minutes, mixing regularly.
4) Samples were centrifuged at 18 000 × *g* for 10 minutes. The supernatant was transferred to a clean tube and mixed with 500µl 100% ethanol to precipitate the DNA.
5) The DNA pellets were washed in 70% ethanol and air-dried for 15 minutes.
6) Samples were resuspended in 50µl TE, overnight, in an orbital incubator at 55°C and stored at -20°C.

Microsatellite selection

For this study I selected primers for five loci identified by Taylor (1998) and Largiadèr *et al.* (1999). The primers were tested using DNA from a selection of stickleback isolates, including those used in this study and samples from Wales and Edinburgh. Each primer was checked to ensure that the microsatellite products were polymorphic. Some primers were rejected, as they did not produce differing products between any individuals, following PCR and electrophoresis on 4% agarose (see conditions below).

PCR

Optimal PCR conditions for amplification of microsatellite fragments were selected, following comparison of PCR performance at different annealing temperatures (a gradient of 45-55°C was used). The concentration of magnesium chloride in the reaction mix was also optimised by comparing a range from 0 to 3.5 mM in 0.5mM increments. A concentration of 2.5mM was found to be optimal and an annealing temperature of 50°C, as these conditions resulted in large amounts of PCR product with all primer sets. Products were visualised by electrophoresis of 5µl aliquots (with 10 × Orange G loading dye) in 4% 10 cm agarose gels (with ethidium bromide staining) in 1 × TBE buffer, at 100V for 20 minutes. A 4µl aliquot of 1kb DNA ladder (Promega) was included in each row of lanes to provide a scale. Gels were then placed on a transilluminator and photographed.

Details of the primers and microsatellite loci used are shown in Table 5.3. Each primer set was used with each DNA sample to find the lengths of all five loci in every individual. One primer from each pair was end-labelled with a 5' ABI

dye (Sigma-genosys, UK). Different coloured dyes (6-FAM or HEX) were used in primers for longer or shorter microsatellites, so that the products from amplification of two loci could be run together in one gel lane and later distinguished by size and the colour of dye fluorescence emission.

PCR

Reactions were carried out in 10µl under the following conditions:

Primers (forward and reverse, 0.1 mg/ml)	0.8 µl
dNTP (10mM)	0.8 µl
10 × PCR reaction buffer (Promega)	1.0 µl
$MgCl_2$ (25mM)	1.0 µl
dH_2O	2.725 µl
Taq (5 units/µl, Promega)	0.075 µl
DNA	2.0 µl

PCR conditions were as follows: 2 minutes at 94°C followed by 30 cycles of 50°C for 30 seconds, 72°C for 20 seconds and 94°C for 30 seconds, then 1 minute at 50°C and 5 minutes at 72°C (extra extension time to increase yield). A negative control for each batch of extracted DNA and a PCR control containing no DNA were included in each PCR to check for extraction or PCR contamination.

Following PCR, 5µl of each sample was run on a 4% agarose gel with a 1kb DNA ladder, in 0.5 × TBE buffer at 120V, for 20-30 minutes to confirm that the PCR had worked, check for contamination, and to estimate the amount of dilution required for each product.

Locus	Repeat Array	Primer Sequences (5'→ 3')	Size Range (bp)	H_E	Genbank Accession no.	Source
Gac 3133PBBE	$(CA)_{21}$	CGCCCAGTTCCTGAACTTAG CATGGTGGGCTGACTGAC	138-150	0.46	AJ010356	Largiadèr *et al.* (1999)
Gac 1116PBBE	$(CA)_{40}$	GGTGTCATGTGGGGGCGAGCAG CCCGAAGCATTGTGGCATCATC	124 -184	0.72	AJ010353	Largiadèr *et al.* (1999)
Gac 4174PBBE	$(CA)_{24}CG(CA)_7$	CCGCGATGATGAGAGTG GTGAAATGCGACAGATGATG	204 - 214	0.72	AJ010358	Largiadèr *et al.* (1999)
Gac 7188PBBE	$(CA)_{45}$	CCCCTCACACATAGTTACAC TTCAATTGGGAGAGAAGC	143-203	0.53	AJ010361	Largiadèr *et al.* (1999)
Gacμ10	$(CA)_{40}$	CCTTCAGATCGGGACATCGATCCC CACCCACACGCAATCCCAGGAATG	148 - 198	0.42-0.90	AFO30257	Taylor (1998)

Table 5.3: Microsatellite loci in the analysis of genetic variation in the Three-spined stickleback, *Gasterosteus aculeatus*. The repeat arrays, primer sequences, allele size range (from reference), expected heterozygosity, Genbank accession number and source reference are given for each locus.

Microsatellite measurement

The PCR products were diluted with distilled water, according to the intensity of ethidium bromide fluorescence shown by DNA bands in the gel. Samples were then loaded onto an acrylamide gel with a 500bp marker (PE Biosystems) and run through an ABI PRISM 373 DNA sequencer (PE Biosystems) by the Molecular Biology Sequencing Unit, GeneScan Service, University of Glasgow.

Analysis of the GeneScan results was carried out using GeneScan analysis software (Applied Biosystems). An example of a digitised gel image is shown in Figure 5.4. and a fluorescence profile of one of the lanes from this gel is shown in Figure 5.5. The peaks show the lengths of two microsatellite loci, measured in base pairs. Data analysis requires the repeat number of each motif, at each locus. This is calculated by subtracting the length of the primers and flanking sequences (available from the Genbank sequence) and dividing the remainder by the length of the repeat, e.g. a CA repeat sequence is divided by two to find the number of repeats.

