ENVIRONMENTAL AND PHYSIOLOGICAL FACTORS INFLUENCING THE FORMATION OF THE EGGSHELL OF THE DOMESTIC FOWL.

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DECLARATION.

I hereby declare that the work presented in this thesis was carried out by me personally, with the exception of figures 2 and 14 which were prepared from scanning electron micrographs taken by Mrs. Sheila Cranstoun.

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SUMMARY.

The thesis begins with a comparison of four egg production systems currently in commercial use within the UK, namely battery, perchery, modified free range and traditional free range. The comparison involved traditional, material and ultrastructural measures and was carried out in order to assess the effect of the various production environments on shell structure and quality. No definitive order of rank was obtained, however, the results obtained indicate that each system has the potential to provide well structured eggshells throughout lay, providing that management of a high standard is maintained. The degree of environmental control offered by the battery system is reflected in terms of stable shell quality performance, while a poorer performance from the perchery is attributed to social order dysfunction. It is, however, acknowledged that many of the behavioural needs of the birds, such as the ability to nest, scratch and perch, are not met in the battery environment. An investigation into battery cage enhancement by means of perch provision and increased space allowance per bird was therefore carried out. Provision of a perch, when all birds had access to that perch, resulted in improved shell ultrastructural quality. If not all birds had access to the perch social stability was disrupted and ultrastructural quality compromised. In addition, force molting was also shown to have a beneficial effect on eggshell quality. This however could not be explained purely in terms of the shells inorganic component.

These investigations were combined with a comprehensive review of the welfare requirements of the laying hen and how they are variously met and compromised within each system. The use of ultrastructural shell quality assessment was shown to provide an insight into the birds homeostatic mechanisms on a daily basis. It has therefore been suggested that it be added to the range of criteria currently used to evaluate the overall welfare status of the laying hen.

As not all of the observations regarding shell quality performance could be explained in terms of the inorganic fraction of the shell alone, the role of the shell's organic matrix was highlighted. In particular, constituent proteins such as keratan sulphate, dermatan sulphate and ovocleiden-17 (OC-17) were investigated. Differences between etched (without membranes) and nonetched (with membranes) shells of good and poor quality were also investigated.

Ultrastructural examination of eggshell matrix revealed a complex architecture that differs within each of the major zones within the shell. Its appearance in areas of the shell associated with crystal nucleation (mammillary cores), rapid growth (palisade layer) and termination (vertical crystal layer and cuticle) indicated a relationship between structure and function. As a result of this investigation a hypothesis has been developed regarding possible roles for the vesicles associated with the organic matrix of the shell.

Extra and intramineral matrix proteins from good and poor quality, etched and nonetched shells were solubilised by sequential extractions using guanidine HCl and EDTA prior to SDS-PAGE. The matrix subunit protein OC-17 was demonstrated in all but the poor quality nonetched extramineral fraction and no differences in profiles were found between etched and nonetched shells. There is evidence to suggest that removal of the eggshell membranes by plasma etching gives a more accurate picture of the composition of the extramineral fraction of the organic matrix than previously obtained.

Addition of soluble extra and intramineral proteins to a metastable solution of calcium carbonate was shown to modify the morphology of calcite crystals formed *in vitro*. This suggested that both these constituents of eggshell matrix are involved in the regulation of calcite crystal growth during the formation of the eggshell, contrary to the findings of previous researchers who have demonstrated an effect solely with the intramineral fraction.

Immunohistological studies demonstrated the presence of OC-17 in the shell membranes, the mammillae, the palisade and vertical crystal layers. The presence of OC-17 in the vertical crystal layer indicated that this region contains an organic matrix component. Little difference in the distribution of OC-17 was demonstrated between good and poor quality shells.

These results provide a foundation for future research on the organic fraction of the eggshell and have reinforced the fact that such studies are in their infancy. They also highlight the fact that this material has, by and large, been ignored in terms of shell quality assessment to date. It was concluded that a better understanding of the mechanisms involved in the biogenesis of the calcified egg shell would benefit the poultry industry at every level and would also pave the way for improvements in many other disciplines.

CHAPTER 1 - GENERAL LITERATURE REVIEW.

1. GENERAL LITERATURE REVIEW.

1.1. THE OVIDUCT.

The oviduct of the domestic fowl is composed of six functionally distinct regions, namely the infundibulum, magnum, isthmus, tubular shell gland (TSG), shell gland pouch (SGP) or uterus, and vagina (Solomon, 1975). It normally takes about 20 weeks for a fully functional and recognisable tract to develop although Fujii (1981), in studies of the immature pullet, found the various regions could be identified at 18 weeks.

In the domestic fowl only the left ovary and oviduct develop and become functional (Watt, 1989). The mature oviduct is normally around 600mm long (Gilbert, 1979), although considerable variation can occur between individuals. Thus, the oviduct is a highly tortuous conducting muscular tube responsible for transporting the ovum from the ovary to the cloaca and providing it *en route* with a supply of nutrients and a protective calcified shell.

The basic structure of the oviduct wall, from the epithelial surface outwards, is as follows. A mucosa with a stratified columnar epithelium and underlying coiled tubular glands in the lamina propria, an inner layer of connective tissue, an inner circular muscle layer, an outer layer of vascularised connective tissue, an outer layer of longitudinal muscle and an outer serosa. The layers of smooth muscle transport the egg along the oviduct (Gilbert, 1979) and increase in thickness caudally. The oviduct is well vascularised and innervated and is discussed in detail by Solomon (1983, 1991).

The time spent by the developing ovum in each region of the oviduct is as follows, 0.25-0.5 hours in the infundibulum, 2-3 hours in the magnum, 1.25 hours in the isthmus, 0.25 to 0.5 hours in the TSG and 18-20 hours in the SGP (Watt, 1985; Solomon, 1991).

1.1.2. THE OVARY.

There is a 24 hour continuous cycle in the oviduct of the domestic fowl, under the control of lutienising hormone from the pituitary (Gilbert, 1971). The ovum and its limiting membranes are known as a follicle and several developing ova are present at any one time at various stages of maturation, forming a follicular hierarchy. Each follicle is attached to the ovary by a narrow stalk. The follicle wall is highly vascularised except at the region known as the stigma, along which it ruptures at ovulation releasing the ovum. Occasionally haemorrhaging occurs resulting in the formation of a blood spot. It takes about 7-9 days for the post ovulatory follicle to regress.

Thirty minutes to one hour has been suggested as the time interval between oviposition of one egg and ovulation of the next (with oviposition being the stimulus for ovulation).

1.1.3. THE INFUNDIBULUM.

Post ovulation, the ovum is engulfed by the fimbriated ends of the funnel-like infundibulum. Within this region fertilisation may take place. The fimbrial lips are composed solely of ciliated cells (Fujii, 1974, 1981) and these cilia beat rhythmically, transporting the ovum on its journey. In this region the ovum is enveloped by a perivitelline membrane consisting of fine proteinaceous rodlets through which sperm pass during fertilisation. In addition, enzymes released here are later triggered by changes in the chemical composition of the oviducal fluid, along with turning of the egg, to encourage chalazae formation (Solomon, 1991). These twisted mucoid fibres stabilise the yolk within the albumen mass. In the cephalic region the mucosal folds are low, becoming more pronounced as the infundibulum narrows (Solomon, 1983) due to the appearance of tubular glands (Aitken, 1971). The mucosal folds run somewhat longitudinally and are covered by approximately equal numbers of ciliated and nonciliated cells.

1.1.4. THE MAGNUM.

On the next phase of the journey the ovum enters the magnum. This is the longest part of the oviduct and is distinguished from the infundibulum by its white colouration, its greater diameter, thicker wall and glandular appearance. In this region up to 40 different proteins are manufactured to form the albumen. This imparts shape to the developing egg which then progresses caudally, blunt end first. The surface of the magnum is lined with tall ciliated and nonciliated epithelial cells and tubular glands in the mucosa are responsible for the manufacture and release of the albumen proteins.

Albumen is a multilayered structure which only achieves its final form after oviposition, containing 80% water at this time. A viscous layer of sulphated mucus is acquired as the ovum exits the caudal portion of the magnum, rendering turgidity and forming a stable foundation for the deposition of the shell membranes. In addition to its nutritional role, albumen also contains antibacterial agents such as lysozyme and avidin and provides a degree of physical protection for the descending ovum.

Mechanical stimulation by the descending egg is the stimulus for albumen release (Amundson and Baker, 1940 and Amundson *et al.,* 1943; cited by Solomon, 1983) and it is believed that the magnum contains enough protein for two eggs at any one time (Solomon, 1991). The magnum is separated from the isthmus by a narrow, aglandular region which can be observed by the naked eye.

1.1.5. THE ISTHMUS.

This region is narrower than the magnum and has less voluminous folds. It is lined by a pseudostratified columnar epithelium of ciliated and non ciliated cells and is responsible for producing the paired shell membranes (Solomon, 1983).

1.1.6. THE TUBULAR SHELL GLAND (TSG).

The TSG shares some ultrastructural features with the isthmus, however, the gland cells lining the TSG are distinguished from the former cells by their complement of glycogen (Wyburn *et al.,* 1973). The TSG is functionally comparable to the shell gland pouch but is specifically responsible for the initial transfer of calcium salts onto the membrane fibres.

In this region calcium deposition is targeted specifically at chemically modified end portions of the membrane fibres known as mammillary cores. The cores and their mantle of calcium salts make up the mammillary bodies. Time spent in this region is relatively short, but nonetheless critical for good shell formation, as it is here that the membrane fibres make close contact with calcium salts in preparation for the main phase of shell formation.

1.1.7. THE SHELL GLAND POUCH (SGP).

The SGP averages 7.3-11cm in length and 3cm in width and serves four separate functions, namely, addition of water or "plumping fluid", secretion of the bulk of material needed for shell calcification, incorporation of pigment and the secretion of the cuticle (Solomon, 1991). The tubular gland cells lining this region are characterised by their content of mitochondria, which vary in number during the laying cycle. Lateral and basal cell walls show complicated infoldings and the luminal surface is covered with microvilli, many of which have swollen tips preceding the phase of calcium transfer.

Prior to the deposition of calcite the egg is "plumped" by the addition of about 15g water into the albumen (Wyburn *et al.,* 1973). This swells the egg and exposes the mammillary bodies.

During its 20 hour stay in the pouch region, the bulk of the true shell is acquired. This consists of 95% calcium carbonate and 5% organic material and is described in detail in section **1.2.**.

Shell pigmentation occurs towards the end of shell formation and is mainly associated with the laying down of the cuticular layer.

The vascular supply to the SGP is profuse and during lay the region appears bright red due to vascular engorgement. Fenestrated capillaries, similar to those found in the renal glomeruli, are present allowing rapid transfer of metabolites, Solomon (1983).

1.1.8. THE VAGINA.

The vagina is separated from the shell gland mucosa by dense connective tissue. The lining mucosal folds are long, slender and "S" shaped with short secondary folds. Ciliated and nonciliated cells are present. Sperm host glands are found at the junction with the SGP. These are fluid filled and sperm may remain viable here for 12-22 days, extracting nutrients from the medium prior to moving anteriorly towards the infundibulum aided by antiperistalsis. Oviposition is brought about by contraction of the SGP which (after egg rotation) pushes the egg into the vagina and through the cloaca pointed end first (Solomon, 1983, 1991).

1.2. FORMATION AND STRUCTURE OF THE EGG SHELL.

1.2.1. INTRODUCTION.

Nature designed the avian eggshell as a microenvironmental chamber for housing the developing embryo. In this capacity it provides physical protection, regulates gas, water and ionic exchange and provides a source of calcium (Arias *et al.,* 1993). These authors described the shell as a multilayered polymer ceramic composite consisting of four layers, the shell membranes, the mammillary layer, the palisade layer (including the vertical crystal layer) and the cuticle (see Figure 1).

1.2.2. THE FABRICATION OF THE EGGSHELL.

The avian eggshell is one of the most rapidly mineralizing biological systems known with 5g of $CaCO_3$ being crystallized in fewer than 20 hours (Simkiss, 1961; cited by Arias *et al.,* 1993). It is produced at the relatively low temperature of 40° C (man-made ceramics require much higher temperatures) and involves the simultaneous deposition of matrix material and calcium carbonate, Arias *et al.,* (1993), Arias and Fernandez (1995). Soluble precursor matrix polypeptides are synthesised in the liver and in the isthmus, the rate of synthesis in the liver being under the control of the sex hormones (Krampitz, 1993). Mineralization takes place in an acellular milieu (the uterine fluid) which contains supersaturated concentrations of calcium and bicarbonate ions. Calcium is transported through the SGP mucosa in association with the presence of the protein calbindin (Nys, 1990).

During lay there is preferential selection of a calcium rich diet by the hen which, however, can only absorb little more than 1g of calcium per day (Tyler, 1940). There is approximately 2g of calcium in the normal eggshell and the labile medullary bone in the femur supplies the excess calcium required at this time (Simkiss, 1975). Thus, there is a state of dynamic equilibrium in existence between dietary calcium in the blood, the medullary bone and the calcifying egg shell.

The carbonate fraction of the crystalline shell originates from the hydration of metabolic carbon dioxide to bicarbonate ions within the shell gland mucosa (Hodges and Lorcher, 1967). This process is under the control of the enzyme carbonic anhydrase which is located within the tubular glands and epithelial cells lining the shell gland (Sturkie, 1986).

All the necessary components accumulate in the SGP prior to shell secretion, the signal for secretion being mechanical stimulation through rotation of the shell free egg (this reaction can be mimicked by rotating a ping pong ball in the SGP), Krampitz (1993).

A detailed working hypothesis for the steps involved in shell mineralization is provided by Fink *et al.,* (1993). These authors describe the eggshell as being fabricated by an "assembly line" method as the egg progresses along the oviduct; thus distinct regions of the oviduct or distinct oviduct cell populations have specialised control over eggshell fabrication. The calcification of the eggshell involves three main steps: (a) fabrication of shell membranes and mammillae by the cells of the isthmus (b) nucleation of calcium carbonate crystals (c) space filling growth of calcite from these first formed crystals accompanied by concomitant matrix deposition in the shell gland pouch. The ordering of the development of each structural feature may be subject to debate, in as much as sequential steps may tend to occur simultaneously at different regions of the developing shell.

Phosphate has been shown to poison calcite formation (Simkiss, 1964). It is also known that premature oviposition can be brought about by intrauterine injection of orthophosphate or pyrophosphate (Ogasawara *et al.,* 1974, 1975; Klingensmith and Hester, 1983, 1985). It has not yet, however, been established whether the end of shell formation is the consequence of an arrest of calcium secretion, changes in uterine fluid composition, or the inhibition of calcite growth, Klingensmith and Hester, (1983).

1.2.3. THE MULTILAYERED SHELL.

The avian eggshell is a composite structure of inorganic (calcium carbonate) and organic matrix material that is rapidly and sequentially fabricated in the oviduct in less than 24 hours. To date, most of the emphasis regarding research into the avian eggshell and specifically into eggshell

quality has been directed towards the inorganic fraction of the shell. The calcified shell comprises three morphologically distinct zones, namely the mammillary layer, the palisade layer and the vertical crystal layer (see Figure 1).

[i] **The Mammillary Layer.** (Figure 1). This corresponds to the innermost region of the shell and is approximately 100μm thick (Arias *et al.*, 1993). The initiation of calcification takes place at this level giving rise to the formation of the basal caps. As the process continues the cone layer is established and with the fusion of these cones the mammillary layer is complete (Watt, 1989).

[ii] **The Palisade Layer.** (Figure 1). Succeeding the initiation process there occurs a rapid phase of calcification during which the palisade layer is formed (Solomon, 1991). This layer makes up the bulk of the shell and is composed of long, fused calcitic columns between 200-350μm thick, Arias *et al.*, (1993). Simons (1971) described the presence of vesicular holes which give this layer its characteristic honeycomb appearance. Solomon (1989; cited by Watt, 1989) hypothesised that these vesicular holes are a naturally occurring phenomenon caused by incomplete fusion during the growth phase of the cubic crystals. Simons (1971), however, considered them to be associated with the organic matrix.

The continuity of the palisade layer is broken only by the presence of gaseous exchange pores running vertically through the entire depth of the shell (Figure 1). These facilitate gas exchange between the internal and external environment during embryonic growth and occur as a consequence of incomplete fusion of adjacent mammillary knobs during the initial stages of calcification (Tullett, 1975, 1987). The egg has 7000-17000 pores ranging from 9-35μm in diameter (Romanoff and Romanoff, 1949). They are more numerous at the broad pole of the shell.

[iii] **The Vertical Crystal Layer.** (Figure 1). According to Creger *et al.*, (1976; cited by Watt, 1985) initial crystal growth is definitive, but then becomes replaced by a much less regular deposition. Towards the outside of the shell the crystals adopt a vertical orientation forming the vertical crystal layer which is between 3 and 8μm thick (Arias *et al.*, 1993). Arias *et al.*, (1992, 1993) and Arias and Fernandez (1993, 1995) have hypothesised that the

reason for this is the absence of an organic matrix framework within this layer.

1.2.4. THE ORGANIC COMPONENTS.

The organic components consist of the shell membranes, the mammillary cores, the shell matrix and the cuticle.

[i] The Shell Membranes. The paired shell membranes are deposited in the isthmus region of the oviduct. The membrane fibres interlace and adhere closely forming a perfuse mat except at the broad pole, where the air space is located. The fibres of the inner component have a width of approximately 2μm and the outer between 2μm and 4μm. Both are composed of a protein core enclosed in a less dense carbohydrate mantle (Simons, 1971). The inner shell membrane lies immediately over the albumen whilst the outer shell membrane is attached to the true shell (Mayes and Takeballi, 1983; cited by Nascimento, 1992).