Microsatellite gel image

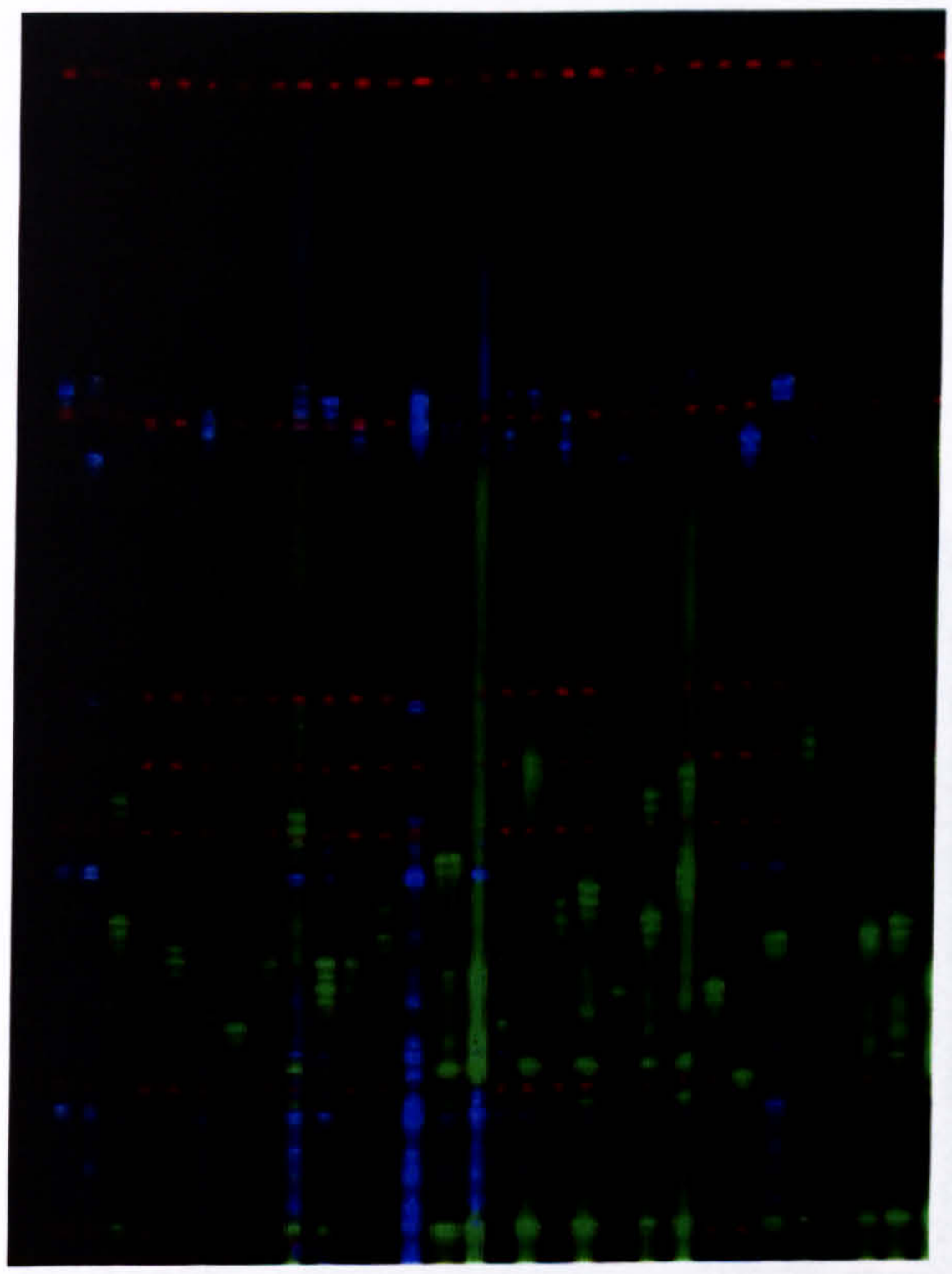

Figure 5.5

Figure 5.4: GeneScan gel image showing microsatellites amplified from two loci: Gac4174PBBE (blue) and Gac116PBBE (green). The base-pair marker is shown in red with lengths on the right.

GeneScan fluorescence profile

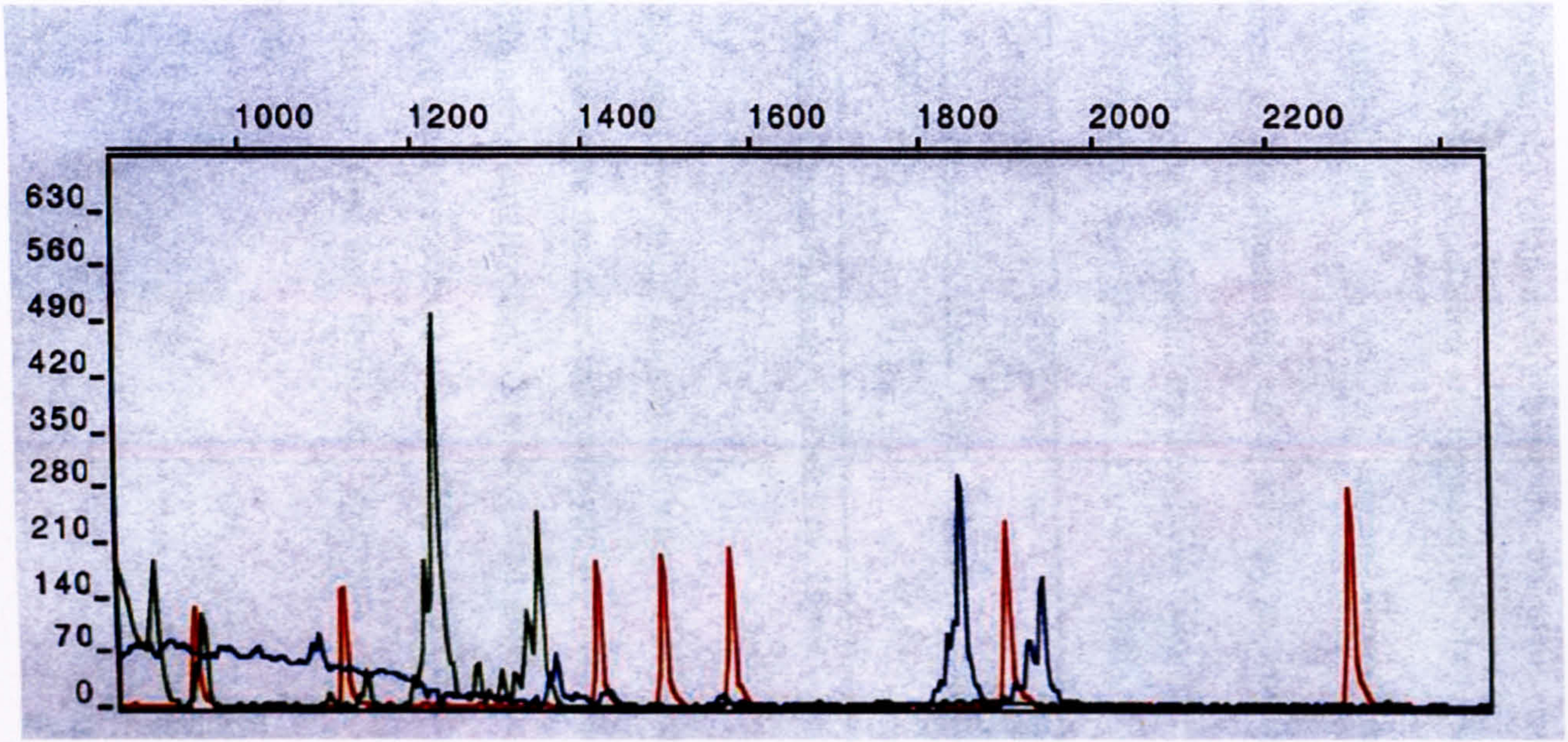

Figure 5.5: Fluorescence profile of the lane indicated by the arrow in Figure 5.4. The peaks shown in red are a kilobase marker scale. The two peaks shown in green are microsatellite alleles at locus Gac116PBBE (112 and 130 base pairs in length) and those in blue are alleles at Gac4174PBBE (193 and 205 base pairs).

5.3 Results

The microsatellite motif repeat number, at each locus, was calculated from the allele lengths for each individual. This genotypic data was analysed using Arlequin software (Schneider *et. al*, 2000)

Variability within populations: Hardy-Weinberg equilibrium

In large populations that exhibit random mating with no selection, mutation or migration, the relationship between the gene frequencies and genotype frequencies of the population are expected to obey Hardy-Weinberg law. For multiple alleles, where A_1 and A_2 are any two of the alleles and they have frequencies in the population of q_1 and q_2 respectively, the expected Hardy-Weinberg genotype frequencies are derived as follows:

Genotype:	A_1A_1	A_1A_2	A_1A_2
Frequency:	q_1^2	$2q_1q_2$	q_2^2

(Adapted from Falconer and McKay, 1996)

The observed allele frequencies within a population, at a given locus can be used to estimate the expected genotype frequencies in the population, under Hardy-Weinberg equilibrium. The observed genotype frequencies can then be compared to those expected using goodness-of-fit methods. A Hardy-Weinberg equilibrium test (Guo and Thomson, 1992) was carried out for each stickleback population.

The observed and expected microsatellite genotype frequencies are shown for each locus in Tables 5.6 - 5.9, along the probability of sampling the observed allele frequencies at each locus, if the population is at Hardy-Weinberg equilibrium.

For most of the loci typed for each population, the probability that the observed allele frequencies have been sampled from a population at Hardy-Weinberg Equilibrium is less than 0.05. The exceptions are the observed allele frequencies at locus Gac7188PBBE in the spined morph sample from Loch Ruthven (Table 5.7) and at two loci Gac1116PBBE and Gac3133PBBE in the sample from the salt marsh (Table 5.8). For these loci, the null hypothesis - that the populations are at Hardy-Weinberg equilibrium, cannot be rejected.

The spined fish sampled from Loch Ruthven were collected at two separate sampling sites. In order to check for an effect of sampling site on population structure, a Hardy-Weinberg test was carried out on the allele frequencies observed in the spined morph sample, sub-divided by site of capture (Tables 5.10 and 5.11). Fish captured at site A showed allele frequencies at three loci, which could have arisen in a population at Hardy-Weinberg equilibrium, with a probability, $p > 0.05$ (Table 5.10). This was also found for one locus from the sample collected at site B (Table 5.11). The occurrence of deviations in the frequency of sampled genotypes from those expected, at Hardy-Weinberg equilibrium could be due to non-random mating within sub-populations. This can result in an excess of homozygotes, a condition known as the Wahlund effect (see discussion).

Hardy-Weinberg test: Loch Ruthven: spineless morph sample

Locus	*No. of Genotypes*	*Obs. Heter.*	*Exp. Heter*	*p. value*
Gac4174PBBE	20	0.55000	0.87444	0.008
Gac1116PBBE	20	0.45000	0.91410	<0.001
Gacμ10	20	0.70000	0.97179	<0.001
Gac3133PBBE	20	0.60000	0.93974	<0.001
Gac7188PBBE	20	0.65000	0.96538	<0.001

Table *5.6*: Number of genotypes and the observed and expected heterozygosities at five loci, from a sample of spineless stickleback morphs from Loch Ruthven (n = 20). The p-value denotes the probability of the observed allele frequencies being encountered in a population at Hardy-Weinberg equilibrium.

Hardy-Weinberg test: Loch Ruthven: spined morph sample

Locus	*No. of Genotypes*	*Obs. Heter.*	*Exp. Heter*	*p. value*
Gac4174PBBE	15	0.40000	0.82299	<0.001
Gac1116PBBE	14	0.42857	0.96296	<0.001
Gacμ10	13	0.61538	0.94769	<0.001
Gac3133PBBE	14	0.71429	0.94709	0.001
Gac7188PBBE	15	0.66667	0.92644	0.073

Table 5.7: Number of Genotypes and the Observed and Expected heterozygosities at five loci, from a sample of spined stickleback morphs from Loch Ruthven (n = 15). The p-value denotes the probability of the observed allele frequencies being encountered in a population at Hardy-Weinberg equilibrium.

Hardy-Weinberg test: salt marsh sample

Locus	*No. of Genotypes*	*Obs. Heter.*	*Exp. Heter*	*p. value*
Gac4174PBBE	17	0.64706	0.90731	0.015
Gac1116PBBE	17	0.88235	0.94652	0.501
Gacμ10	17	0.52941	0.91087	<0.001
Gac3133PBBE	17	0.76471	0.90374	0.053
Gac7188PBBE	17	0.58824	0.93048	<0.001

Table 5.8: Number of genotypes and the observed and expected heterozygosities at five loci, from a sample of stickleback from a salt marsh population (n = 17). The p-value denotes the probability the observed allele frequencies being encountered in a population at Hardy-Weinberg equilibrium.

Hardy-Weinberg test: Loch a' Choire sample

Locus	*No. of Genotypes*	*Obs. Heter.*	*Exp. Heter*	*p. value*
Gac4174PBBE	22	0.59091	0.66596	<0.001
Gac1116PBBE	22	0.45455	0.93763	<0.001
Gacμ10	22	0.68182	0.87209	0.002
Gac3133PBBE	22	0.40909	0.69345	0.007
Gac7188PBBE	22	0.63636	0.94080	<0.001

Table 5.9: Number of genotypes and the observed and expected heterozygosities at five loci, from a sample of stickleback from Loch a' Choire (n = 22). The p-value denotes the probability the observed allele frequencies being encountered in a population at Hardy-Weinberg equilibrium.

Hardy-Weinberg test: Loch Ruthven, spined morph, sampling site A

Locus	*No. of Genotypes*	*Obs. Heter.*	*Exp. Heter*	*p. value*
Gac4174PBBE	8	0.75000	0.85833	0.125
Gac1116PBBE	8	0.50000	1.00000	<0.001
Gacμ10	6	0.83333	1.00000	0.120
Gac3133PBBE	8	0.75000	0.89167	0.408
Gac7188PBBE	8	0.50000	0.85833	0.018

Table 5.10: Number of genotypes and the observed and expected heterozygosities at five loci, from a sample of spined stickleback from Loch Ruthven (n = 8) collected at site A. The p-value denotes the probability the observed allele frequencies being encountered in a population at Hardy-Weinberg equilibrium.

Hardy-Weinberg test: Loch Ruthven, spined morph, sampling site B

Locus	*No. of Genotypes*	*Obs. Heter.*	*Exp. Heter*	*p. value*
Gac4174PBBE	7	0.00000	0.78022	0.001
Gac1116PBBE	6	0.33333	0.95455	<0.001
Gacμ10	7	0.42857	0.94505	0.003
Gac3133PBBE	6	0.66667	0.93939	0.012
Gac7188PBBE	7	0.85714	0.92308	0.490

Table 5.11: Number of genotypes and the observed and expected heterozygosities at five loci, from a sample of spined stickleback from Loch Ruthven (n = 7) collected at site B. The p-value denotes the probability the observed allele frequencies being encountered in a population at Hardy-Weinberg equilibrium.

Variability between populations: Population pair-wise comparisons

F_{ST} and R_{ST} statistics were calculated summarise the degree of differentiation among samples (Tables 5.12 and 5.13). The p-values (shown bellow each statistic in parentheses) denote the probabilities, associated with each statistic, that the genetic difference between each pair of populations is zero.

Population pair-wise F_{ST} values

Population	*Ruthven (spineless)*	*Ruthven (spined)*	*salt marsh*	*Loch a' Choire*
Ruthven (Spineless)	0.000			
Ruthven (Spined)	0.018 (0.198)	0.000		
salt marsh	0.050 (<0.001)	0.049 (<0.001)	0.000	
Loch a' Choire	0.082 (<0.001)	0.082 (<0.001)	0.080 (<0.001)	0.000

Table 5.12: Population pair-wise F_{ST} values, calculated from the allele frequencies from each sub-population relative to the whole population. Each statistic represents the genetic difference between the sub-populations heading each row and column. P-values (shown in parentheses) denote the probability of observing the sampled genetic difference between each population pair, if $F_{ST} = 0$.