The predominant components of the shell membranes are highly insoluble proteins which are resistant to typical extraction techniques (Krampitz *et al.,* 1972, 1974; cited by Arias *et al.,* 1993). Early research indicated a keratinous nature for the membranes because of their similar amino acid composition. Later studies based on structure, amino acid analysis, solubilities and the absence of epitopes recognised by anti-keratin antibodies, showed this to be inaccurate, (Arias *et al.,* 1993). The detection of hydroxyproline (Balch and Cooke, 1970), hydroxylysine (Candlish and Scougall, 1969) and lysine derived cross links (Crombie *et al.,* 1981) gave an indication to the collagenous nature of the proteins (Wong *et al.,* 1984). It was also believed that elastin could be a component of the membranes, however, the protein involved proved resistant to elastase (Leach *et al.,* 1981; cited by Arias *et al.,* 1993).

Immunohistochemical studies have confirmed the collagenous nature of the shell membranes, which contain mainly type X collagen. Additionally they contain types I and V collagen, type I is found mainly in the outer shell membrane while type V is associated mainly with the inner (Arias *et al.,* 1992, 1993; Arias and Fernandez 1993, 1995). These authors have also demonstrated the presence of the proteoglycan keratan sulphate in the outer

shell membrane. Proteoglycans are known to be calcium binding polyionic molecules which can modulate phosphate and carbonate precipitation (Wu *et al.*, 1992, 1994; Arias and Fernandez, 1995).

The outer shell membrane is essential for normal calcification. Indirect evidence suggests that the structure of the shell membranes guides the pattern of crystal deposition. Meenashki *et al.,* (1971) showed that the pattern of mineral deposited by the snail Otala (species not identified), after replacement of a piece of its shell with hen's shell membrane, corresponded closely to that of the hen's shell and not of the snails. It has also been shown that no shell is formed in their absence, Krampitz (1993). The membranes do not mineralize completely but contain uniformly distributed nucleation sites for crystal deposition and growth. These are known as mammillary cores.

[ii] **The Mammillary Cores.** These are the epitactic nucleation sites which initiate crystal deposition and growth (Watt, 1989). They are chemically bound to the outer shell membrane through disulphide links (King and Robinson, 1972; cited by Watt, 1989). From here crystals grow radially in all directions to enclose the membrane fibres and thus firmly bind the calcified part of the shell to the shell membranes, Nascimento (1992). An account of the biochemical composition of the cores is provided by Arias *et al.,* (1992, 1993). In brief, they are known to be rich in neutral glycosaminoglycans (mucopolysaccharides), hexosamines, hexoses, sialic acid. Protein-acid polysaccharide complexes and keratan sulphate have also been histochemically detected. The detailed chemical composition of the mammillary core components, however, remains unresolved.

[iii] **The Organic Matrix.** As in the case of other mineralized tissues eggshells are created by living cells which simultaneously deposit an organic matrix with an inorganic crystalline fraction. The eggshell is an example of heterogeneous nucleation, that is, crystal nucleation onto a substrate that is chemically different from the crystal that is being nucleated (Arias *et al.,* 1993). In contrast to other examples of biomineralization such as bone, tooth and mollusc shell, however, the cells involved in eggshell formation are not intrinsic components of the resulting structure.

The mechanisms by which matrix macromolecules influence calcite crystallization are not well understood, but may involve acceleration or inhibition of crystal growth on specific crystallographic planes, due to specific interactions between binding sites and calcium carbonate (Krampitz and Grasser, 1988; Krampitz, 1993). Recent studies have indeed shown that unpurified matrix material can affect the morphology and rate of calcium carbonate crystals *in vitro* (Wheeler and Sikes, 1984; Gautron *et al.,* 1993, 1995 in press; Gautron, 1994). The role of specific matrix proteins in the biomineralization process, however, remains undetermined. The presence of matrix proteins is also thought to influence the strength and shape of the final structure of calcium carbonate in the calcified eggshell, Silyn-Roberts and Sharp (1986).

Biochemical studies have demonstrated that the matrix consists of a mixture of 70% proteins and glycoproteins with 11% polysaccharides. It is rich in neutral polysaccharides, hexoses, hexosamines, sialic acid and protein-acid polysaccharide complexes. In addition, the proteoglycans keratan sulphate and dermatan sulphate have also been detected histochemically, Arias *et al.,* (1992, 1993) Arias and Fernandez (1993, 1995). The proteoglycans are a class of glycoproteins widely found in animal connective tissues. Their common structural characteristic is that they contain linear polysaccharide chains which have a repeating unit containing an amino sugar derivative. These carbohydrate chains are known as glycosaminoglycans which all carry a strong negative charge at neutral pH, due to the presence of uronic acid (not keratan sulphate), sulphate substituents or both. This high charge has a marked effect on the physiochemical features of these molecules and influences their calcium binding properties in organic matrices. Proteoglycans usually contain many glycosaminoglycans linked to a protein core (Beeley, 1985). According to Arias *et al.,* (1992, 1993) and Arias and Fernandez (1993, 1995) keratan sulphate relates to crystal nucleation and the maintenance of "calcium reserve bodies", which constitute the primary source of calcium for the developing embryo (Diekert *et al.*, 1989a and 1989b). Dermatan sulphate is, in contrast, more polyionic than keratan sulphate and appears at the end of mammillary knob formation. Thus, it relates to crystal growth and the establishment of preferential orientation of calcite crystals in the palisade layer.

Using chemical extraction techniques the matrix can be subdivided into soluble (40%) and insoluble (60%) fractions (Krampitz, 1993; Gautron *et al.,* 1993; Gautron, 1994). The soluble fraction is primarily intracrystalline (extramineral) while the insoluble fraction is intercrystalline (intramineral) (Gautron *et al.,* 1993; Gautron, 1994).

Krampitz *et al.,* (1974; cited by Arias *et al.,* 1993) and Schade (1987, cited by Gautron, 1994) postulated the hypothesis that the soluble matrix could be a supramolecular aggregate of molecular weight up to 500kDa. Krampitz *et al.,* (1980) identified a protein with abundant amino acid residues which also contained γ-carboxyglutamic acid. These authors named this protein ovocalcin. Schade (1987, cited by Gautron, 1994) described one of the building blocks of soluble matrix as a polypeptide structure with several attached polysaccharide chains (resembling proteoglycans). According to Eckert *et al.,* (1986) the molecular weight of these subunits was estimated to be between 15 and 19kDa.

Using soluble matrix proteins characterised by chromatography and SDS-PAGE, Hincke *et al.,* (1992) demonstrated that mineralized layers of the shell possess a complex array of distinct proteins which are differentially distributed between the mammillary and palisade layers of the eggshell. The most abundant shell matrix protein was found to have a molecular weight of 17kDa and was named ovocleidin-17 [ovum, Latin - egg; kleidoun, Greek, to lock in] (Hincke *et al.,* 1993). This protein was purified to homogeneity by a combination of anionic exchange and hydroxyapatite chromatographies. Antibodies to this protein were raised in rabbits, thus enabling western blotting and immunohistochemical studies to be carried out (Hincke *et al.,* 1993, 1995). These authors found that whilst the OC-17 antigen was found in the shell gland mucosa only the tubular gland cells were positive. These cells also contain calbindin and are therefore thought to be responsible for calcium transport into the lumen of the shell gland (Wasserman *et al.,* 1991). Hincke *et al.,* (1993, 1995) have therefore hypothesised that calcium and OC-17 are secreted at the same cellular source and that the protein becomes trapped within the mineral during shell formation.

Immunohistochemical studies have indicated that OC-17 is uniformly distributed throughout the shell matrix but appears to be concentrated in the mammillary bodies (Hincke *et al.,* 1993). These results, however, relate to pooled eggs of unknown provenance and so it is uncertain if the distribution of this specific protein varies in eggs of good and poor quality.

More recently, Hincke (1995) has identified a 43kDa protein found in eggshell matrix extract as ovalbumin. In contrast to OC-17, this specific protein appears to be found only in the mammillary bodies and is not distributed throughout the shell matrix. It is as yet unclear as to whether ovalbumin plays a specific role in shell mineralization.

By treating the insoluble matrix with sodium dodecyl sulphate (SDS) it can be split into subunits which can then be fractionated (Eckert *et al.,* 1986; Schade, 1987; cited by Gautron, 1994). These authors found the insoluble matrix to be composed of a limited number of polypeptides (50, 27, 16 and 14 kDa). Multiple modifications of these polypeptides are possible, due to the presence of sulphated oligosaccharides (Krampitz, 1993).

Given that current hypotheses depend mainly upon information from experiments using large organic molecules (Arias *et al.*, 1992, 1993; Arias and Fernandez 1993, 1995; Wu *et al.,* 1992, 1994) and mixtures of proteins (Wheeler and Sikes, 1984; Addadi and Weiner, 1992) then further testing of these hypotheses requires that properties of purified eggshell matrix proteins, such as OC-17 and ovalbumin, be studied.

[iii] **The Cuticle.** This proteinaceous layer covers the shell to a depth of about 10μm, although its presence can be irregular and varied (Parsons, 1982). It contains fine particles of an inorganic phosphate and is known to be composed of mucin and phosphorus in the form of glycoproteins of high molecular weight with hundreds of carbohydrate side chains. According to Arias *et al.,* (1992, 1993) and Arias and Fernandez (1993, 1995) organic matrix is absent in this layer.

The cuticle acts as a barrier to pathogenic invasion by plugging exposed pore sites, although Nascimento (1992) has shown that often these cuticular pores remain unplugged. Board and Halls (1973) found that the cuticle is also occasionally totally absent.

Integration with porphyrin pigment complexes provide a function other than a physical barrier for the cuticle. Indeed, the presence of an insoluble organic phosphorous compound associated with its deposition (Nys *et al.,* 1991) and the presence of cuticular vesicles which appear to contain high concentrations of hydroxyapatite ($Ca_{10}PO_4OH_2$) (Fink *et al.,* 1993), all support the role of phosphorous in the termination of shell formation. The latter authors have hypothesised that the secretion of a phosphate-rich phase within the cuticle competitively poisons the calcite growth process, although the exact mode of action remains unclear.

1.2.5. STRUCTURAL AND ULTRASTRUCTURAL VARIATIONS.

The actual mineralization process is influenced by a number of factors such as pH, rate of deposition and the supersaturation level of the calcium carbonate (Solomon, 1991). The fact that the oviducal environment is not static results in the production of certain structural and ultrastructural variations.

At any time during the laying year, an individual hen may spontaneously produce abnormal eggshells which are usually selected out during routine grading. Such eggs include not only shells which display changes in cuticular appearance (eg. pink, lilac, calcium splash, fine dusted) but also soft shelled eggs, wrinkled eggs, slab sided eggs and eggs with an equatorial bulge (Van Middlekoop, 1971; Watt, 1989). The factors responsible for such variation are reviewed in section **1.3.**.

To date, 12 ultrastructural variations have been identified and they are all associated with the mammillary layer, Reid (1984), Watt (1985, 1989), Mohumed (1986), Bain (1990), Solomon (1991) and Nascimento (1992). These variations can occur randomly in eggshells passed as grade 1 marketable by traditional measures of shell quality and are discussed as follows. They should be considered with reference to the "normal" mammillary pattern (Figure 2) and are illustrated in Figures 3-18.

[i] **Changed Membrane.** (Figures 3, 4 and 6). The sulphate groups associated with the membrane fibres are a small but significant part of their composition; in the normal egg, plasma etching removes these groups along with the ash of the organic layer. However, many eggs examined after plasma treatment still possess an abnormally sulphur rich mat of "changed membrane" (CM) adhering to the mammillary layer.

[ii] **Cap Quality.** (Figure 5). The mammillary caps arise from the initial seeding of calcium carbonate onto and around the membrane fibres. Good quality caps (GC) should show, after plasma etching, deep channels formerly occupied by the latter and as such provide evidence of the firm bonding between the organic and the inorganic components of the shell. Poor quality caps (PC) show little evidence of such bonding.

[iii] **Type A Mammillary Bodies.** (Figure 6). These can occur singly or in groups (A). Their contact with the membrane fibres is minimal and they have a conical appearance at the level of the mammillary layer. Growth of the cone and palisade layer does occur but the columns so formed rarely contribute to the entire thickness of the shell.

[iv] **Type B Mammillary Bodies.** (Figures 7 and 8). Rounded type B bodies (B) occur more frequently in the mammillary layer than type A bodies. Although they make contact with the membrane fibres to varying degrees, they make no contribution to the true thickness of the shell.

[v] **Cuffing.** (Figure 9). Extracrystalline cuffs (CU) at the junction of the cone and palisade layers assist in the early fusion of the palisade columns and thereby enhance the shells resistance to cracking (Bain, 1990).

[vi] **Confluence.** (Figure 10). Mammillary caps are not always spatially discrete. Eggs laid by young birds invariably demonstrate large areas of fusion at the cap sites. Confluence (CO) alters pore distribution and influences the formation of the palisade layer, ie. the crystal columns which constitute the bulk of the shell.

[vii] **Aragonite.** (Figures 11 and 12). The normal shell consists of calcium carbonate in its calcite modification. Calcite is only one of three polymorphic variations, the others being aragonite and vaterite. The environmental

conditions which encourage the growth of these different forms are quite distinct. The hard calcite eggs of the domestic fowl contrast sharply with the softer aragonite eggs of many reptiles, reflecting the varying demands placed upon each during embryonic development. Variations from the norm do occur in nature, with aragonite (AG) sometimes being present in the avian eggshell and calcite in the reptilian shell.

[vii] **Cubics.** (Figure 13). Given available space, calcium carbonate will grow preferentially as a cubic form. Under conditions of stress and when the formation of normal palisade columns is impaired, individual cubic (CC) crystals frequently occupy intermammillary sites.

[ix] **Early Fusion and Late Fusion.** (Figures 14 and 15). These terms describe the growth of adjacent palisade columns. If the latter fuse early (EF) then the effective thickness (see **2.3.2.**) of the shell is increased and *vice versa* (LF).

[x] **Alignment.** (Figure 16). This relates to the ordered (or otherwise) spatial arrangement of the mammillary surface. Aligned mammillae offer a low resistance to crack growth.

[xi] **Depressions.** (Figure 17). These are areas which display a concave distortion to the normal mammillary layer appearance (D). They arise through the accumulation of oviducal debris on the shell membranes, which in turn either interferes with the distribution of nucleation sites, or masks their presence.

[xii] **Erosion.** (Figure 18). This feature relates to pitting of the mammillary surface to varying depths. Erosions (E) typically lack normal basal cap and cone structures thus exposing the underlying palisade layer.

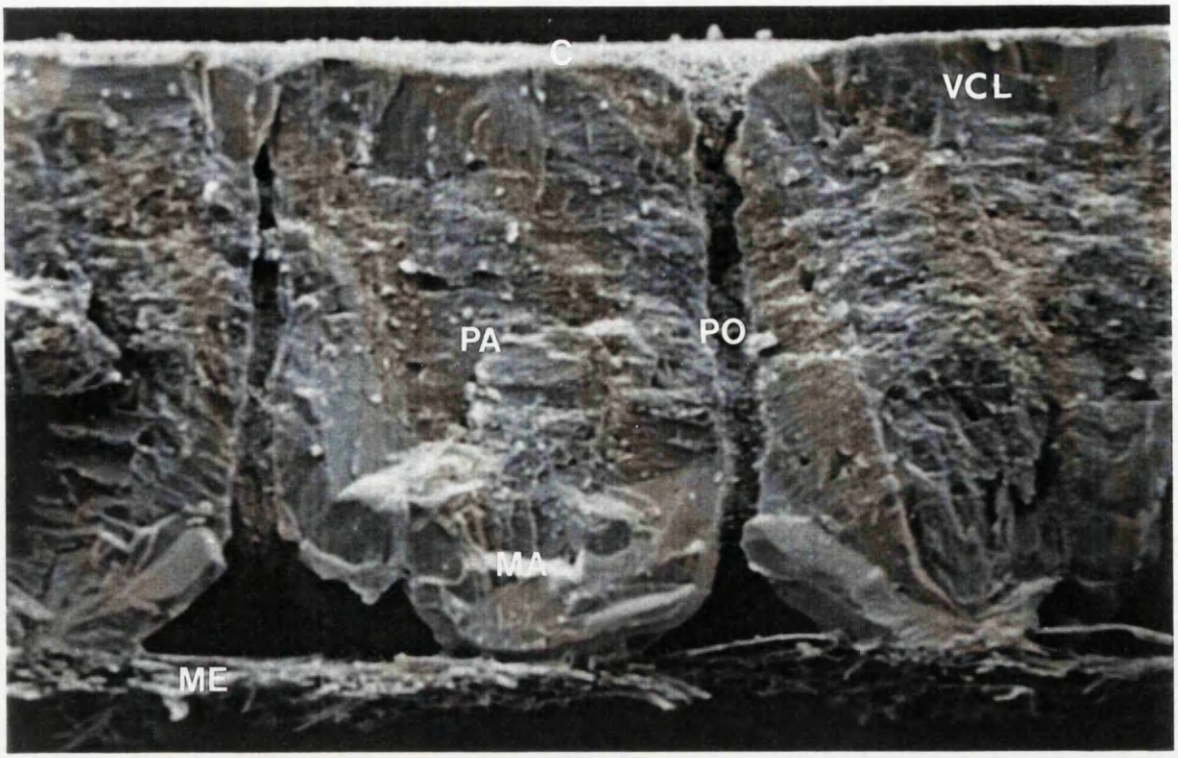


Figure 1: SEM micrograph of a transverse section of a fully formed shell (x720). ME=membranes, MA=mammillary layer, PA=palisade layer VCL=vertical crystal layer, C=cuticle, PO=gaseous exchange pore.