Population pair-wise R_{ST} values

Population	*Ruthven (spineless)*	*Ruthven (spined)*	*salt marsh*	*Loch a' Choire*
Ruthven (spineless)	0.000			
Ruthven (spined)	0.054 (0.090)	0.000		
Salt marsh	0.435 (<0.001)	0.315 (<0.001)	0.000	
Loch a' Choire	0.461 (<0.001)	0.390 (<0.001)	0.255 (<0.001)	0.000

Table 5.13: Population pair-wise R_{ST} values, calculated from the variance in allele sizes of each sub-population. Each statistic represents the genetic difference between the sub-populations heading each row and column.

Both F_{ST} and R_{ST} values show similar differences between the sub-populations. Each population pair are significantly different from one another ($p \leq 0.05$), with the exception of the Loch Ruthven morphs. The genetic difference between the spined and spineless population samples, based upon F_{ST}, is not significant ($p = 0.198$). The R_{ST} values are considerably larger but agree that the genetic difference between morphs is not significant ($p = 0.090$).

Possible evolutionary correlates of genetic differentiation

Linearized F_{ST} (Rousset 1997), as a measure of genetic distance, was compared to geographical and morphological population parameters. Genetic distances were calculated for each population, from the F_{ST} value relating to the putative ancestral (salt marsh) population, as follows:

Linearized $F_{ST} = F^{ST}/ (1 - F_{ST})$.

The relationships between genetic distance and population altitude (above sea level) and geographical distance from the salt marsh population are shown in Figures 5.14 and 5.15.

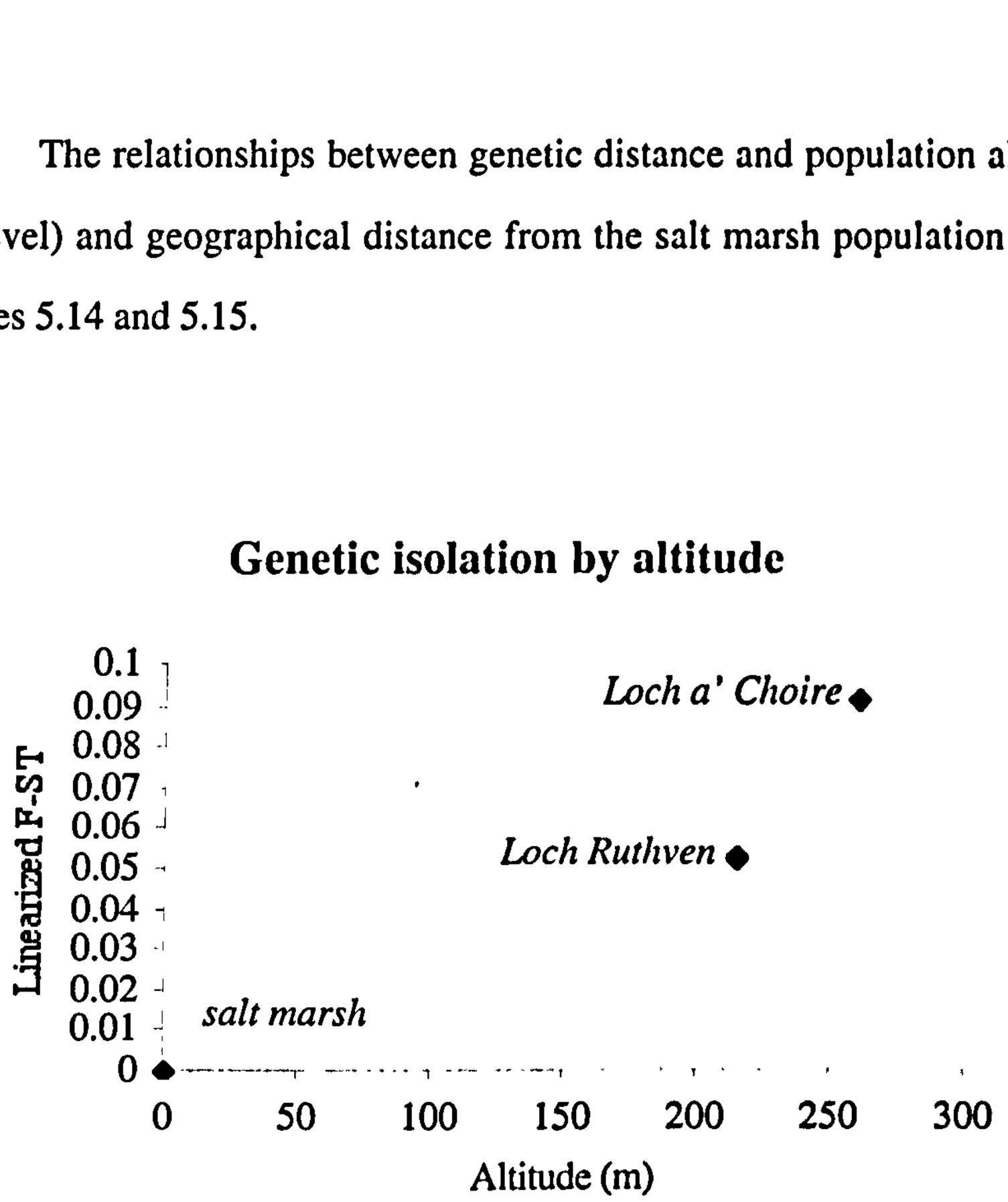

Figure 5.14: Linearized F_{ST} between each stickleback sub-population and the marine form plotted against population altitude

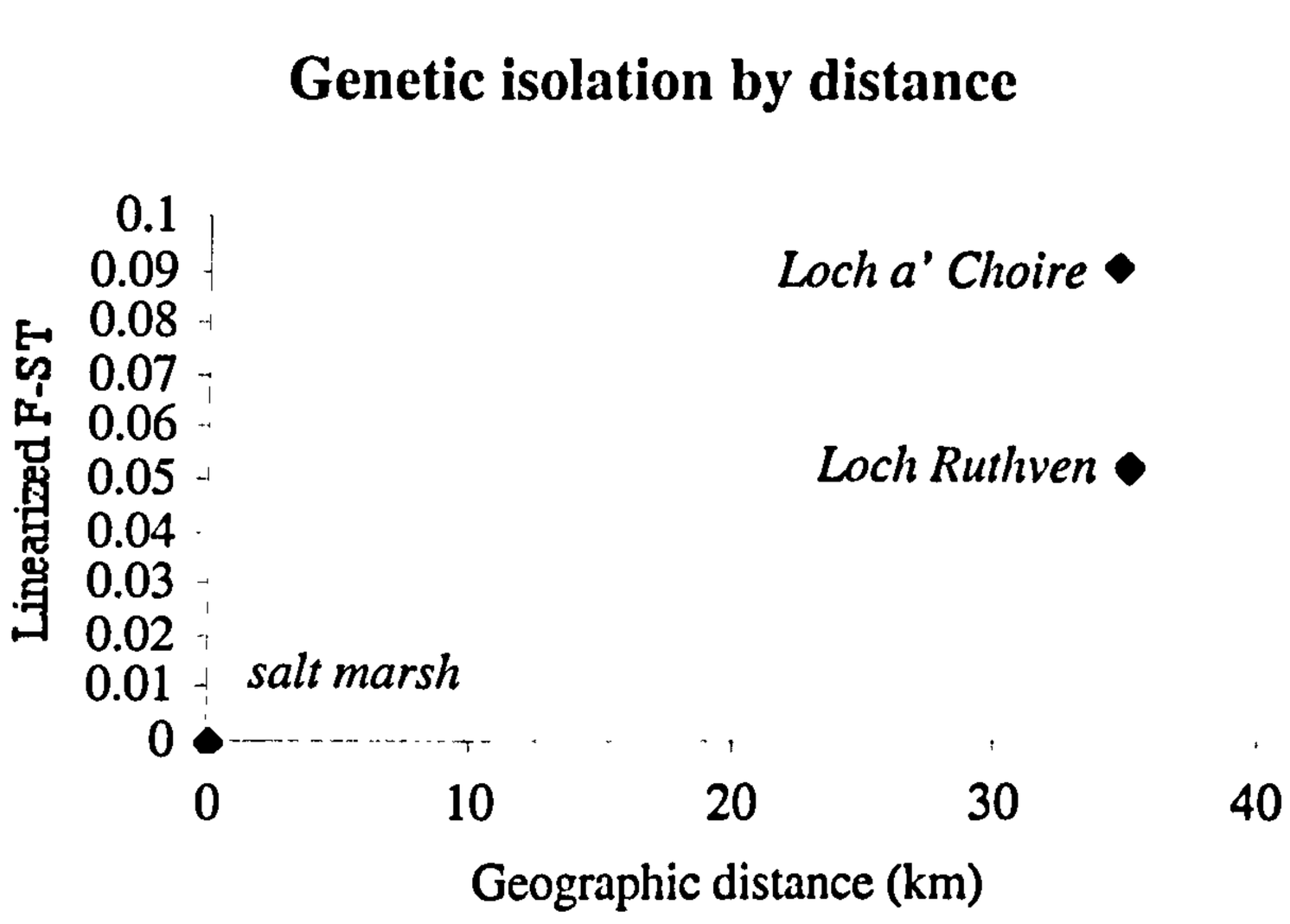

Figure 5.15: Linearized F_{ST} between each stickleback sub-population and the marine form plotted against the distance from the salt marsh sampling site.

There appears to be a positive relationship between the genetic distance and the of populations from the marine sampling site and height above sea level (Figure 5.16). Principle component analysis was carried out upon residuals of the measured morphological variables, standardised by length by Arnott, Pagnon and Barber (unpublished). They found that morphological variation correlated significantly with population altitude. Of the morphological variables, length corrected body depth and body mass were found to correlate strongly with altitude (Female body depth: $r = -0.8333$, $p < 0.001$; Male body depth: $r = 0.831$, $p < 0.002$; Female body mass: $r = -0.795$, $p < 0.003$; Male body mass $r = -0.888$, $p < 0.0001$). Thus, fish from populations at high altitude are slimmer bodied than those found nearer sea level.

In order to examine the relationship between morphological and genetic variation, linearised F_{ST} was compared to the divergence of each population, in mean length corrected body mass and body depth, from the marine form (Figures 5.16 and 5.17). Morphological measurements were provided by Arnott, Pagnon and Barber (unpublished). Divergence in body mass and depth was calculated as the magnitude of the decrease in mean length corrected body mass and mean length corrected body depth between each population sample and the salt marsh sample (marine form).

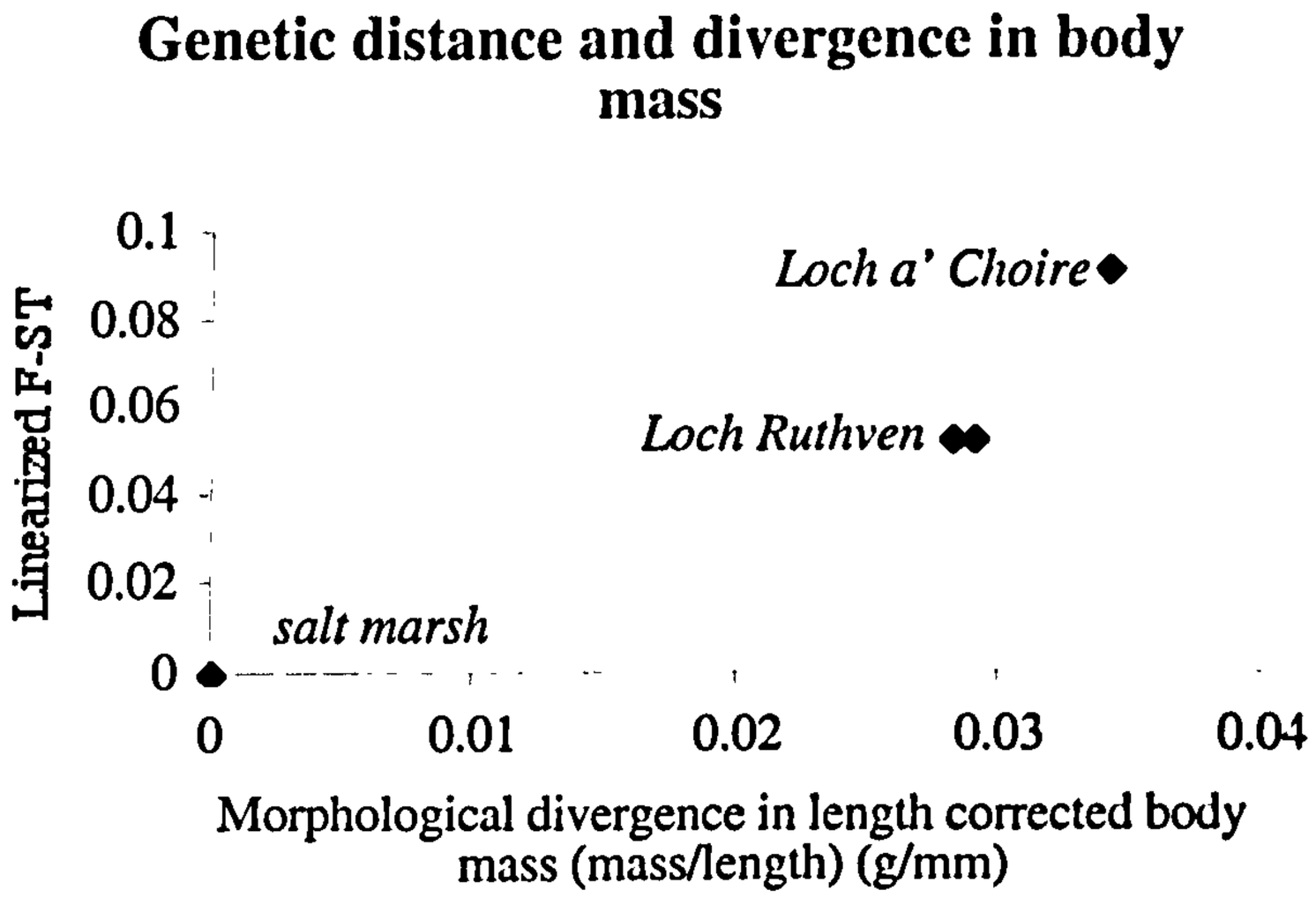

Figure 5.16: Linearized F_{ST} between each sub-population and the marine form plotted against divergence in mean length corrected body mass. Divergence was calculated as the magnitude of the decrease in mean length corrected body mass between each population and the marine form (g/mm).