Figure 2: Mammillary surface of "normal shell" after membrane removal by plasma etching (x720).

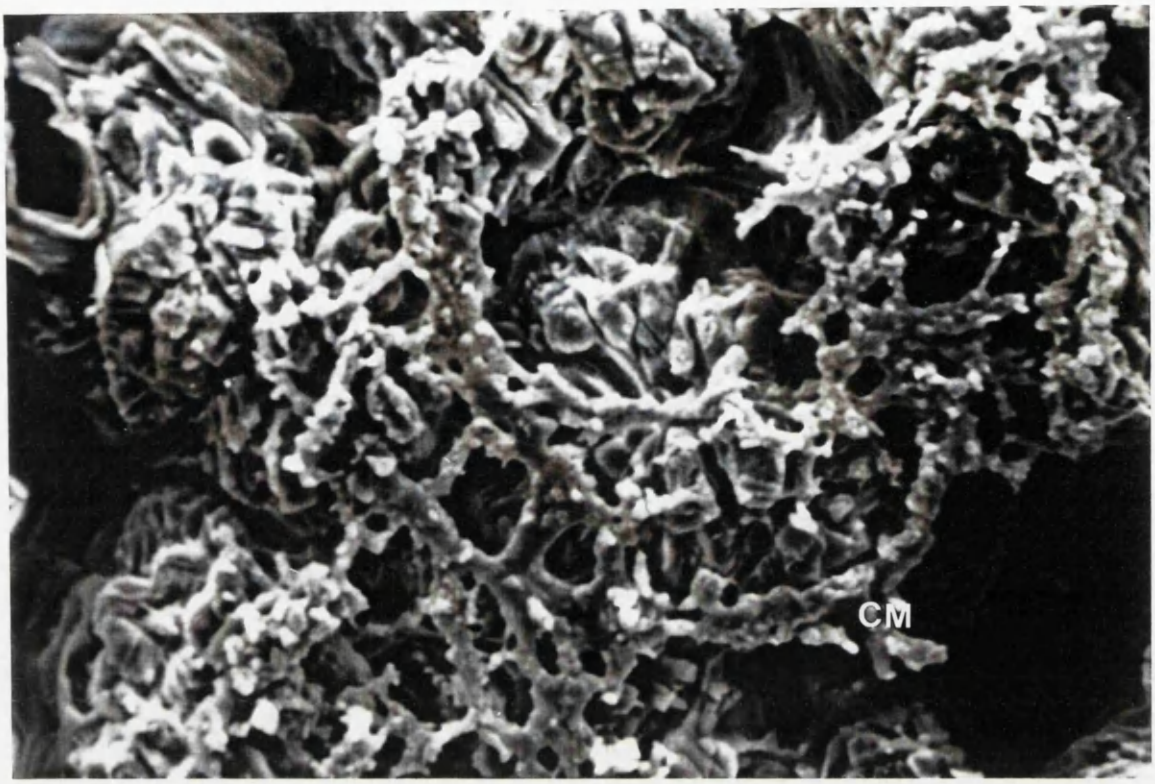


Figure 3: Sulphur rich changed membrane (CM) on the surface of the mammillary layer (x1440).

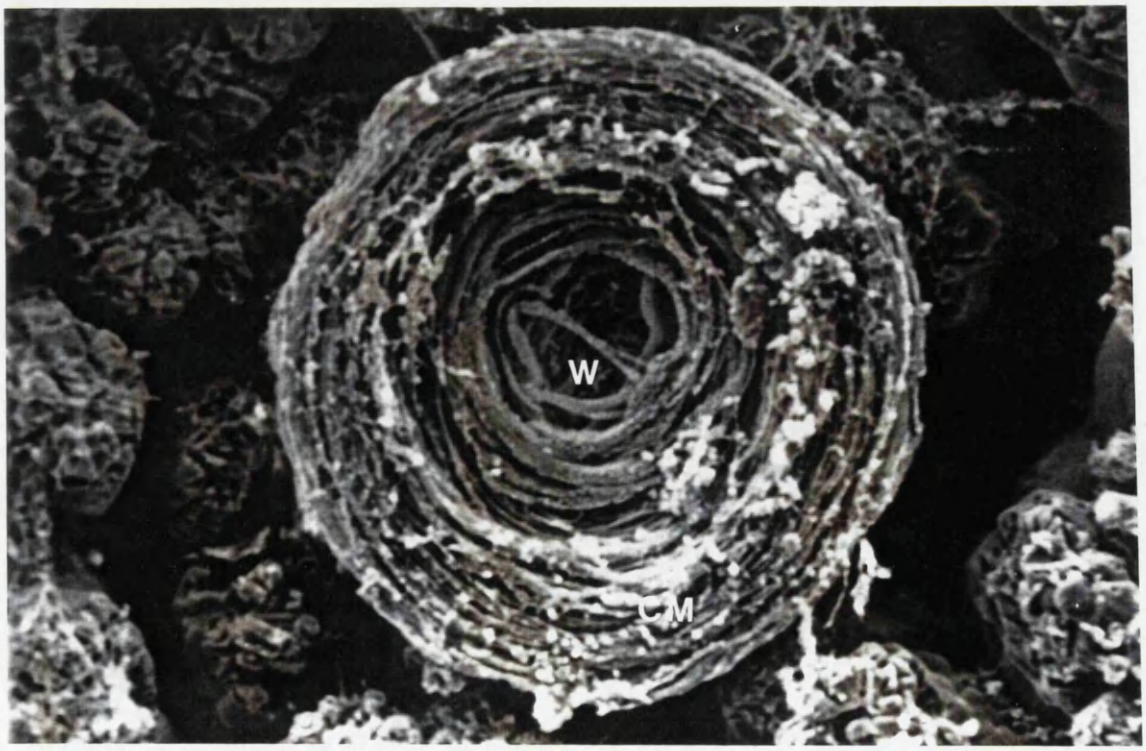


Figure 4: Changed membrane (CM) in the form of a membrane whorl (W) (x720).

Figure 5: Poor quality caps (PC) displaying minimal areas of attachment, lying between good quality caps (GC) (x720).

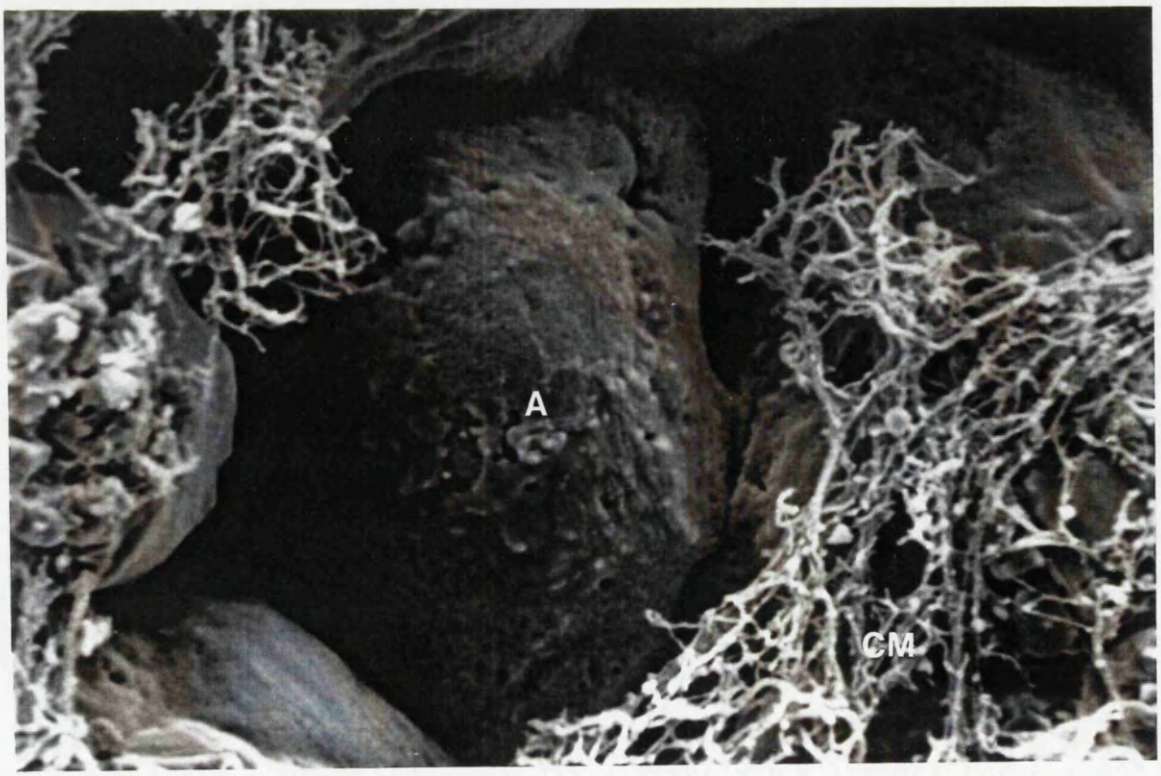


Figure 6: Type A mammillary bodies (A) and changed membrane (CM) (x1440).

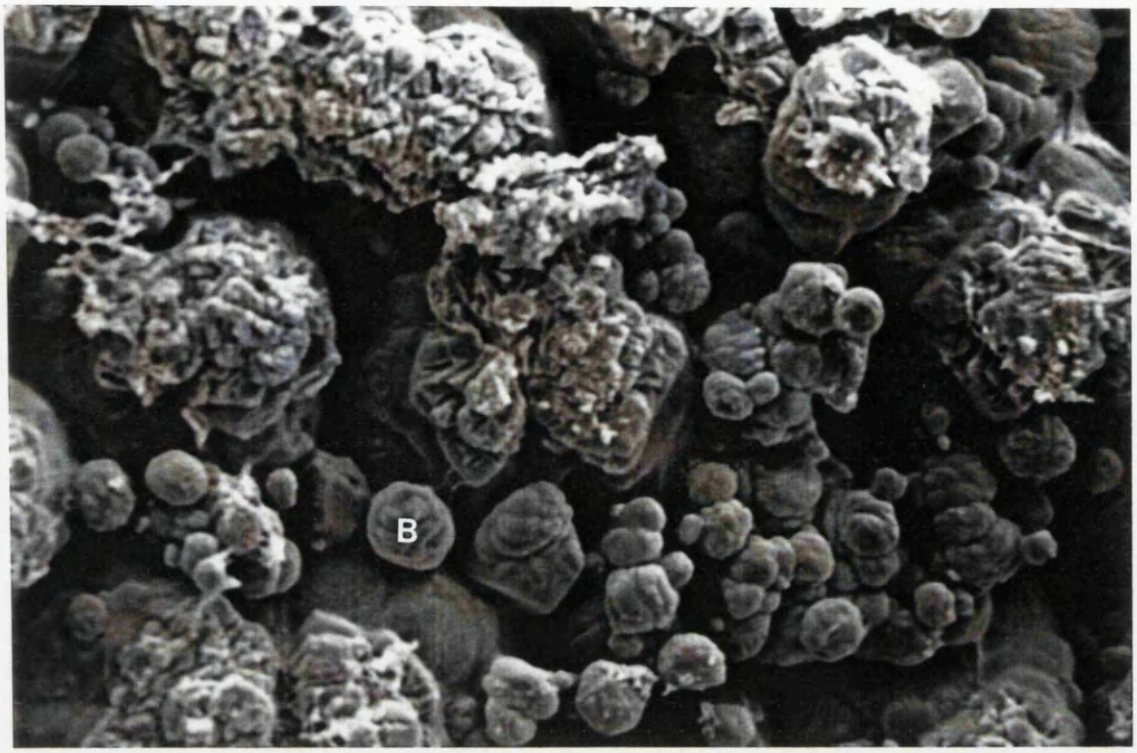


Figure 7: Type B mammillary bodies (B) loosely filling spaces between adjacent mammillae (x720).

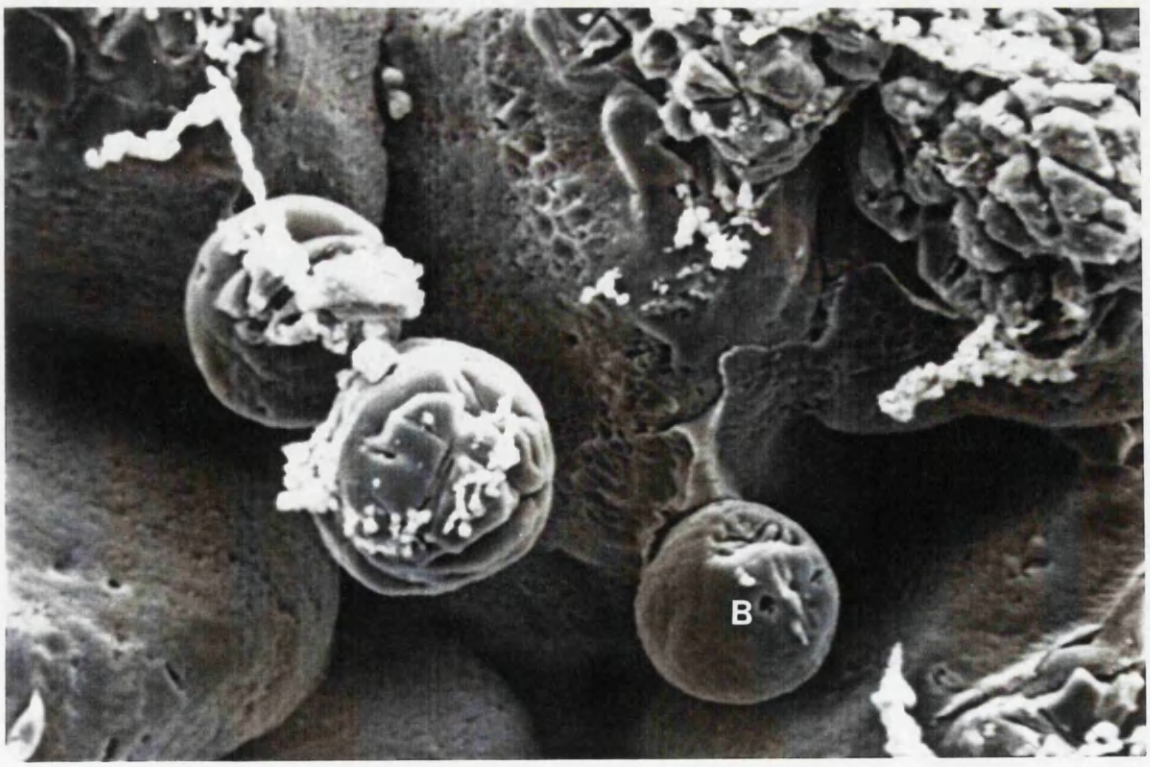


Figure 8: Type B mammillary bodies (B) which make no contribution to the palisade layer (x1440).

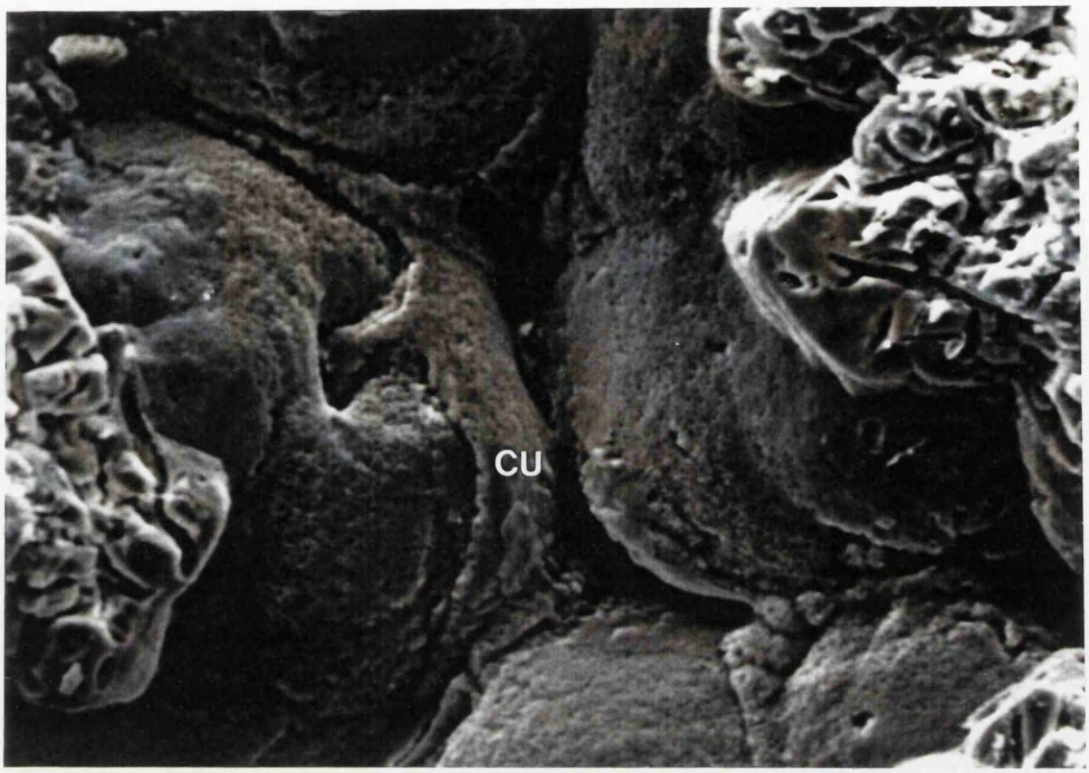


Figure 9: Cuffing (CU) constitutes a secondary nucleation of calcium carbonate between adjacent mammillary knobs (x1440).

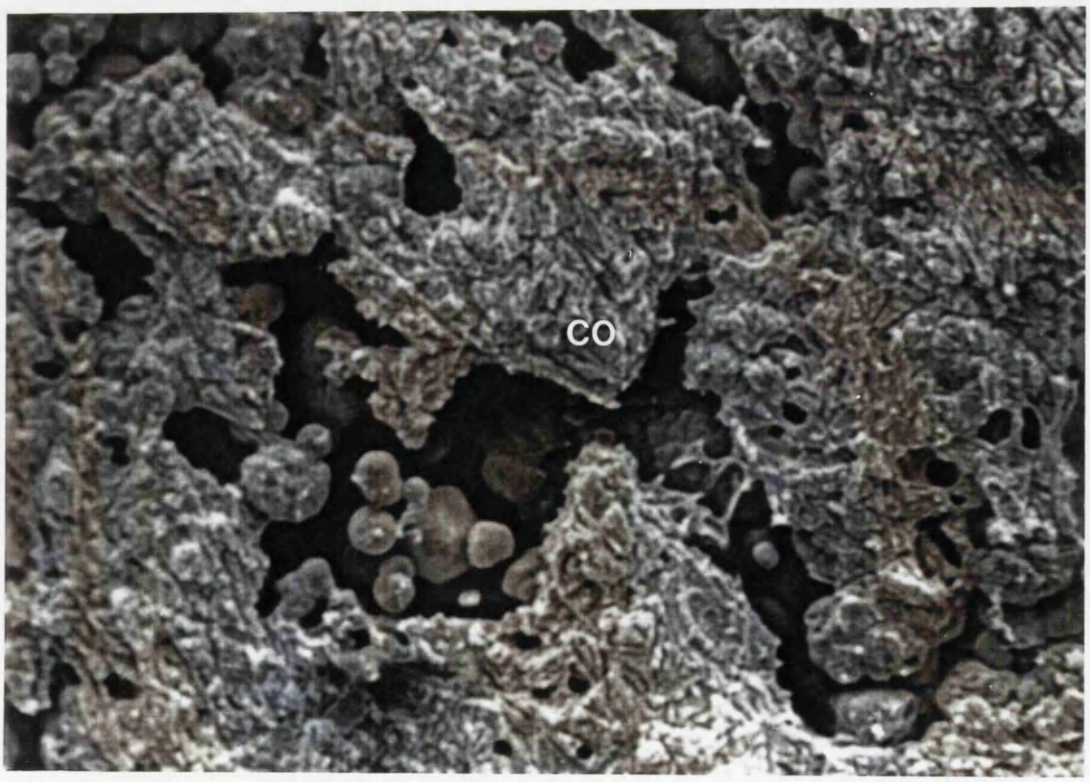


Figure 10: Confluence (CO) resulting from fusion at the cap sites. Type B's (B) are also present in the inter-mammillary spaces (x360).

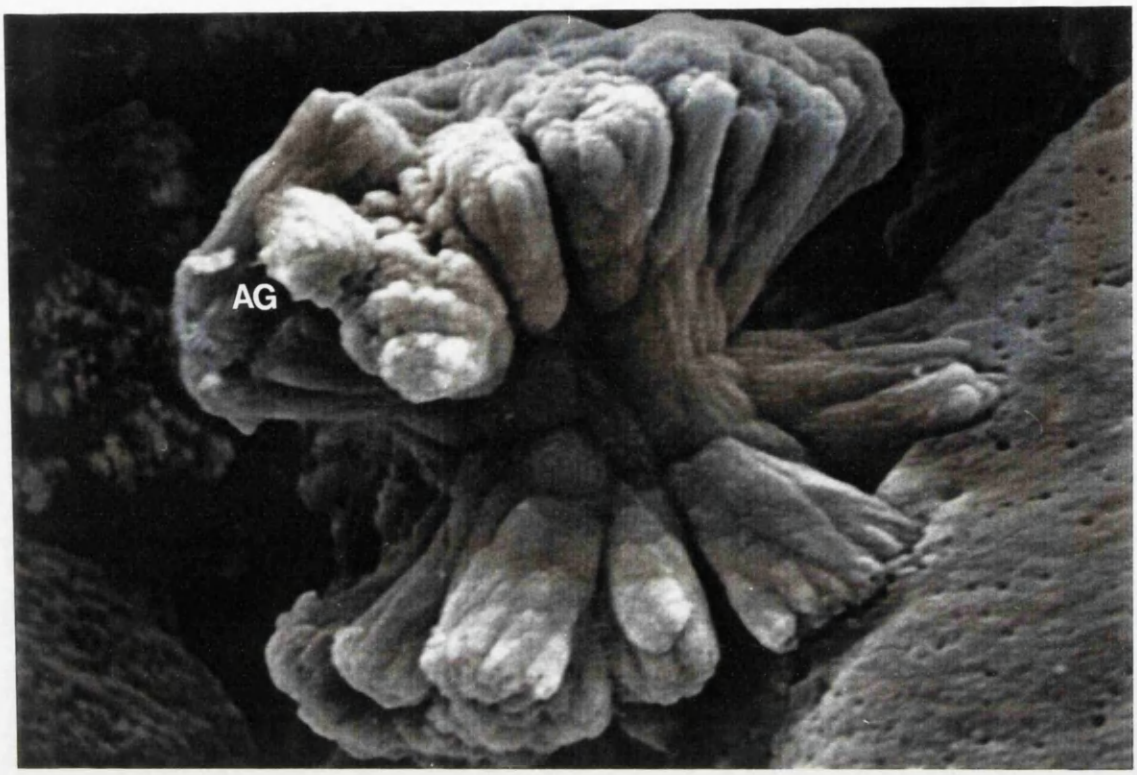


Figure 11 : Aragonite crystal (AG) in typical "wheatsheaf" formation (x2800).

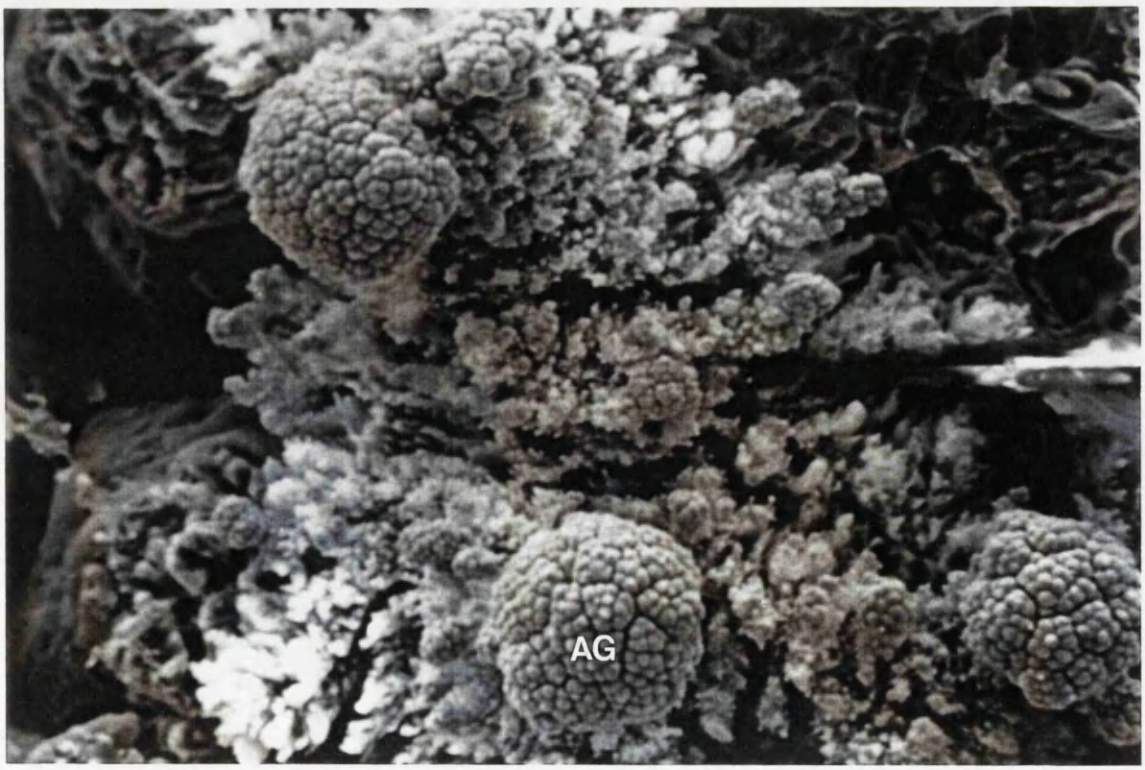


Figure 12: Aragonite crystals (AG) in less common " floret" form (x720).

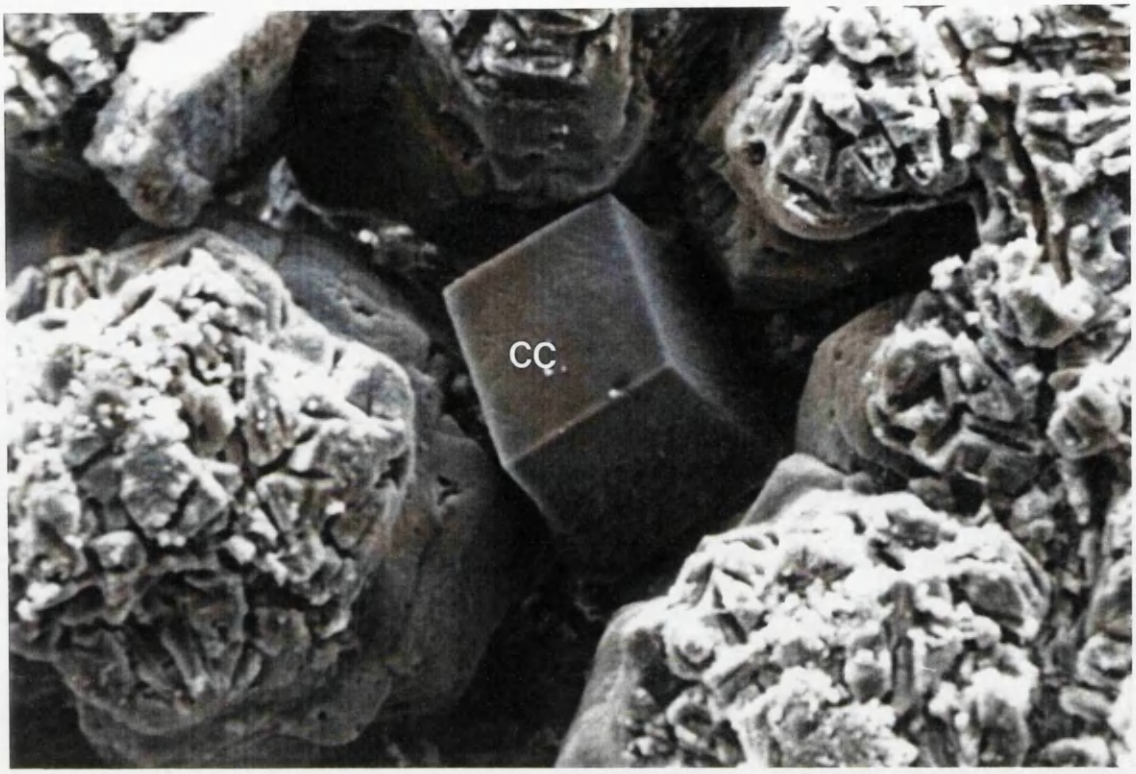


Figure 13: Cubic crystal (CC) located in an inter-mammillary space (x1440).

Figure 14: Early fusion (EF) of mammillary caps (x720).

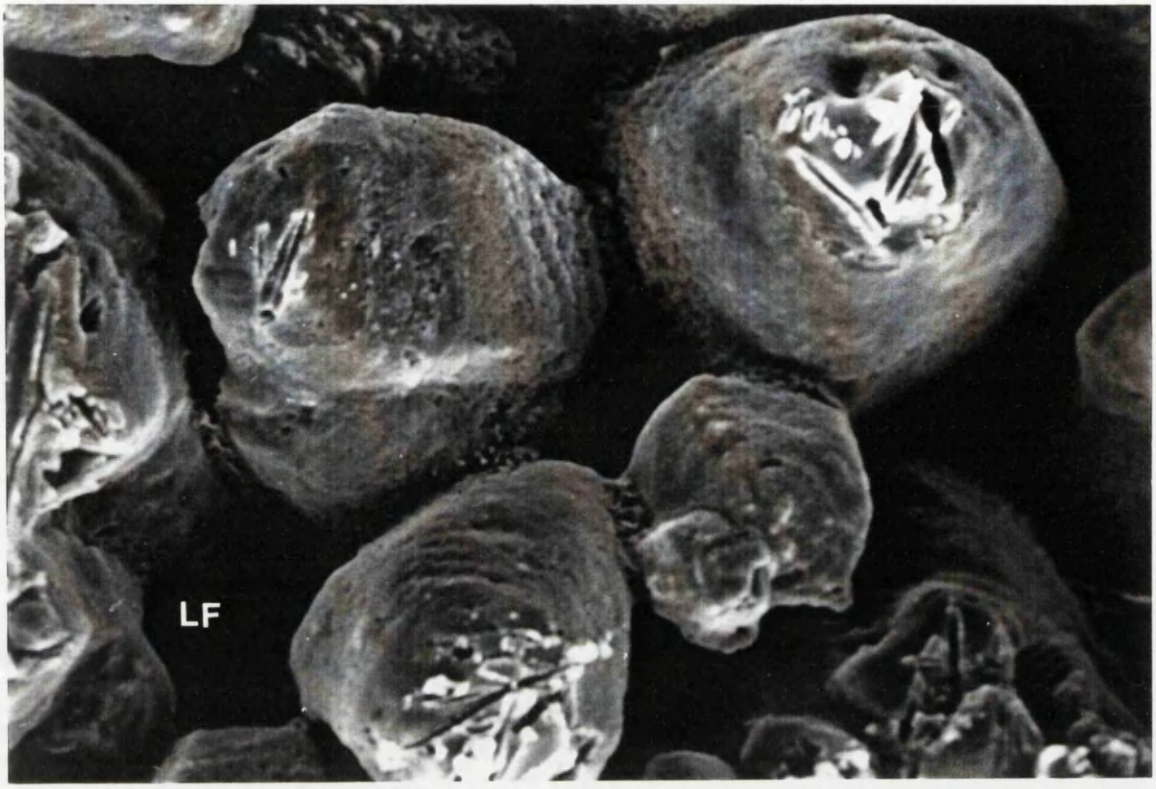


Figure 15: Late fusion (LF) of mammillary caps (x1440).

Figure 16: Alignment of mammillae (x90).

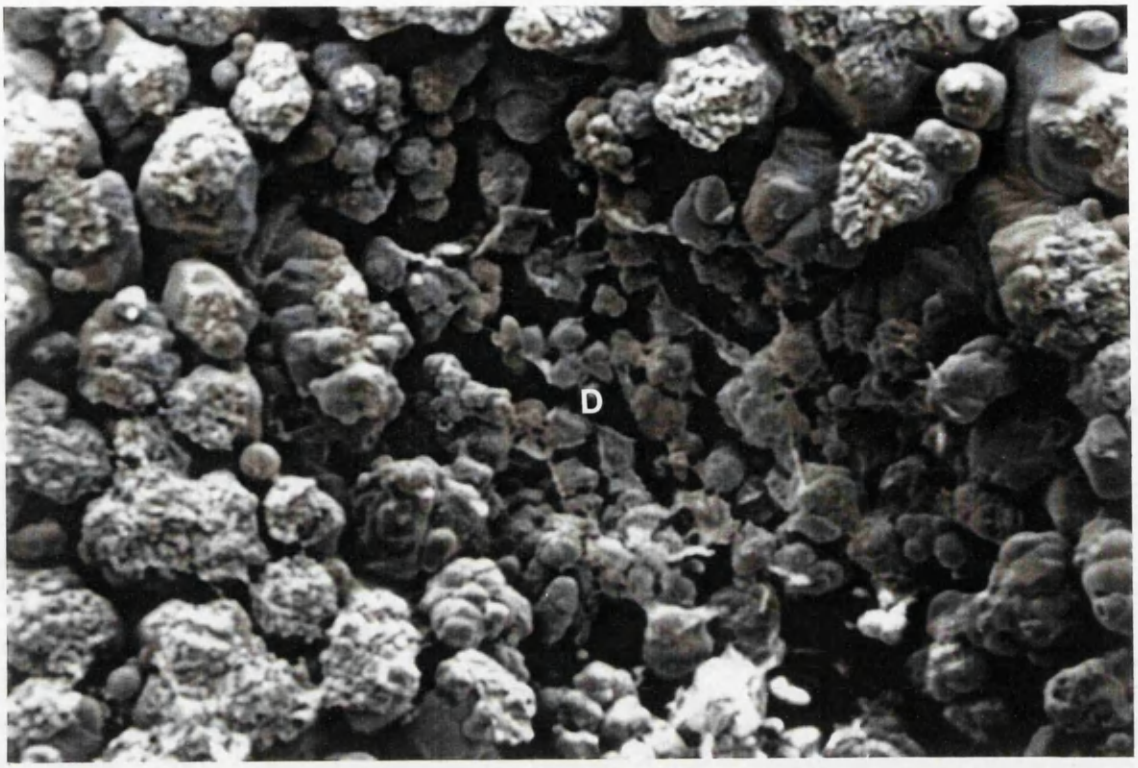


Figure 17: A depression (D) in the mammillary surface (x360).