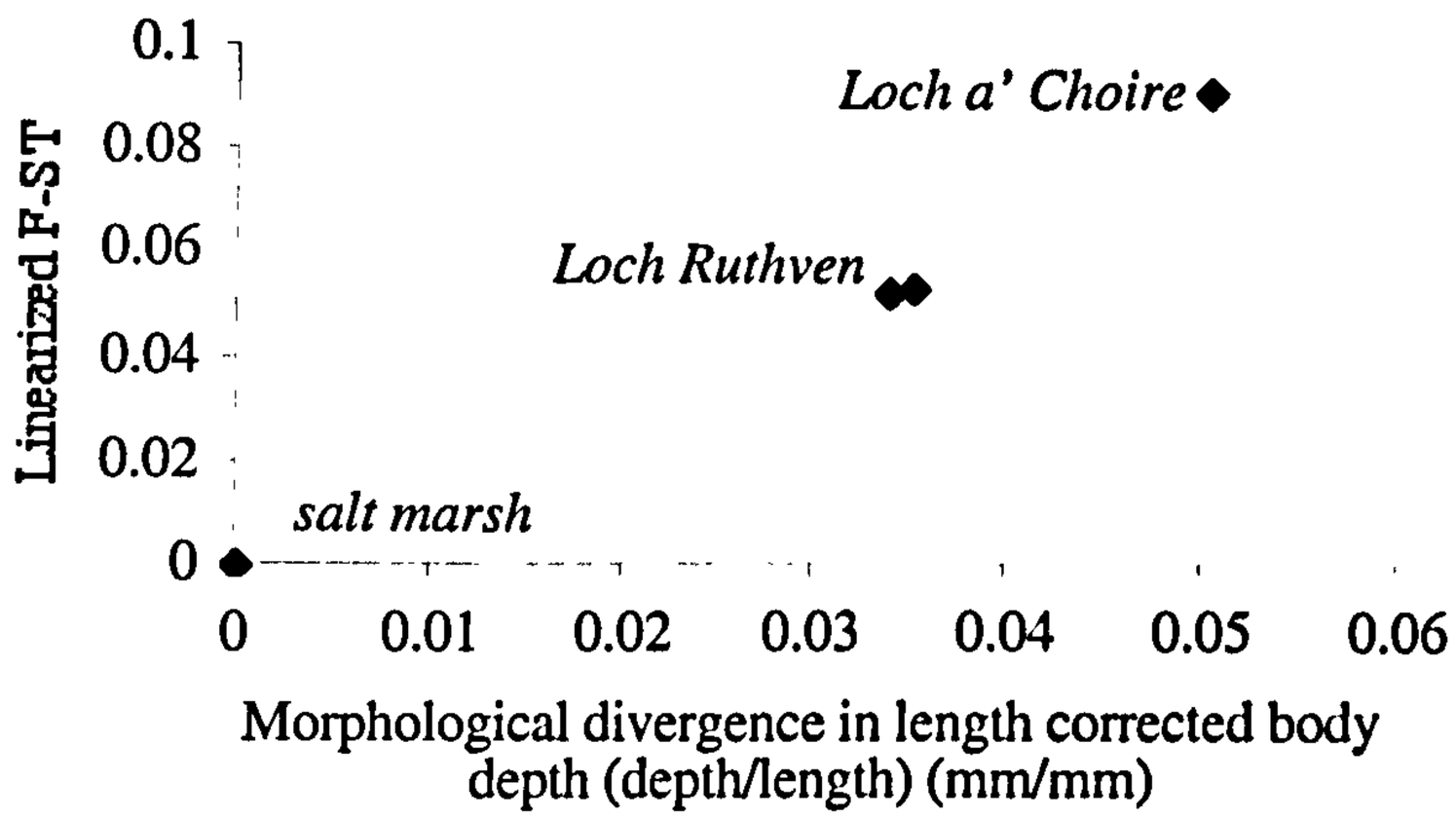

Figure 5.17: Linearized F_{ST} between each stickleback sub-population and the marine form and divergence in mean length corrected body depth. Divergence was calculated as the magnitude of the decrease in mean length corrected body depth between each population and the marine form (mm/mm).

There appears to be a positive relationship between genetic distance from the salt marsh population and the extent of the reduction in mean length corrected body mass and body depth (between each population and the salt marsh population). Unfortunately, the relationships between morphology, altitude and genetic divergence cannot be analysed statistically as the number of populations studied is too small.

Assignment test

An assignment test was carried out that uses the microsatellite repeat number data at each locus from each individual of a population to calculate the

probability of its genotype in the assigned population and the probability of its genotype in every population. A matrix was calculated, giving, for each pair of populations, the number of individuals sampled in the first but assigned to the second (Table 5.18). An assignment calculator was used on the web-site: <http://www.biology.ualberta.ca/jbrzusto/Doh.php>. The calculations used are described by Paetkau *et al.* (1995).

Assignment test

Population name (to) *(from)* ↴ ↳	*Ruthven (Spineless)*	*Ruthven (Spined)*	*salt marsh*	*Loch a' Choire*
Ruthven (Spineless)	8	9	3	0
Ruthven (Spined)	7	5	1	2
salt marsh	0	2	15	0
Loch a' Choire	1	0	1	18

Table 5.18: Population level assignment matrix showing the number of individuals sampled from the first population (row headings) and assigned to the second (column headings).

The assignment test gives an indication of the genetic isolation of each population. Most of the individuals sampled from the salt marsh and Loch a' Choire are assigned to their population of origin. Individuals sampled from Loch Ruthven are usually assigned to Loch Ruthven, but each morph is often assigned by genotype, to the other morphological group.

5.4 Discussion

Hardy-Weinberg proportions

Under Wahlund's principle (Wahlund, 1928; in Wallace 1981), the sub-division of a large population into a series of smaller ones will result in a higher frequency of homozygotes, at the expense of heterozygotes. Therefore, the excess of homozygotes above that expected from Hardy-Weinberg proportions in the populations studied may be attributed to a Wahlund effect.

Should non-random mating occur due to population sub-division, it would be expected that the heterozygosity of the two groups of Spined morphs from separate sampling sites at Loch Ruthven would be closer to the expected proportions under Hardy-Weinberg equilibrium. This is the case for the sub-population sampled at site A, for which three out of five loci are at Hardy-Weinberg equilibrium, in contrast to the spined population considered as a whole, which has the expected proportion of heterozygotes at only one locus.

Lower than expected heterozygosity might also have been found as a result of sampling loci that exhibit null alleles. Null or non-amplifying alleles can arise as result of mutations in one of the priming sites in the flanking sequences adjacent to the microsatellite repeat (Pemberton *et al.*, 1995). This would result in loci appearing to be homozygous when, in fact, a second heterozygous microsatellite allele was present, but was not amplified in the PCR. However, the probability of mutation in flanking sequences is lowered when loci are used for analysis that were characterised for the species of study (Scribner and Pearce, 2000). Also, a survey of the results of the Hardy-Weinberg analysis shows that, although the observed heterozygosity is often lower that expected, all of the loci used show frequencies of heterozygotes that could be expected at Hardy-Weinberg equilibrium in at least one of the population samples.

Genetic distance measures

The R_{ST} values are significantly greater than zero for all possible population pair-wise comparisons, as are the F_{ST} values except that between the pair of morphs in Loch Ruthven. The R_{ST} statistics exceed F_{ST} for all of the populations studied. This might be due to a high mutation rate, which lowers F_{ST} (Balloux and Goudet, 2002). F_{ST} is also more sensitive to mutation rate when migration is low (Balloux and Lugon-Moulin, 2002). It is likely that there is little migration between the populations studied (except those within Loch Ruthven) due to geographical barriers. Under a strict step-wise mutation model, R_{ST} is independent of mutation rate so may be a better measure of genetic differentiation, in this case. However, R_{ST} has a high variance (Balloux and Lugon-Moulin, 2002).