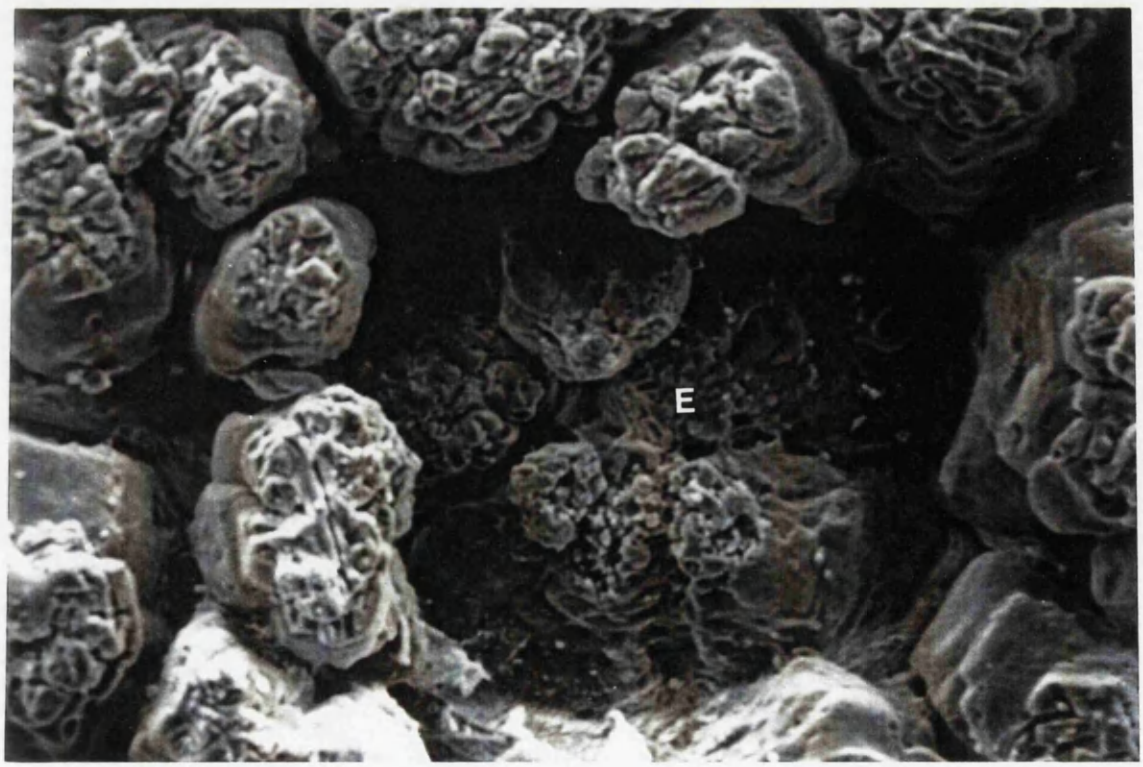


Figure 18: An erosion (E) of the mammillary caps (x720).

1.3. FACTORS KNOWN TO INFLUENCE EGGSHELL QUALITY.

1.3.1. INTRODUCTION.

The progenitor of the domestic fowl is the wild jungle fowl (*Gallus gallus*) of S.E. Asia which lays on average 22-26 eggs *per annum*. Todays commercial hybrid fowl lays on an almost daily basis producing upwards of 300 eggs *per annum*. Most modern strains come into lay at about 20-22 weeks of age, with peak production occurring at 24-28 weeks. Commercial strains lay for approximately 40 weeks until they are aged between 70 and 72 weeks. After this time egg production is no longer economical and flocks are replaced.

The table egg has no function as an embryonic chamber (unlike the hatching egg) although factors such as shell strength, shell thickness, shell colour and egg size are very important, with well identified consumer preferences. Eggs which fall short of these criteria are worth less to the producer as they may be downgraded at the packing station or rejected by the consumer.

In the commercial environment, eggshell quality and strength are routinely assessed on farm and in company packing stations. Such measurements may be direct, as in the case of shell compression and deformation tests, or indirect, such as measurements of egg weight, egg mass, shell thickness, shell colour and specific gravity (Watt, 1985; Bain, 1990). All table eggs are also graded and candled to remove any hairline cracks not visible to the naked eye. The methods used to measure shell quality commercially are not intended to identify specific shell faults, they are used to establish trends and identify problems, thereby enabling quality to be monitored and maintained (Belyavin *et al.*, 1991).

However, shell performance is not merely a reflection of its thickness. Application of finite element analysis techniques to the eggshell under load enabled Bain (1990) to demonstrate that material properties, namely the stiffness of the shell and its resistance to crack growth, influence its ultimate strength.

Although the mechanisms involved are currently not well understood, the relationship between the organic and inorganic fractions of the eggshell are also believed to play an important role in terms of shell strength and quality (Arias *et al.,* 1992, 1993; Arias and Fernandez, 1993, 1995; Gautron *et al.,* 1993, 1995 in press; Gautron, 1994; Hincke *et al.*, 1992, 1993, 1995; Hincke, 1995).

Eggshell production and formation is not a static phenomenon (see **1.2.6.**) and eggshell quality is influenced by many factors (Petersen, 1965). These include: the age of the bird, its genotype, the position and number of eggs laid within a clutch, nutrition, disease and the environmental conditions pertaining during egg production.

1.3.2. AGE.

After peak production egg numbers decline and eggshell quality also deteriorates (Bain, 1990). Brooks (1971) found total breakages of 2.75% of all eggs laid in the first month with this figure rising to 13.5% in the fifteenth month. Boorman *et al.,* (1985) found a general downward trend with age with considerable variation amongst individual birds, whilst Bain (1992) reported a linear decline in shell breaking strength with age in battery cage flocks. This results in an overall increase in downgrading due to shell damage and faults with increasing flock age (Watt, 1989). Belyavin (1986) considered this to be one of the greatest problems associated with commercial laying flocks. Roland *et al.,* (1975) and Roland (1979, 1981) reported that with increasing flock age, the amount of shell deposited either did not change or increased slightly. As egg weight increased with flock age, this resulted in thinner shells.

1.3.3. GENOTYPE.

Most modern laying hens are genetic hybrids arising from crosses of pure bred lines such as Rhode Island Red, Plymouth Rock, White Leghorn and White Cornish with each breeding company developing its own strains tailored to meet specific market requirements, Tullett (*pers comm*). Solomon (industry report) found significant ultrastructural differences in the quality of shells both within and between the four main strains of commercial hybrid stock used in the UK (all these being brown shelled strains). According to Potts and Washburn (1983) the average breaking strength of eggs is

consistently higher in white strains than in brown strains.

Although genetic variation in shell quality is well established, little is known about the biochemical mechanisms which determine specific shell quality characteristics (Bulfield and McKay, 1985; cited by Bain 1990).

1.3.4. TIME OF OVIPOSITION.

The time interval between successive ovipositions ranges between 24 and 28 hours. The results of studies by Washburn and Potts (1975), Roland (1981), Arafa *et al.*, (1982) and Belyavin *et al.,* (1985) indicate that eggs laid in the afternoon exhibit better shell characteristics than eggs laid in the morning.

Within any one clutch shell strength varies and can be quite erratic in long sequences (Belyavin, 1986). The latter author maintains that the first egg in the sequence has a weaker shell than the second.

It is also possible for birds to have two ovipositions per day (Watt, 1989). In this situation the first egg is retained past its normal oviposition time and the following egg, upon its arrival in the shell gland, is then pressed against the egg being retained causing the formation of a characteristic "slab sided" appearance. Both these eggs would be downgraded in the commercial situation.

1.3.5. NUTRITION.

The laying hen eats to satisfy its need for energy and consequently all other nutritional requirements are formulated to be in balance with energy levels (Dun, *pers comm*). With the exception of water, the most important factors in terms of shell quality are calcium and phosphorus. According to Bain (1990) the literature on dietary manipulation of these elements is extensive, yet there appears to be no consensus of opinion as to how they affect shell quality.

Levels of manganese, bicarbonate, chloride, sodium and vitamin D_3 in the diet can also significantly influence shell quality (Hurwitz, 1985; cited by Bain, 1990).

1.3.6. DISEASE.

Several disease conditions can result in the production of poor quality egg shells. These include Newcastle Disease, Infectious Bronchitis, and Egg Drop Syndrome (EDS-76) (Spackman, 1985). These diseases are now controlled by routine vaccination procedure and effective hygiene policies within commercial flocks, although breakdowns in both vaccine and procedure can result in serious disease outbreaks from time to time.

1.3.7. ENVIRONMENT.

Various environmental factors are known to influence shell quality (Brown, 1967). These include:

[i] Temperature. Egg production, egg weight and shell thickness are decreased by heat stress (Nordstrom, 1973) and the effect is even more pronounced if the relative humidity is high (Sauveur and Picard, 1985).

[ii] Gaseous Environment. Ammonia gas at concentrations greater than 20µl/l has been reported to decrease production and affect eggshell quality (Nagaraji *et al.*, 1983; cited by Watt, 1989).

[iii] Photoperiod. Belyavin (1986) suggested that although laying hens are managed under twenty four hour cycles in the majority of cases they do not cycle on a twenty four hour basis. As the flock ages it becomes less compatible with lighting management, resulting in decreased egg shell quality.

Yannakopoulos and Morris (1979) reported that a twenty eight hour ahemeral light cycle resulted in a reduction in rate of lay of 4.5%, an increase in egg weight of 5.8% and an increase in shell thickness of 9.4%. The percentage of eggs with shell faults at candling was also reduced from 4.1% to 2.8%. These authors reported that a twenty eight hour light/dark cycle resulted in a longer and more uniform interval between consecutive ovipositions, giving reliable increases in shell thickness. These increases being large enough to reduce the proportion of cracked eggs in many practical situations.

[iv] Housing. Hughes and McCorquodale (1985) reported a higher proportion of cracked eggs from battery cages compared with range over the laying year, with the difference increasing as the flocks aged. The differences reported in measures of shell strength, although real, were small and it is likely that environmental insults were responsible for a larger contribution to crack incidence than shell strength.

Mohumed (1986) reported the results of an investigation into the effects of housing on shell structure, towards the end of lay, in one strain of bird housed under three different systems viz. free range, strawyard and battery cages. The battery system gave the poorest results in terms of physical quality, this being in agreement with the findings of Hughes and McCorquodale (1985). However, ultrastructural examination revealed the presence of significantly greater ultrastructural variants in the extensive systems. Mohumed (1986) also found that the incidence of surface splashing was more evident in the free range and strawyard birds than in their caged counterparts.

Bain (1992) has also reported observations on the effect of housing on shell quality using eggs from a single strain of hen housed in battery cages, on range or in a perchery. Eggs from the range birds showed better physical characteristics at the end of lay than the battery or perchery birds. The incidence of variations in shell ultrastructure, however, was found to be more pronounced in eggs from the two alternative systems.

The wide range of production regimes currently in use or being developed means that the housing systems in which domestic poultry are kept are more varied than for any other domestic species. It is not surprising therefore that the interactions between environmental factors and poultry biology are complex and that these should be reflected in measurable difference in shell quality in eggs from the various systems. However, the bulk of information to date has relied on information gleaned from experimental situations that do not properly reflect the commercial environment. In addition, the "traditional" technology used to assess shell quality in such experiments has concentrated mainly on the physical properties of the shell.

1.4. CURRENT HOUSING SYSTEMS FOR LAYING HENS.

1.4.1. GENERAL INTRODUCTION.

The egg of the domestic fowl has long been recognised as a valuable asset to human nutrition and remains almost unsurpassed in terms of dietary acceptability and culinary diversity. Domestic fowl have been kept by man as a source of eggs (and meat) for many centuries and until the latter part of the 20th century egg production was limited to extensive systems, with seasonal production linked to the birds photoperiodic reproductive response.

The general shift towards intensification and mechanisation in agricultural production at this time, combined with a shrinking labour force, exacerbated some of the inherent problems in systems such as free range and deep litter. In particular, those associated with disease, parasitic infection, predators and aggressive behaviour escalated as flock sizes increased and available labour input fell. As a result of this the welfare of birds kept under such systems was seen to decline. This, together with an ever increasing demand for eggs and egg products, meant it was clear that production systems would have to undergo some form of radical evolutionary change in order to meet the requirements of a new era. Such a change occurred with the advent of the battery cage in the early 1960's.

1.4.2. THE BATTERY SYSTEM.

The battery system was initially developed to overcome husbandry and management problems and not to enhance profit for the egg producer (Dun, 1992). Indeed, when first introduced the capital costs were much higher than in existing systems as was labour input, due to the absence of automation. Battery units, however, evolved rapidly into the most efficient and cost effective method of producing eggs. Birds are kept in a clean, low disease risk environment which separates them from their faeces, greatly reducing the risk of parasitic infection. Food and water are freely available and the small colony size minimises aggressive interaction. Overall stocking densities are high with 20000 or more birds stocked 4 or 9 to a cage, a space allowance of 450cm^2 per bird and the cages stacked in three or four tiers. This enables one stockperson to effectively manage very large numbers of birds on a relatively

small area of land.

Changes in agricultural production during the 1960's were accompanied by sociological and economic improvements within society as a whole, resulting in a more affluent and generally better educated consumer. The majority of the population was now urban based and far removed from the practicalities of livestock production and the general perception of the farmyard was of an idyllic country environment for both man and animals alike. As the reality of the situation became apparent, the ethics of intensive of animal production began to be questioned by the consumer. The battery system was at the forefront of such criticism.

In addition to increasing public concern, the European Parliament Resolution on Farm Animal Welfare Policy (1987) stated that the use of the battery cage for the housing of laying hens contravened the Council of Europe Convention (1976) on the Welfare of Animals kept for Farming Purposes as welfare was considered unsatisfactory. As far as specific legislation regarding the protection of hens in battery cages within the EC is concerned the most important legal constraint has been the directive adopted in 1986 and amended in 1988 (CEC, 1989). This directive was then translated into national laws; in the UK the Welfare of Battery Hens Regulations were introduced in 1987, requiring all new cages to meet these standards by 1988 and all cages from Jan 1995. The main requirements were as follows (Hoelgaard, 1992).

[i] For 4 birds or more a minimum area of 450 cm^2/bird should be provided on a horizontal plane.

[ii] The cage should be at least 40cm high over 65% of the minimum cage area and not less than 35cm anywhere in that area.

[iii] The slope must not exceed 14% or 8° with rectangular mesh floors.

[iv] There should be a minimum feed trough space of 10cm/bird.

[v] A minimum 2 nipple drinkers or cups within reach of each cage or 10cm trough/bird should be provided.

Despite this legislation a review of the scientific evidence concerning laying hen welfare indicates many of the behavioural needs of the bird are still not being met by the battery cage.

Areas of concern are ability to exercise (Broom, 1992), space availability (Dawkins, 1989; Baxter, 1994), the maintenance of bone structure and normal bone function (Smith and Gilligan, 1989; cited by Baxter 1994; Knowles and Broom, 1990) and cage design (Carter, 1971a; Tauson, 1980, 1986, 1989; Gregory and Wilkins, 1989; Hughes and Appleby. 1989a; Appleby, 1991; Baxter, 1994). In behavioural terms absence of litter material for foraging (Bareham, 1976; Dawkins, 1989; Appleby *et al.,* 1992) and dust bathing Vestergaard (1982, 1989; Broom, 1992; Baxter, 1994) are considered to have an adverse effect on bird welfare. A suitable nest site is now acknowledged as an essential welfare requirement for laying hens (Wood-Gush, 1954b, 1969, 1972; Perry *et al.,* 1971; Duncan *et al.,* 1978, 1979b; Kite, 1985; Duncan and Kite, 1989; Ramos and Craig, 1988; Appleby, 1991; Reed and Nicol, 1992; Baxter, 1994) whilst inability to express perching behaviour is also considered detrimental to welfare (Ruzler and Quisenberry, 1970b; Dorminey and Arscott, 1971; Tauson 1984, 1989; Braastad, 1990; Appleby *et al.,* 1992; Duncan *et al.,* 1992).

1.4.3. ALTERNATIVE HOUSING SYSTEMS.

Both public concern and the scientific evidence relating to the welfare of laying hens in battery cages has led to a resurgence of traditional and alternative systems of egg production. The main alternative systems of egg production currently in use are listed below and will be discussed in order from extensive to most intensive. The conditions for the classification of each system, according to EC egg marketing regulations (Qvist, 1992) are also given.

1.4.3.1. TRADITIONAL FREE RANGE.

In the past, free range was a general description indicating only that poultry were allowed to range over the fields. There was no legal restriction on stocking density. Poultry fitted well into a mixed farming economy and their foraging helped control pests such as leather jackets. They frequently followed cattle which were moved on as larger grasses were eaten, leaving short new growth available for the hens.

Today, free range is a specific term and flocks described as such must meet the following criteria. The EC specifications state that inside the housing, stocking density should not be greater than 25 birds m^2 with an outdoor stocking density no greater than 1000 birds/ha of available ground (1 hen/ $10m^2$). Birds should have continuous access to open air runs and the ground to which they have access must be mainly covered with vegetation.

The type of buildings used will depend on the scale of operations with basically two options, static or movable. Within these two categories there is a wide choice, including the use of existing and refurbished buildings, purpose built units and houses made of polythene and even straw bales. The entire area should be well fenced to keep out predators. It should be remembered that birds and stockmen alike are at the mercy of the elements and any real environmental control is impossible, although supplemental light in the winter time will boost production. A very high degree of stockmanship and management is required. Fittings normally include nest boxes, perches, pop holes, feeders and drinkers. Ideally the land used should be light, free draining, fertile and capable of producing and maintaining a healthy sward. The hens should be rotated over a series of paddocks to prevent a build up of parasitic infection and prevent the land becoming "fowl sick". This can be achieved by either portable housing or movement of fenced off areas.

It should not, however, be assumed that the term free range is synonymous with improved welfare since problems such as feather pecking and cannibalism are common especially in larger flocks. Hughes and Dun (1985) for example reported a significantly higher incidence of cannibalism in free range birds compared to those kept in cages, whilst Dun (1992) reported a mortality rate of 15.5% in a free range flock compared to 4.1% with caged

layers, the additional losses in free range birds being the result of cannibalism. Dun (1992) also reported a floor egg problem associated with free range birds.

1.4.3.2. SEMI INTENSIVE (MODIFIED FREE RANGE).

For these systems the EC regulations are as for free range, with the exception that the maximum outdoor stocking density should not be greater than 4000 hens/ha of land available (1 hen/2.5m^2)

1.4.3.3. DEEP LITTER.

An egg producer who wants to market eggs with the label "deep litter" must fulfil the following conditions. The maximum stocking density may not be greater than 7 hens/m^2 of available floor space (1400 cm^2/bird) with at least one third of this area being covered with litter material such as straw, wood shavings, sand or turf. A sufficiently large part of the floor area should be available for the collection of droppings.

This was the first widely used method of housing hens intensively. The litter breaks down rapidly by bacterial activity which is exothermic, the heat produced inhibiting harmful bacteria and pathogens and also contributing to house temperature. Except where land is freely draining, a concrete floor is advised which, although adding to the capital cost, allows for thorough cleaning and disinfection. Droppings accumulate through slats into a pit which is cleaned out at the end of each laying period. Feeders and drinkers may be located on the slats to encourage birds to use them. Food distribution and egg collection can be automated as can environmental control. The perchery and aviary systems discussed later are in effect modifications of the deep litter system.

1.4.3.4. PERCHERY AND AVIARY SYSTEMS.

Eggs from these systems are often marketed as barn eggs and as such must meet the following specifications. The maximum stocking density shall not be more than 25 birds/m^2 of available floor space (400cm^2/ bird) and the interior of the building must supply at least 15 cm perch space/hen.

The aim here is to seek to make use of the vertical dimensions of the deep litter house by introducing perches (perchery) or platforms (aviary) which are located down the centre of the house. These areas are supplied with drinkers and feed troughs at regular intervals. With the same number of birds per house the area per bird can be increased by up to 50%, alternatively keeping the same floor area per bird the bird numbers can be increased by 50%. Examples of such systems include the A frame perchery (Michie and Wilson, 1984, 1985), the Hans Kier System developed in Scandinavia (Norgaard-Nielsen, 1989), the Gleadthorpe Aviary (Hill, 1982, 1983 a, b), the Swiss Voltage System (Hultgren, 1989) and the Tiered Wire Frame System (Ehlhardt et al., 1989).

1.4.3.5. MODIFIED CAGES.

These cages offer the hen a more complex environment whilst retaining the advantages of small colony size, hygiene and economics of the battery system.

Bird area specifications are identical to battery cages in modified cages (at least 450cm^2 of cage area per bird, and 10cm/bird of trough space with an adequate water supply).

Elson (1976) and Bareham (1976) evaluated changes in the design or structure of conventional cages by enlarging the dimensions of the commercial battery cage, increasing bird numbers and by including features such as perches, nests and sandbaths. Both authors obtained a considerable improvement in the behaviour of birds in such cages without any significant loss in hygiene or economy. The higher risk of vices such as feather pecking or cannibalism with increased group size was apparently reduced by having perches at different levels, which afforded subordinate birds the opportunity to

avoid confrontation with their superiors, hence the name "get away" cage.

A pilot study on modified cages was undertaken by Robertson *et al.,* (1989). Five prototype cages were designed each with a specialised nesting and perching area and some with additional dustbathing and scratching areas. All were adaptations of existing battery cage design containing between 3 and 10 birds. This resulted in the Edinburgh Modified Cage System (Appleby, 1994; cited by Sherwin, 1994), containing 5 birds, a perch, sandbath and nest site. Other examples include the Litter Shelf System (Dun *et al.,* 1986; Keeling 1989) and the Elson Tiered Terrace (Elson 1989). Modified cages for laying hens are more fully reviewed by Sherwin (1994).

Studies on modified cage design are for, the most part, in the developmental stage and have not yet been put into commercial practice. This means that a gap in current knowledge exists in terms of how these systems will perform in a business environment. However, any cage designed for commercial use must take into account the financial requirements of the industry as well as the welfare requirements of the birds.

1.5. SCIENTIFIC ASSESSMENT OF WELFARE.

1.5.1. INTRODUCTION.

Ethical views as to the treatment of animals vary from the concept of complete domination by man over lower creatures, through an intermediate position whereby man has a duty to treat animals well (ie. a good life whilst living and a quick humane death when necessary), to the idea that animals have total rights which must be fully respected (Singer, 1976). Most people would consider the intermediate position satisfactory, although an increasing number of animal rights activist organisations are emerging, some with fanatical elements, which are capable of civil disobedience and indeed terrorism to make themselves heard.

1.5.2. DEFINING WELFARE.

The first problem to overcome when considering welfare is one of definition, a variety exist in the literature. The Brambell Committee (1965) provided a general working description: "Welfare is a wide term that embraces both the physical and mental wellbeing of the animal. Any attempt to evaluate welfare must therefore take into account the scientific evidence available concerning the feelings of animals which can be derived from their structure and function and also their behaviour." Hughes (1976) subsequently considered welfare to be "a state of complete mental and physical health, where the animal is in harmony with its environment," whilst Broom (1988) regarded the welfare of an individual as being "its state as regards its attempt to cope with its environment".

Thus, no precise and unambiguous definition of welfare exists. Nevertheless, in any consideration of the subject the "Five Freedoms" listed by the Farm Animal Welfare Council (cited by Dun, 1992) should be taken into account. These are listed as follows.

[i] Freedom from hunger and thirst.

[ii] Freedom of movement.

[iii] Freedom from pain, fear and distress.

[iv] Freedom from injury and disease.

[v] Freedom to exercise most normal patterns of behaviour.

It is the opinion of the present author that these freedoms should only be perceived as a general guideline in animal production systems. Thus, within the battery cage total freedom of movement cannot be accommodated and the freedom to exercise normal behaviour is compromised, although modifications of the system can enrich the environment. The other statements could be perceived as dictates, fairly easy to voice but much more difficult to apply given the vagaries of commercial production systems.

1.5.3. INDICATORS OF WELFARE STATUS.

A variety of indicators used in the assessment of welfare status exist. These include.

[i] **Behaviour.** One of the most simple and direct methods of obtaining information on welfare is to observe the animal in its natural or production environment and there are many behavioural measures which allow some assessment of welfare status. (Broadhurst, 1960; Fox, 1968; Fraser, 1968; Meyer-Holzappfel, 1968; Wood-Gush *et al.,* 1978; Wiepkema *et al.,* 1985; Dawkins, 1989).

Stereotypic behaviour involves a relatively invariate sequence of movements occurring so frequently in a particular context that it cannot be considered to form part of the normal functioning of the animal. The general consensus of opinion at present is that this kind of behaviour is a means of coping with difficult conditions via the production of analgesic peptides such, as β-endorphin and metenkephalin, which enable the animal to self narcotise. Stereotyped movements form part of the normal behavioural repertoire of

animals but the occurrence of prolonged stereotypies indicates that conditions are adverse to the individual (Broom, 1983, 1988). An example of such behaviour is the stereotyped pacing exhibited by certain hens in battery cages due to the absence of a suitable nest site (Duncan, 1970; Reed, 1991). Other behavioural indicators of welfare status include vacuum behaviour (Lorenz, 1976; Duncan 1979a+b; Vestergaard, 1982, 1989) and displacement preening (Duncan and Wood Gush, 1972a).

Another means of using an animals behavioural responses to try and evaluate welfare status is the use of preference tests. The use of preference testing in welfare studies has been reviewed fully by Duncan (1981) and Reed (1991).

[ii] **Health.** Obviously any situation which results in a decline in health status will result in a decrease in animal welfare. Animals which are ill can normally be identified, enabling therapeutic measures to be taken. Of course, animals may suffer despite appearing to be in good health. Some physiological disease conditions may result in changed hormone levels which can be used as indicators of reduced welfare (Baldwin and Stephens, 1973; Hails, 1978; Kilgour and Delangen, 1970; Wood-Gush *et al.*, 1975). See **[iv]**.

[iii] **Productivity.** Often the commercial farmer/producer will argue that an animal which is producing well cannot be suffering or be experiencing a poor welfare status. Productivity in itself is a rather unambiguous term. It may be calculated on an individual basis, on a flock/herd basis, or on a farm basis. With products such as fatty livers from geese productivity maybe high yet the welfare of the birds in question is obviously poor.

Whilst an inability to grow or reproduce when given a suitable partner may indicate that welfare is poor, the reverse need not apply (Broom, 1988). An animal which is growing and reproducing may only be able to do so by extensive use of behavioural and psychological coping procedures and may be very susceptible to disease should a challenge occur. To conclude, productivity has certain scientific merit as a measure of welfare but should always be interpreted with caution.

[iv] Physiological measures. The use of physiological measures of welfare status is linked to the animals stress response. In his formulation of the "General Adaption Syndrome" Seyle (1956) showed the role of glucocorticoids in coping with stressors. Adrenal-cortical activity is not, however, only confined to adverse conditions, it also occurs during courtship, mating and seeking food, Broom (1988).

Initially, the very process of collecting blood samples for analysis exposed the host to stressors which influenced results. Many of the associated problems have now been overcome as techniques become more refined (Draper and Lake, 1967; Beuving and Vonder, 1977; Duncan and Filshie, 1979; Duncan, 1981).

Ganong (1963) has stated that "Stress is certainly one of the most grandly imprecise terms in the lexicon of science" and Seyle (1973) maintained that "Everybody knows what stress is and nobody knows what stress is". Freeman (1987) acknowledged that its meaning changes from situation to situation and from user to user.

As in the case of welfare, various definitions exist in the literature (Seyle, 1950; Ganong, 1963; Broom, 1983). Amoroso (1967; cited by Freeman, 1987) devised the mnemonic Situations That Release Emergency Signals necessary for Survival. This is useful as it illustrates the concept that stress is a means of adaptation and restoration of homeostasis in an organism.

However, the term stress is currently being questioned in the field of animal science because of the confusion surrounding its definition. "Discomfort" has been suggested as a substitute (Mills, *pers comm*).

In modern poultry production birds are routinely exposed to behavioural and physical stressors, the effects of which will be dependent upon the severity and duration of the stressor, the age, sex and genetic make up of the bird. No individual is completely free from the effects of stressors and it may be that health status *per se* may be a stressor.

The influence of pharmacological stress (injection of adrenaline) and environmental stress (mixing birds normally caged separately) on shell structure and quality has been described by Hughes and Gilbert (1984), Hughes *et al.,* (1986) and Watt (1989). The results obtained by these authors, although useful, arise from studies on small groups of birds housed under conditions very different to those found in commercial practice.

1.6. AIMS OF CURRENT WORK.

Within the constraints of the academic environment, it is relatively simple to establish a research protocol which will provide information on aspects of bird welfare and environment. The translation of these results to the commercial situation can be fraught with difficulties, however, given that in the latter the influence of economies of scale and diversity of management input are immeasurable. Nevertheless, this thesis sets out in the first instance, to address shell quality within the industrial sector by comparing and contrasting eggs produced within the battery, perchery, modified free range and free range systems where management, strain of bird and experimental procedure were all standardised. Following this broad spectrum approach the thesis then addresses the following specific areas:

[i] The influence of an enriched environment in the battery system on shell quality and structure.

[ii] The influence of two different stocking densities within this environment in respect of current and proposed EC legislation regarding space allowance per bird.

Given the diversity of ultrastructure observed within the inorganic fraction of the shell, detailed consideration is thereafter given to the organic matrix in its role as a reinforcing framework. This begins with an analysis of the morphology and distribution of the matrix in shells collected at the beginning, middle and end of lay and then seeks to elucidate the composition and role of soluble and insoluble matrix fractions from eggshells classified as good and poor in terms of ultrastructural quality. The effect of plasma etching, in order to dissociate the shell membranes, was also examined.

Current evidence suggests that specific matrix proteins may be involved in the process of shell mineralization. As the wealth of literature is in favour of the matrix proteins directing the growth pattern of the mineral phase, the penultimate experimental chapter of this thesis is devoted to a study of the influence of matrix proteins extracted from good and poor quality shells on the crystallization of calcium carbonate *in vitro*.

The final experimental chapter of the thesis constitutes an investigation into the distribution and localisation of a specific matrix protein, in good and poor quality shells, using immunohistochemical techniques. This was carried out in an attempt to ascertain whether the presence or absence of this protein correlates with observed ultrastructural modifications in the inorganic aspects of the shell.

CHAPTER 2 - A COMPARISON OF THE COMMERCIAL BATTERY CAGE AND THREE ALTERNATIVE SYSTEMS OF EGG PRODUCTION IN TERMS OF SHELL QUALITY AND STRUCTURE.

2. A COMPARISON OF THE COMMERCIAL BATTERY CAGE WITH THREE ALTERNATIVE SYSTEMS OF EGG PRODUCTION IN TERMS OF SHELL QUALITY AND STRUCTURE.

2.1. INTRODUCTION.

Despite the widespread criticism that it attracts, it should be remembered that initially, the battery cage was a response to management problems associated with the wider environment provided by free range and deep litter systems (Dun, 1992). Cost effectiveness and ease of management are not, however, the sole criteria to be considered when opting for a particular production system. One need only examine the research of Baxter (1994) to realise that these features have been achieved at the expense of certain behavioural aspects of bird welfare. Combined with increasing public concern and political intervention, this has led to a resurgence of interest in traditional (deep litter, straw yard, free range) and new alternative systems (perchery, aviary, colony cages and modified cages) of egg production.

Nevertheless, alternative systems should not be interpreted as the panacea for welfare related problems, as they too are known to present behavioural disorders such as feather pecking and cannibalism (Appleby, 1991). Studies of alternative systems have shown that perceived potential advantages are not always achieved in practice, this often being linked to increasing stocking density in an attempt to enable economic competition with cage producers (Dun 1992; Scott, 1995). Indeed, Harrison (1989) has expressed concern that, although many alternative systems show promising results under experimental use, they deteriorate under commercial conditions; thus the comparative performance and shell quality trials such as those conducted by Hughes and Dun (1985) and Mohumed (1986) were operated under research conditions with small flock sizes and under exceptional management procedure. Although yielding useful data, the results of such trials do not accurately reflect the commercial environment. In addition, much of the data currently available on shell quality from different housing systems have arisen from trials including many heterogeneous variables such as different strains of bird, different management and nutrition. The current research was therefore designed to minimise such variability by comparing systems housing an identical brown egg laying strain of bird and managed by the same commercial egg producing company.

Bain (1992) also reports that the conventional assessment of shell quality is often based on the erroneous assumption that shell thickness is the single most important quality characteristic and that the eggshell is homogeneous throughout its thickness. In view of this information, the assessment of quality used in the present thesis takes into consideration the fact that the eggshell is a multilayered structure, in which the different components all contribute to its performance in the field (after Bain, 1990).

2.2. MATERIALS AND METHODS.

The systems under comparison were:

System 1. Commercial battery cages (BAT), birds stocked 5 to a cage (450 cm^2/bird). Cages were stacked 4 tiers high and there were 20000 birds per house.

System 2. Perchery (PER) birds stocked at 15.5/m^2, colony size 15125 birds.

System 3. Modified free range (MFR), birds stocked at 11.7/m^2, colony size 4700 birds.

System 4. Traditional free range (TFR), birds stocked at 7/m^2, colony size 4000.

For systems 3 and 4 stocking densities refer to in house, outdoor stocking densities relating to current EC legislation (**1.4.3.**).

30 clean (unwashed) ungraded eggs were selected at random from each of the systems, at times corresponding to beginning (24 weeks), middle (46 weeks) and end of lay (72 weeks). All eggs were subjected to traditional measurements of quality and subsequently prepared for scanning electron microscopy. The resulting data were analysed using a two way analysis of variance.

2.2.1. TRADITIONAL QUALITY MEASUREMENTS.

[i] Egg Size and Shape. The eggs were first weighed to the nearest 0.1g. Length and breadth measurements were then taken, to the nearest 0.1mm, using hand callipers. These measurements were subsequently used to determine the shape index (see Bain, 1990) for each egg by dividing length/breadth.

[ii] Deformation. Using a commercial nondestructive (ND) deformation apparatus (as manufactured by Mauris N.V., Hollantleen 18, Utrecht, Netherlands) measurements of deformation were taken from three areas on the equatorial surface of each shell and a mean value calculated to the nearest μm.

[iii] Shell Breaking Strength and Stiffness. A quasistatic compression test (QSC) was carried out on each egg using a J.J. Lloyd screw driven testing machine. Each egg was placed with its major axis perpendicular to the plates so that the force was applied to the equator. All tests in the current study were carried out with a compression speed of 5cm/minute. The force exerted on the shell and the resulting deformation was recorded by means of a 100N load cell and a displacement measuring transducer coupled to an X Y chart recorder. The egg was compressed until the point of failure as indicated by an audible crack and a sharp drop in the force (F) vs. deformation (d) curve. At this point, the crosshead was reversed. The slope of the force vs. deformation line provides a direct measure of shell stiffness (Bain, 1990).

2.2.2. SCANNING ELECTRON MICROSCOPY (SEM).

2.2.2.1. GENERAL TREATMENT.

Each egg was emptied by removing the pointed pole end of the shell and shaking out the contents. Following this $1cm^2$ pieces of shell were cut from the equator using a diamond tipped circular saw mounted on a dentist's drill. Each piece of shell was then soaked in distilled water for two minutes, after which the loosely attached inner shell membranes were manually removed.

2.2.2.2. ASSESSMENT OF THICKNESS PROFILES.

After drying at room temperature the samples were snapped in two and each piece mounted vertically on a grooved aluminium stub with conductive silver paint. Each transverse section was then coated with gold/palladium for four minutes in an Emscope sputter coater SC 500 (Ashford, Kent, England) and subsequently viewed using a Philips 501B scanning electron microscope at 15 Kv.