Balloux and Goudet (2002) carried out simulations of R_{ST} and F_{ST}, under different levels of gene flow, mutation rates, population number and sizes and show that no statistic is best overall. However, R_{ST} is recommended when there is low gene flow between populations and F_{ST} when there is high gene exchange. Slatkin (1995) concludes that F_{ST} measures show too much genetic similarity when the coalescence time between the populations studied is large and should only be used when the time scale involved is tens or hundreds of generations.

The colonisation of the Lochs studied is thought to have occurred post-glacially, during marine incursion, around 10, 000 years ago. Allowing for a similar number of stickleback generations since isolation, coupled with low migration, we would expect the R_{ST} statistics to provide a more accurate measure

of genetic differentiation. However, the Loch Ruthven morphs, should they constitute separate populations, have an unknown coalescence time and no obvious geographical barriers to migration, so that F_{ST} may give a better description of their genetic differentiation.

Speciation

The significant genetic differences between stickleback populations are interesting. Although an R_{ST} or F_{ST} value greater than zero does not tell us whether or not the populations are reproductively isolated, the genetic differentiation of stickleback populations provides insight into evolutionary divergence of the species.

There appears to be a trend towards increased genetic divergence from the marine form with increasing altitude. This might reflect the time since isolation, assuming that the colonisation of freshwater habitats took place during post-glacial marine incursion. Receding sea levels would be expected to isolate populations at higher altitudes (e.g. Loch a' Choire) earlier than those nearer present sea level (e.g. Loch Ruthven).

Analysis of morphological data by Arnott, Pagnon and Barber (unpublished) showed a significant correlation of length corrected body mass and body depth with altitude of the14 populations studied. Linearized F_{ST} appears to increase with divergence in mean body mass and depth, from the marine form, suggesting that genetic and morphological differentiation co-vary. However, the populations that were genotyped constitute a fraction of those for which morphological variation has been extensively investigated. The interaction between genetic distance and morphological divergence with altitude and possibly

time since isolation could be resolved further by following the methods of this chapter with the remaining populations.

There is little evidence from this study to suggest that genetic isolation is related to the geographical distance from the founder population. This is in contrast to the findings of Reusch *et al.* (2001). Genetic distance was measured from microsatellite variation of sticklebacks sampled from a variety of habitats in Germany. Populations followed an isolation by distance model, within lake and estuarine habitat types. Again, extending the genetic analysis, to the other populations in the locality of those in our study, would provide further insight into the effect of geographic isolation.

Sympatric morphs

Although the F_{ST} comparison between the spined and spineless stickleback morphs in Loch Ruthven indicates no significant genetic difference between the two, there are striking morphological differences. In addition to lacking pelvic spines, the spineless morph fish exhibited a reduction in pelvic girdle length, and increased pelvic girdle asymmetry, when compared to the spined fish and the population sample from Loch a' Choire (Arnott, Pagnon and Barber, unpublished). There were no significant differences between the morphs in length-corrected body depth (two tailed t-test, $p = 0.65$) and body mass (two tailed t-test, $p= 0.40$).

Should the morphs have constituted discrete, reproductively isolated populations, like those found in British Columbia, their mechanism of divergence might have been revealed by genotypic variation. If each morph was more closely related to the other than to the putative ancestor then sympatric speciation may have been invoked. It is probable that the morphological differences observed

have indeed arisen in sympatry, given that the value of F_{ST} is not significantly greater than zero for the difference between morphs, yet significant for differences between each morph and the putative marine ancestor. Nevertheless, care should be taken when interpreting genetic data in this way. Although the mtDNA divergence of morphs in the British Columbian lakes suggests that speciation has occurred in sympatry, a similar pattern of monophyly between morphs would arise if speciation reached an advanced stage in allopatry, followed by a second invasion of marine stickleback, with subsequent gene flow during secondary contact (Taylor *et al.*, 1997).

The pair-wise F_{ST} value for genetic difference between the Loch Ruthven morphs, at 0.018, is considerably smaller than values found between samples of benthic and limnetic pairs found in British Columbia. F_{ST} values for Emily, Enos, Paxton and Priest lakes are approximately ten-fold greater, ranging from 0.209 to 0.336, and were all highly significant at $p < 0.001$ (Taylor and McPhail, 2000).

It would be interesting to carry out behavioural studies on the Loch Ruthven morphs, such as mate choice trials (described in the previous chapter) which would reveal any divergence in mating preferences between the morphs and in turn, might indicate the evolution of prezygotic reproductive isolation and incipient speciation. However, the morph specific mating preferences of the sympatric morphs found in the British Columbian lakes appeared to be based upon divergence in body size (Nagel and Schluter, 1998) and there is no significant size difference between the morphs sampled from Loch Ruthven.

Gene flow

Methods of assignment testing have been evaluated, under different conditions, by Cornuet *et al.* (1999) using simulated population data. Likelihood methods based upon the likelihood that an individual's multilocus genotype will occur in two or more candidate populations, were found to perform better than genetic distance measures in the assignment of individuals to the correct population of origin. The genotypic frequency likelihood based method of assignment (Paetkau *et. al.*, 1995), used in the present study, performed well (80-100% accuracy) when population differentiation estimated by F_{ST} ranged from 0.05 to 0.1 (Cornuet *et al.*, 1999). The F_{ST} values obtained for the populations in this study lie within this range, with the exception of the pair-wise value between the Loch Ruthven morphs. However, Cornuet *et al'* s (1999) assessment of accuracy is based upon larger sample sizes (30 individuals per population) sampled at ten loci. Also the frequency method used assumes that populations are at Hardy-Weinberg equilibrium and linkage equilibrium (alleles are randomly associated).

Given that the genotypic frequencies of the populations sampled showed deviation from Hardy-Weinberg expectations, it might have been better to have used the Bayesian assignment method described by Cornuet *et al* (1999), which does not assume Hardy-Weinberg equilibrium or linkage equilibrium.

The assignment testing placed 2 out of 17 of the salt marsh genotypes in the Ruthven spined population. This genetic similarity cannot be ascribed to gene flow between the two groups, owing to geographical isolation and must instead be attributed to the retention of ancestral alleles in the Ruthven population.

It is possible that the spined fish captured in Loch Ruthven, originate from the Loch a' Choire population and have been washed downstream via the small fast-flowing burn that connects the two Lochs. It is, however, unlikely that fish

could migrate upstream from Loch Ruthven, as the gradient is steep. If downstream migration occurred, then Loch Ruthven spined morphs would be expected to be genetically more similar to the Loch a' Choire population sample than the spineless morphs. Relatively few fish, outwith the indigenous population, are assigned to or from Loch a' Choire, which in addition to the high pair-wise F_{ST} values obtained between this population and both of the Loch Ruthven morphs, suggests that gene flow is minimal.

Stickleback evolution

The results of this study of stickleback populations of the Highlands of Scotland further illustrate the rapid divergence of freshwater colonists that characterises the evolution of this species (Bell and Foster, 1994). Rates of speciation in post-glacial fishes are high due to the increased availability of novel niches within newly formed lakes, which lack competing species (Schluter, 1996, Smith and Skulason, 1996).