Examination of each sample was carried out at a magnification of x160 at a constant working distance of 13, with the sample being tilted until neither the mammillary nor cuticular surfaces were visible. Following the methodology outlined by Bain (1990) the total thickness, mammillary thickness and effective thickness (t effective) were determined. The latter

comprises the point from where the palisade columns fuse, to the level of the cuticular layer.

2.2.2.3. ULTRASTRUCTURAL ASSESSMENT OF THE MAMMILLARY LAYER.

Additional 1cm^2 samples were cut from each shell. Following manual removal of the inner membranes, each sample was placed on a glass petri dish and dried in a 60°C oven for 20 minutes. In order to remove the more tightly attached outer membrane fibres the samples were then subjected to the nondestructive technique of plasma etching (see Reid, 1983) for four hours .

The Nanotech 100 Plasma Chemistry Unit utilises a low temperature activated plasma to remove the remaining tightly adhering membrane fibres from the shell surface. The specimens are placed inner surface uppermost in an atmosphere of oxygen gas at 133.3 Pascals made reactive by applying a radio frequency of 100 ohms. Volatilisation removes the organic content of the membrane fibres and any residual ash is blown away with a jet pressure duster leaving the mammillary surface of the shell intact.

Following plasma etching, the samples were individually mounted mammillary layer uppermost on aluminium stubs using conductive silver paint. They were allowed to dry in a 60°C oven and then coated with gold/palladium, prior to scanning electron microscopy.

Analysis of shell ultrastructure was carried out using the methodology as described by Reid (1984), Watt (1985, 1989), Bain (1990) Solomon (1991) and Nascimento (1992). The incidence of confluence, depressions, erosion and mammillary alignment (see **1.2.5.**) in each sample was evaluated at a magnification of x40. The remaining features were assessed at a magnification of x320. The presence or absence of changed membrane was confirmed by means of an EDAX analysis (see Watt, 1985, 1989).

Bain (1990) described a scoring system for quantifying the incidence of structural variants in the eggshells of commercial layers. In brief, each variant was assigned a range of possible scores weighted in terms of whether a high or low incidence of that particular feature contributed to or detracted from the

shells performance. Following a reinterpretation of the data, Bain (*pers comm*) has since developed an updated version of this scoring system and this has been applied in the current study (see Figure 19).

2.2.2.4. SPECIALISED ASSESSMENT OF THE MATERIAL PROPERTIES OF EGGSHELLS.

[i] Elastic Modulus. The elastic or Young's modulus of a material describes the unique relationship that exists between the stresses and strains that are produced when the egg is subjected to moderate loads. It is in turn related to the stiffness characteristics of the shell.

The values for effective thickness together with length, breadth and stiffness (F/d) were used in each case to calculate the elastic modulus (E. shell) of each shell as outlined by Bain (1990) where,

$$E.\ shell = \frac{C\ F\ R}{d\ t^2 effective}$$

C=compliance (dependent on shape)
R=radius of curvature (breadth/2)
F/d=stiffness

[ii] Fracture Toughness. The fracture toughness (Kc) provides a quantitative relationship between the applied force necessary to cause failure and the size of any defects that may be present in the shell. For each egg, Kc was calculated using the method described by Bain (1990) where,

$$Kc = Knd.\ \frac{F}{t^{3/2}}$$

$$Knd = 0.777(2.388+2.934\ \text{ø})^{1/2}$$

and

$$\text{ø} = \frac{\text{critical crack length}}{\text{radius of curvature}}$$

Force (F) was taken to equal the force at fracture (breaking strength), the thickness (t) as the effective thickness, and the critical crack length was assumed to equal 6mm, this is the point at which crack growth becomes unstable (Bain, 1990).

2.3. RESULTS.

2.3.1. TRADITIONAL ASSESSMENT OF SHELL QUALITY.

Mean values ± standard deviations are presented in Table 1. Statistically significant differences between and within systems are indicated in Table 2, as are any significant interactive effects.

All of the parameters measured showed significant differences both between and within systems, with the exception of shape index, which only demonstrated a within systems difference. The most relevant parameters to the producer viz. egg weight, shape, deformation, breaking strength and stiffness, are illustrated in Graphs 1-5.

2.3.2. SEM ASSESSMENT OF SHELL QUALITY.

[i] Thickness profiles. Total, mammillary and effective thickness measurements all showed significant differences both between and within systems (see Tables 1-2 and Graph 6).

[ii] Ultrastructural assessment of the mammillary layer.

The overall ultrastructural integrity of the shell can be represented in terms of a total ultrastructural score. This comprises the summation of a series of weighted values assigned to each of the 12 ultrastructural variants found in the mammillary layer (**2.3.3.**).

Mean values ± standard deviations are presented in Table 3.

A statistically significant difference was demonstrated between the systems in terms of total ultrastructural score (see Tables 3-4 and Graph 9), and specifically confluence, type B's, depressions, cubics, type A's, cuffing (see Tables 3-4 and Graphs 10-15), cap quality, and fusion (see Tables 3-4).

No significant difference was observed in terms of total ultrastructural score within each system, however, a significant difference was demonstrated for confluence, cubics, type A's, cuffing, aragonite (see Tables 3-4 and Graph 16), cap quality and fusion (see Tables 3-4). Significant interactive effects are also indicated in Table 4.

2.3.3. MATERIAL ASSESSMENT OF SHELL QUALITY.

The material properties of the shells examined showed significant differences between and within systems viz. elastic modulus (see Tables 1, 2 and Graph 7) and fracture toughness (Graph 8).

2.4. DISCUSSION.

The traditional measurements of quality assessment indicate that differences exist both between and within (in relation to increasing bird age) the systems under investigation, although no consistent differences were apparent throughout the laying period (see Tables 1-2 and Graphs 1-5). At the beginning of lay, the eggs from the MFR were significantly larger (in terms of weight, length and breadth, Graphs 1-2) than those laid within the other systems and at the end of lay, the perchery system encouraged the production of significantly smaller eggs. The root cause or causes for these differences are probably many and varied; for example it was shown by Hughes and Dun (1985) that increased egg weight on range was linked to a higher food intake as a result of the birds consuming pasture. In the current trial, it is possible that access to grass in the MFR flock has resulted in increased food consumption and therefore heavier eggs from this system at the beginning of lay. The fact that this was not the case at the middle and end of lay may be explained by an acclimatisation to this supplementary food source by the birds, or perhaps to a deterioration in pasture quality.

If access to vegetation is the major factor influencing food intake and thus egg weight, one might have anticipated that the eggs laid within the TFR would also be larger. This was not the case. At the beginning of lay, the eggs from the TFR were comparable to those laid within the battery and perchery systems while at mid lay, they were the largest. At the end of lay, although following the traditional pattern of increasing egg weight with increasing bird age (Bain, 1990; Solomon *pers comm*), the eggs from the TFR were smaller than those produced by the MFR and battery systems. It is tempting to speculate that the smaller egg size observed at various stages of the egg laying cycle within the TFR is a direct reflection of the lowered stocking density in operation within this system. Under these conditions it is feasible that more energy is expended in the process of foraging, resulting in the production of smaller eggs.

Attention is now drawn to the egg size within the perchery system. At the beginning of lay, egg size is comparable with those from the battery and TFR systems. However, as these birds age and egg size increases, the eggs associated with the perchery remain appreciably smaller than those from the other systems. The perchery system affords the birds a certain degree of

freedom (in terms of a third dimension) and therefore the ability to expend energy. It, however, denies the bird access to vegetation and so it is feasible that this use of energy, in conjunction with the absence of dietary supplementation, is the reason for the production of smaller eggs.

According to traditional measures of shell quality a high ND deformation or stiffness value signifies a weaker shell. Bain (1990) has challenged this assumption and has shown that it is not always proven to be the case. The latter author has also demonstrated that factors other than those which influence the stiffness characteristics of an eggshell may affect its performance under load.

The nature of the organic/inorganic complex which constitutes the avian eggshell gives it a certain degree of flexibility or deformation, a property that is necessary if the egg is to withstand the traumas imposed upon it by the external environment. At the beginning of lay, the eggs from the perchery displayed significantly higher deformation values than the other three systems (see Tables 1-2 and Graph 3) yet according to their breaking strength values, ie. their ability to withstand load, the values from all systems were comparable (see Tables 1-2 and Graph 4). At mid lay eggs, from all the systems performed similarly with respect to deformation and breaking strength values. It was only at the end of lay that significant variation in response to these tests began to appear. Thus, at this time, the eggs from the perchery system displayed similar deformation values to those from the battery system. However, with respect to breaking strength values, the eggs from the perchery system were significantly weaker than those from all the systems under study.

According to Roland (1981) eggshells become progressively thinner with increasing bird age as the same amount of calcium carbonate is spread over a larger surface area. Thomson (*pers comm*), however, reported thicker shells at the end of lay from free range birds and the results of the present study are in agreement with these findings. Indeed, the total thickness increased from the beginning to the end of lay in all the systems assessed and therefore cannot account for the observed changes in breaking strength or stiffness (see Tables 1-2 and Graphs 4-5).

Shell thickness is a traditional measurement of shell quality in the field. Using this as an indicator of quality one would rank the eggs from the TFR first at the beginning of lay, with the eggs from the perchery occupying bottom place. At mid lay, no significant difference was apparent between the systems. However, at the end of lay, eggs from the perchery were significantly thinner than those from the other three systems. How then does this translate to the effective thickness of the shells? The effective thickness of the shell (a true expression of the depth of shell which can make a meaningful contribution to the withstanding of trauma) also increased with increasing flock age with the exception of the TFR (see Tables 1-2 and Graph 6). During the main phase of shell growth the palisade columns, which form the bulk of the true shell, fuse at varying depths. Structural anomalies within the inner layers of the shell, in particular at the level of the mammillary layer, all conspire to interfere with this fusion process. At the beginning of lay, when shell formation is a new and potentially stressful phenomenon, intrashell defects are common. Likewise at the end of lay, as age takes its toll, variations in morphology are a consistent feature of the shells produced at this time (Solomon, 1991). Within the perchery at the beginning of lay, the total thickness values were mirrored by effective thickness values. At mid lay, when all total thickness values were comparable, the eggs from the MFR system displayed a greater effective thickness than the other three and at the end of lay, these eggs maintained their increased effective thickness values. These differences highlight the need to be aware of the structural variations which exist within the normal eggshell. Measurements of total thickness make no recognition of these subtle variations which ultimately affect shell performance.

The elastic modulus is the material property relating to the shells elasticity. Unlike the ND deformation test it takes into consideration the radius of curvature, the length and breadth characteristics and the effective thickness of the shell. This refined technology revealed that the elastic properties of the shells under investigation showed a high degree of variability in the alternative systems compared to the battery system.

In general terms, the elastic modulus values (see Tables 1-2 and Graph 7) reflect the observed variations in stiffness values (see Tables 1-2 and Graph 5). Having ruled out the curvature, length and breadth characteristics and effective thickness of the eggshell as variables, this then reflects two things:

[i] the relationship between the organic and inorganic fractions of the shell is not consistent from the beginning to end of lay.

[ii] the inconsistency in this relationship appears to be greater within the alternative systems of egg production.

The fracture toughness of an eggshell is a measure of its' resistance to unstable crack growth (Bain, 1990). According to the latter author as shell quality declines the fracture toughness values will correspondingly decrease, a feature observed within all of the systems under analysis. The greatest decline in fracture toughness was found in the perchery system in the current study (see Tables 1-2 and Graph 8).

Crack initiation and subsequent growth begins at the level of the mammillary layer and thereafter proceeds, at varying rates, throughout the depth of the shell. The rate of crack growth to a large extent reflects the morphology of the nucleation layer. Thus, according to Bain (1990) the rate of fusion between adjacent mammillary bodies will directly influence crack initiation whilst an ordered arrangement of mammillary bodies will, by virtue of that alignment, facilitate subsequent crack propagation. Moreover, the various aberrant crystal forms contained within the mammillary layer will either impede or encourage the fault lines. To this end the ultrastructural integrity of the eggshell is of paramount importance. To date, most of the literature dealing with such variation within the eggshell has concentrated on the inorganic fraction of the shell (Reid, 1983, 1984; Watt, 1985, 1989; Mohumed, 1986; Solomon, 1987, 1991, 1993; Bain, 1990, 1992; Bain and Fraser, 1993; Bain *et al.,* 1992; Nascimento, 1992 and Roberts and Brackpool, 1994).

Ultrastructural analysis has revealed a total of 12 variations from the norm with reference to the morphology of the mammillary layer. All eggs contain structural variations, it is the nature and incidence of these variations which ultimately determines the response of this inner layer to load. Not all of the variations are detrimental in the table egg, thus confluence, cuffing and well formed caps all improve the relationship between the mammillary layer

and shell membranes. Furthermore, early fusion of the palisade columns serves to increase the effective thickness of the shell. In the analysis of the shell, the presence or absence of these 12 variants is accounted for and the summation of the values (total ultrastructural score) is a reflection of their inclusion or otherwise. In general terms, total ultrastructural score values increase (ultrastructural quality becomes poorer) at the end of lay (Mohumed, 1986; Bain, 1990; Nascimento, 1992) after an improvement during the mid lay period (Solomon, *pers comm*). They have also been shown to increase under conditions of physiological and environmental stress (Watt, 1985, 1989). Within the current research no significant decline in shell ultrastructure at the end of lay was demonstrable within any of the systems, although a trend for increased total ultrastructural score values was observed at this time in the TFR and perchery (see Tables 3-4 and Graph 9).

The beginning of lay total ultrastructural score values reported in Tables 3-4 for the battery, MFR, and perchery systems are significantly higher than reported by Solomon (*pers comm*) for birds of similar age. As stated previously, however, the beginning of lay is a time of potential stress. One of the daily indicators of the birds response to its environment is to be found within the ultrastructure of its shell (Watt, 1985, 1989). The question to be posed at this time is, whether acclimatising birds from their rearing conditions to the more confined battery environment and the stresses involved in re-establishing a stable social order within the MFR, perchery and TFR systems, are reflected in terms of shell ultrastructure. If this is the case, then the TFR would appear to be providing the environment most conducive to shell formation at the beginning of lay. The results obtained by Solomon (*pers comm*) refer to birds housed individually in cages (provided with perches) where stress due to social order formation would not be manifest. It is suggested that this may account for the lower total ultrastructural scores observed by the latter author.

If birds become adapted to the traumas of early lay then shell quality as, evidenced by a lower total ultrastructural score, should improve during mid lay. Within three of the systems (TFR, MFR and perchery) the values at mid lay were consistent with the beginning of lay. In the case of the battery system, however, the total ultrastructural score values decreased signifying an improvement in shell ultrastructure. This trend for improved shell quality within the battery system at mid lay has been observed previously (Solomon,

pers comm). It is hypothesised that this feature reflects the birds adaptation to an environment which offers stability with reference to daily management input.

In terms of the individual variants which combine to provide the total ultrastructural score values, Tables 3-4 list their incidence and significance. The lower total ultrastructural score values for the eggs from the TFR systems at the beginning of lay reflect the lower levels of those inclusions acknowledged by Watt (1985, 1989) to occur in response to stress. Of these, mention should be made of the relative absence of type A and B bodies, cubics and depressions. All of these variants encourage the lack of bonding between the true shell and the shell membranes and as such represent points of weakness within the eggshell. At mid lay, the eggs from the TFR still displayed the lowest total ultrastructural score.

At the end of lay, the nature of the individual shell characteristics changed and the slight decrease in shell quality at this time within the TFR can be accounted for by a decline in the quality of the mammillary caps (see Tables 3-4). A similar reasoning for the observed variation in quality throughout lay for the MFR and battery can be explained with reference to specific ultrastructural characteristics.

In the attempt to devise a ranking order the TFR system would, with respect to its total ultrastructural score, come first in the list. Nevertheless, using traditional and material measures of quality this was not always the case. This serves to underline the fact that in this protocol to date consideration has only been given to the inorganic fraction of the shell.

The perchery system did display the traditional decline in ultrastructural shell quality at the end of lay, with shells containing many of the detrimental characteristics associated with this period in the birds laying life viz. type A and B bodies, cubics and aragonite. The shells from this system also had the lowest fracture toughness at this time (see Tables 1-2 and Graph 8), indicating the influence of the ultrastructural variables in assisting the process of crack initiation and propagation. Indeed, as there is no apparent relationship between the ultrastructural criteria and fracture toughness values in any of the other systems under investigation, one must question whether the ultrastructural organisation of the mammillary layer alone influences this

material property as hypothesised by Bain (1990).

This investigation has revealed marginal differences in traditional and ultrastructural shell quality between and within four commonly used egg production systems. The observed stability (with reference to product quality) irrespective of bird age and system is not common and credit must be given to the company under whose control the birds were reared and managed. The low total ultrastructural scores also indicate the absence of disease factors such as Infectious Bronchitis and Egg Drop Syndrome. The desired ranking order has not emerged with reference to any one system being better or worse throughout the laying period. All of the systems analysed peaked with respect to one facet of shell structure or performance at some point within the period of the study. Unpredictability in product quality should be minimised in the commercial environment and the poorer performance of the birds housed in the perchery system might give cause to reconsider their managerial protocol.

The free range systems will always have a role to play because of the emotive forces created by the term "intensive rearing". Nevertheless, the battery system will probably remain the prime source of egg production in the EC. In the following chapter detailed consideration is therefore given to the enrichment of the battery environment under commercial conditions.