The high rate of evolution in the stickleback is counteracted by a high probability of rapid extinction (Bell and Foster, 1994). One of the species pairs extant in British Columbia may be, at present, undergoing collapse. An increased number of hybrids in Enos Lake, in addition to the observation that the gill raker number in limnetic morphs is being lowered towards an intermediate state, suggests that selection pressures are changing (Kraak *et al.*, 2001).

The fate of the sympatric morphs of this study, whether speciation or introgression, will depend upon the future costs and benefits of maintaining divergent phenotypic traits. It is interesting that none of the populations sampled seem to contain allele frequencies at all loci that are in agreement with Hardy-

Weinberg equilibrium. The occurrence of non-random mating within lochs might serve to increase the rate of future adaptive radiation.

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Appendix I: Reagents

This section describes the reagents used and the method of preparation of their stock form, if applicable. Unless otherwise stated, reagents were the purest grades available from Sigma-aldrich, or BDH (Merk-Eurolab), stored at room temperature. Aqueous solutions were made up in either autoclaved distilled water or distilled water, as appropriate.

ABI dye labelled primers	Supplied by Sigma-genosys, stored at -20°C. Primer stock and PCR products containing labelled primers were wrapped in aluminium foil to prevent contact with light.
Agarose	Prepared fresh, for each gel between 0.8 and 4% (w/v) was dissolved in TBE buffer.
AluI	Supplied by Promega with 10 × reaction buffer, stored at -20°C.
BSA	Fraction V, stored dry at 4°C and as a 10mg/ml solution in water at -20°C.
Chelex100	Supplied by Biorad, 5% suspension in autoclaved distilled water used for DNA extraction, stored at 4°C.
Chloroform	Chloroform:propan-2-ol (24:1).
Degraded salmon sperm DNA	10mg/ml aqueous solution, supplied by Sigma-aldrich, stored at -20°C. Used at a concentration of 100µg/ml in hybridisation

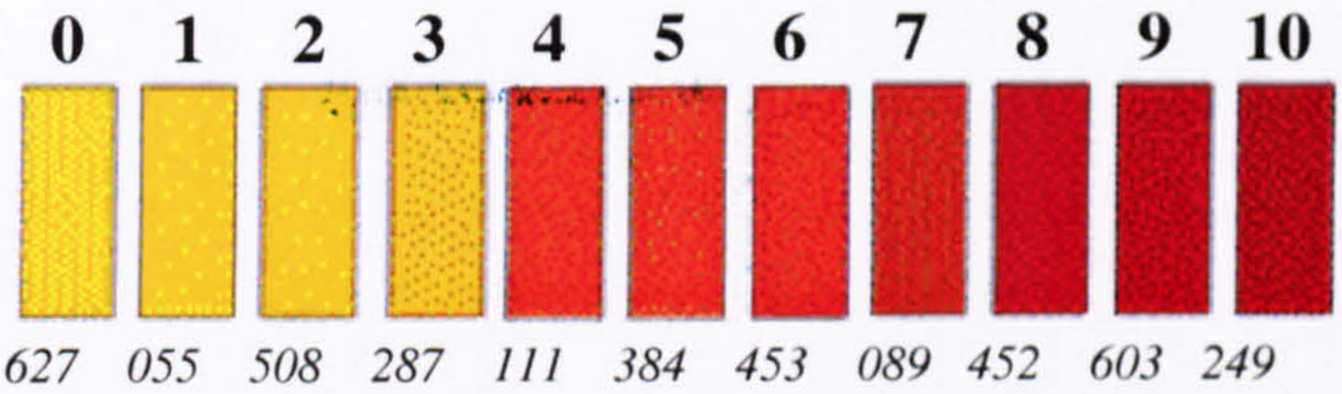
	solution.
Denhardts solution	50 × concentrate supplied by Sigma-aldrich, stored at - 20°C. 5 × Denhardts solution added to hybridisation solution.
Digestion solution	20mM EDTA,50mM Tris[hydroxymethyl] aminomethane, 20mM NaCl, adjusted to pH8 with 0.1N HCl, autoclaved and 1.5%SDS added.
dNTP	ATP, CTP, GTP, TTP (100mM) stock nucleotides, supplied by Promega, stored at - 20°C. 10µl of each added to 360µl autoclaved distilled water to make dNTP mix used in PCR, stored at -20°C.
λ *EcoR I* marker	Supplied by Promega. Diluted to 0.2µg/ul, supplied with 6 × loading dye, stored at -20°C.
HinfI	Supplied by Promega with 10 × reaction buffer, stored at -20°C.
Hybond	Hybond-N nylon membrane supplied by Amersham-pharmacia, single-stranded DNA binding capacity: 600µg/cm^2.
Hybridisation buffer	0.5M Na_2HPO_4, 5% SDS.
1kb DNA ladder	1kb DNA ladder supplied by Promega or BRL. Diluted to 0.2µg/ul, stored at -20°C.
Orange G loading dye (10 ×)	Glycerol (50%v/v), 10mM Tris[hydroxymethyl] aminomethane

	(adjusted to pH8 with 0.1N HCl), 25mM EDTA, Orange G. Filtered (0.2μm).
γ^{32}P ATP	*Isoblue*™ γ^{32}P ATP, 10Ci/ml, specific activity 3000Ci/mmol supplied by ICN,stored at -20°C.
γ^{33}P ATP	*Isoblue*™ γ^{33}P ATP, 10Ci/ml, specific activity 3000Ci/mmol supplied by ICN, stored at -20°C.
Phenol	Re-distilled phenol was equilibrated with 0.5 volumes of TE (pH7.6) and 0.1% SDS. The aqueous layer was removed and m-Cresol (0.05vols), 2-mercaptoethanol (0.002vols) and 8-hydroquinoline (0.1%w/v) added. Stored at -20°C or in foil wrapped tubes for immediate use.
Phenol/Chloroform	Phenol:chloroform:propan-2-ol (25:24:1).
Proteinase K	Supplied by Promega, stored at -20°C.
SDS	Sodium dodecyl sulphate (10%w/v).
SET	100mM NaCl, 1mM EDTA, 100mM Tris[hydroxymethyl] aminomethane (adjusted to pH8 with 0.1N HCl). Autoclaved.
SSC (20 ×)	3M NaCl, 0.3M sodium citrate (adjusted to pH7 with 0.1N NaOH).
Taq DNA polymerase	Supplied by Promega with 10 × reaction buffer, stored at - 20°C.
TBE (1 ×)	89mM Tris[hydroxymethyl] aminomethane,

	89mM Boric acid, 2mM EDTA (adjusted to pH8 with 0.1N HCl)..
TE	10mM Tris[hydroxymethyl] aminomethane (adjusted to pH8 with 0.1N HCl), 1mM EDTA. Autoclaved.
T_4PNK	Supplied by Promega with 10 × kinase buffer, stored at - 20°C.

Appendix II: Stickleback colour chart

The colour chart used as an index of male stickleback nuptial colouration, in mate choice trials and the analysis of stickleback offspring sex ratio (Chapter 3), is shown below:



This colour chart is a section of the Winsor and Newton Designers Gouache Colour Range. The manufacturers colour codes are given in italics and the colour scores, assigned to male sticklebacks with a matching intensity of red, are given in bold. The colour range can be downloaded at the following web-site:

<http://www.winsornewton.com/Main/Sitesections/ColorChartsAll/colorchartmainpage.html>.