		BEG LAY		MID LAY		END LAY	
CATEGORY	SYSTEM	MEAN	± S.D	MEAN	± S.D	MEAN	± S.D
WEIGHT (g)	BAT	54.4	3.8	63.7	3.4	69.2	6.7
	PER	55.0	5.6	60.8	3.5	63.3	6.5
	MFR	60.3	8.7	63.6	4.7	69.5	4.9
	TFR	54.2	3.1	65.3	5.5	67.1	4.9
LENGTH (mm)	BAT	55.2	2.1	58.3	2.3	60.8	2.9
	PER	54.6	2.4	57.7	2.0	60.7	2.2
	MFR	57.3	3.4	58.6	2.3	61.5	2.6
	TFR	53.9	1.1	59.0	2.3	60.4	4.9
BREADTH (mm)	BAT	42.1	1.1	44.3	2.3	44.9	1.5
	PER	42.0	1.5	43.4	0.9	44.4	1.3
	MFR	44.3	2.9	43.6	1.3	44.6	1.4
	TFR	41.8	1.0	43.9	1.4	44.0	1.3
DEFORMATION (µm)	BAT	20	3	20	3	21	3
	PER	22	5	20	2	21	3
	MFR	19	3	20	2	19	3
	TFR	20	4	21	2	18	2
SHAPE INDEX -	BAT	1.31	0.01	1.32	0.05	1.36	0.06
	PER	1.30	0.05	1.33	0.05	1.37	0.05
	MFR	1.30	0.09	1.34	0.06	1.38	0.05
	TFR	1.29	0.03	1.35	0.05	1.37	0.01
STRENGTH (N)	BAT	35.4	4.5	31.8	6.4	31.0	6.2
	PER	35.2	6.1	31.1	5.2	25.3	6.2
	MFR	34.1	6.8	32.0	5.3	33.0	4.7
	TFR	34.5	10.5	31.2	4.6	29.7	4.6
STIFFNESS (N/mm)	BAT	170	27	166	26	176	36
	PER	177	34	181	30	155	25
	MFR	178	32	175	27	193	32
	TFR	211	95	172	31	139	21
THICKNESS (mm)	BAT	0.327	0.019	0.333	0.022	0.357	0.023
	PER	0.310	0.025	0.335	0.021	0.332	0.025
	MFR	0.326	0.019	0.333	0.022	0.357	0.023
	TFR	0.337	0.027	0.327	0.019	0.348	0.015
T. mam (mm)	BAT	0.074	0.012	0.067	0.008	0.071	0.010
	PER	0.072	0.012	0.079	0.011	0.075	0.012
	MFR	0.084	0.014	0.064	0.007	0.078	0.012
	TFR	0.071	0.013	0.071	0.008	0.083	0.010
T. eff (mm)	BAT	0.253	0.021	0.256	0.025	0.267	0.022
	PER	0.238	0.022	0.256	0.019	0.256	0.023
	MFR	0.241	0.022	0.269	0.021	0.280	0.021
	TFR	0.267	0.013	0.256	0.008	0.256	0.017
Kc (N/mm -3/2)	BAT	715	81	618	105	568	112
	PER	778	108	612	112	493	124
	MFR	737	177	583	100	562	68
	TFR	648	201	610	79	551	93
E. shell (N/mm -2)	BAT	31525	3960	31755	5208	31349	5347
	PER	37227	9513	34247	5661	30624	5794
	MFR	38032	7704	30427	4437	32214	4360
	TFR	34771	10328	32954	4400	25434	5209

Table 1. Traditional measures, thickness profiles and material properties. Means ± standard deviations.

CATEGORY	FACTOR	P VALUE	SIGNIFICANCE
WEIGHT	AGE	0.000	***
(g)	BET. SYSTEMS	0.000	***
	INTERACTION	0.001	***
LENGTH	AGE	0.000	***
(mm)	BET. SYSTEMS	0.000	***
	INTERACTION	0.003	**
BREADTH	AGE	0.000	***
(mm)	BET. SYSTEMS	0.000	***
	INTERACTION	0.000	***
DEFORMATION	AGE	0.430	N.S
(μm)	BET. SYSTEMS	0.031	*
	INTERACTION	0.002	**
SHAPE INDEX	AGE	0.000	***
-	BET. SYSTEMS	0.202	N.S
	INTERACTION	0.509	N.S
STRENGTH	AGE	0.000	***
(N)	BET. SYSTEMS	0.007	**
	INTERACTION	0.001	***
STIFFNESS	AGE	0.005	**
(N/mm)	BET. SYSTEMS	0.004	**
	INTERACTION	0.000	***
THICKNESS	AGE	0.000	***
(mm)	BET. SYSTEMS	0.000	***
	INTERACTION	0.000	***
T. mam	AGE	0.000	***
(mm)	BET. SYSTEMS	0.006	**
	INTERACTION	0.000	***
T. eff	AGE	0.000	***
(mm)	BET. SYSTEMS	0.000	***
	INTERACTION	0.000	***
Kc	AGE	0.000	***
(N/mm -3/2)	BET. SYSTEMS	0.000	***
	INTERACTION	0.000	***
E. shell	AGE	0.000	***
(N/mm -2)	BET. SYSTEMS	0.038	*
	INTERACTION	0.000	***

Table 2. Traditional measures, thickness profiles and material properties.
P values and significance levels.
$P \leq 0.001$ = ***, $P \leq 0.01$= **, $P \leq 0.1$= *, N.S = non significant.

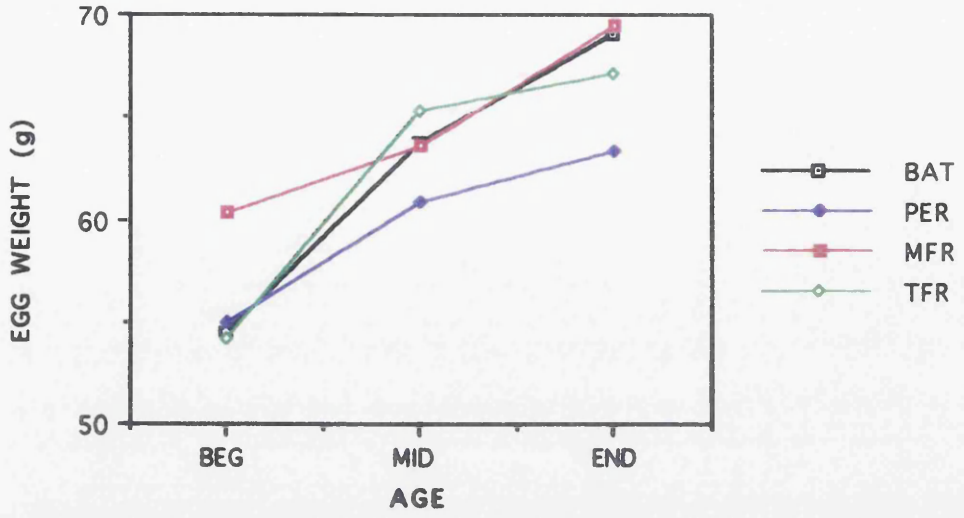
		BEG LAY		MID LAY		END LAY	
CATEGORY	SYSTEM	MEAN	± S.D	MEAN	± S.D	MEAN	± S.D
CONFLUENCE	BAT	4.1	2.2	2.3	1.8	1.7	1.3
	PER	3.9	2.5	2.3	1.3	1.2	0.6
	MFR	4.1	2.2	3.3	2.3	1.3	0.7
	TFR	1.7	1.0	1.5	0.9	2.2	1.9
FUSION	BAT	6.5	1.8	5.9	1.4	7.6	1.8
	PER	6.5	1.5	6.2	1.0	7.5	1.8
	MFR	6.5	1.8	6.2	1.7	6.6	1.7
	TFR	6.7	1.6	7.3	1.6	7.5	1.2
CUFFING	BAT	6.5	1.3	6.9	0.7	6.6	1.2
	PER	4.7	2.1	6.3	1.6	5.9	1.8
	MFR	6.5	1.3	6.7	1.0	6.3	1.5
	TFR	6.1	1.7	5.5	2.1	1.5	0.9
ALIGNMENT	BAT	3.2	1.5	2.9	0.9	2.8	0.8
	PER	2.9	1.3	3.3	0.7	3.1	0.6
	MFR	3.2	1.5	2.9	0.7	2.9	0.4
	TFR	1.1	0.5	2.8	0.8	3.0	0.5
TYPE B	BAT	2.0	1.6	1.5	0.9	1.8	1.0
	PER	1.7	1.0	1.8	1.3	2.3	1.6
	MFR	2.0	1.6	2.4	1.6	1.8	1.0
	TFR	1.1	0.5	1.5	1.3	1.9	1.6
DEPRESSIONS	BAT	1.5	0.9	1.1	0.3	1.3	0.8
	PER	1.1	1.0	1.0	0.2	1.5	1.0
	MFR	1.5	0.9	1.2	0.6	1.2	0.6
	TFR	1.0	0.2	1.0	0.2	1.4	0.9
ERROSIONS	BAT	1.5	0.9	1.0	0.0	1.1	0.4
	PER	1.0	0.0	1.1	0.4	1.1	0.5
	MFR	1.1	0.5	1.0	0.0	1.0	0.0
	TFR	1.0	0.0	1.0	0.0	1.0	0.0
CUBICS	BAT	1.1	0.5	1.1	0.4	1.1	0.5
	PER	1.4	1.2	1.1	0.4	1.1	0.5
	MFR	1.5	0.9	1.4	0.8	1.1	0.4
	TFR	1.0	0.0	1.1	0.4	1.1	0.4
ARAGONITE	BAT	1.1	0.5	1.8	1.3	1.2	0.6
	PER	1.3	0.8	1.4	0.8	2.0	1.4
	MFR	1.1	0.5	1.9	1.4	1.8	1.0
	TFR	1.3	0.8	1.4	1.2	1.4	0.8
CAP QUALITY	BAT	2.3	1.3	2.5	1.3	3.0	1.1
	PER	2.5	1.2	3.1	1.2	3.8	1.5
	MFR	2.3	1.3	2.8	1.2	3.4	1.2
	TFR	2.3	1.0	2.5	1.0	4.1	1.4
TYPE A'S	BAT	1.9	1.4	1.5	0.9	1.5	0.9
	PER	1.8	1.3	1.2	0.6	2.1	1.4
	MFR	1.9	1.4	1.9	1.4	1.7	1.0
	TFR	1.4	1.3	1.5	0.9	1.9	1.0
CH. MEMBRANE	BAT	1.1	0.5	1.1	0.5	1.0	0.0
	PER	1.7	1.0	1.2	0.6	2.0	1.6
	MFR	1.3	1.0	1.1	0.5	1.2	0.8
	TFR	1.8	1.3	1.1	0.5	3.0	1.4
TOTAL SCORE	BAT	32.9	5.6	29.6	4.1	30.5	4.1
	PER	30.5	5.8	30.0	4.6	33.7	6.0
	MFR	32.9	5.6	32.8	5.6	30.3	3.5
	TFR	28.6	3.2	28.3	5.2	30.1	4.2

Table 3. Mean ultrastructural scores ± standard deviations.

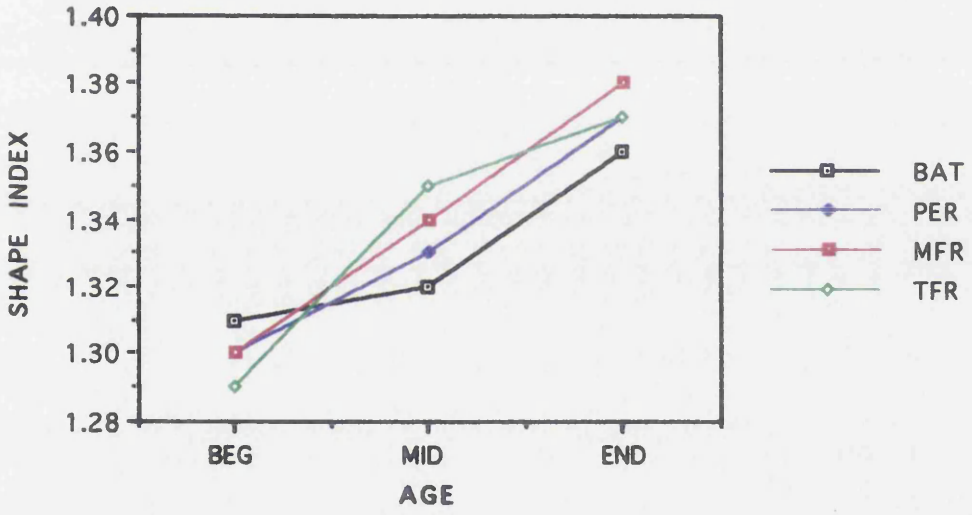
CATEGORY	FACTOR	P VALUE	SIGNIFICANCE
CONFLUENCE	AGE	0.000	***
	BET. SYSTEMS	0.000	***
	INTERACTION	0.000	***
FUSION	AGE	0.000	***
	BET. SYSTEMS	0.000	***
	INTERACTION	0.000	***
CUFFING	AGE	0.000	***
	BET. SYSTEMS	0.000	***
	INTERACTION	0.000	***
ALIGNMENT	AGE	0.072	N.S
	BET. SYSTEMS	0.946	N.S
	INTERACTION	0.176	N.S
TYPE B	AGE	0.179	N.S
	BET. SYSTEMS	0.029	*
	INTERACTION	0.192	N.S
DEPRESSIONS	AGE	0.455	N.S
	BET. SYSTEMS	0.008	**
	INTERACTION	0.034	*
EROSIONS	AGE	0.630	N.S
	BET. SYSTEMS	0.562	N.S
	INTERACTION	0.360	N.S
CUBICS	AGE	0.013	*
	BET. SYSTEMS	0.014	*
	INTERACTION	0.204	N.S
ARAGONITE	AGE	0.005	**
	BET. SYSTEMS	0.239	N.S
	INTERACTION	0.006	**
CAP QUALITY	AGE	0.002	**
	BET. SYSTEMS	0.000	***
	INTERACTION	0.201	N.S
TYPE A'S	AGE	0.000	***
	BET. SYSTEMS	0.000	***
	INTERACTION	0.000	***
CH. MEMBRANE	AGE	0.172	N.S
	BET. SYSTEMS	0.252	N.S
	INTERACTION	0.127	N.S
TOTAL SCORE	AGE	0.189	N.S
	BET. SYSTEMS	0.000	***
	INTERACTION	0.002	**

Table 4. Ultrastructural score P values and significance levels.
$P \leq 0.001$ = ***, $P \leq 0.01$= **, $P \leq 0.1$= *, N.S= non significant.

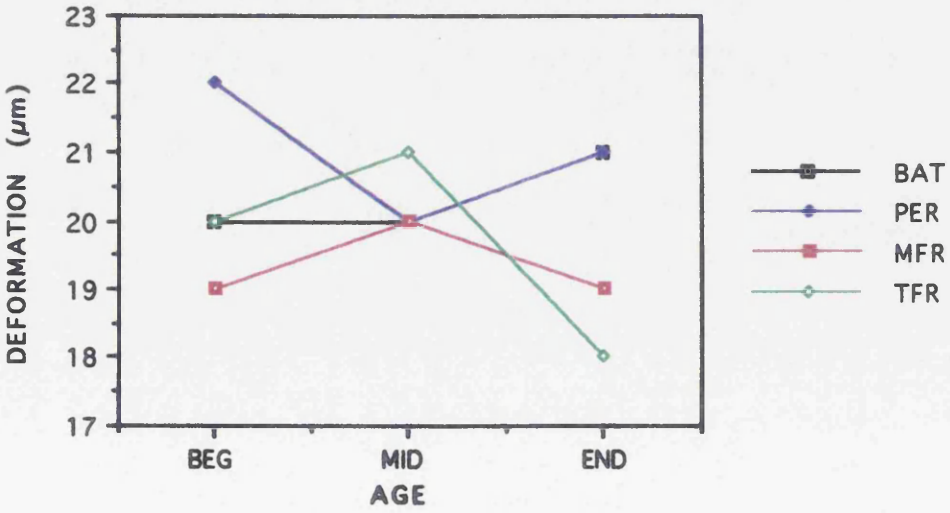
GRAPH 1. MEAN EGG WEIGHT ± S.D



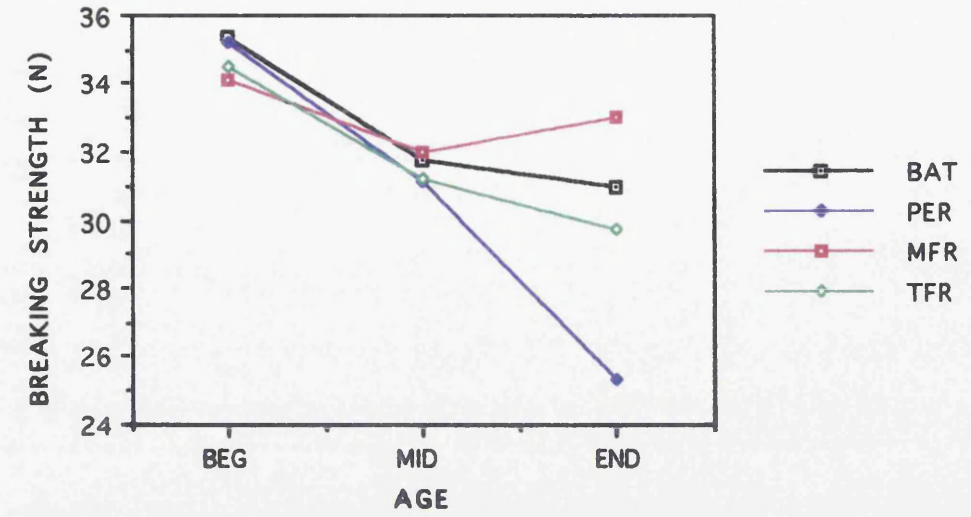
GRAPH 2. MEAN SHAPE INDEX ± S.D



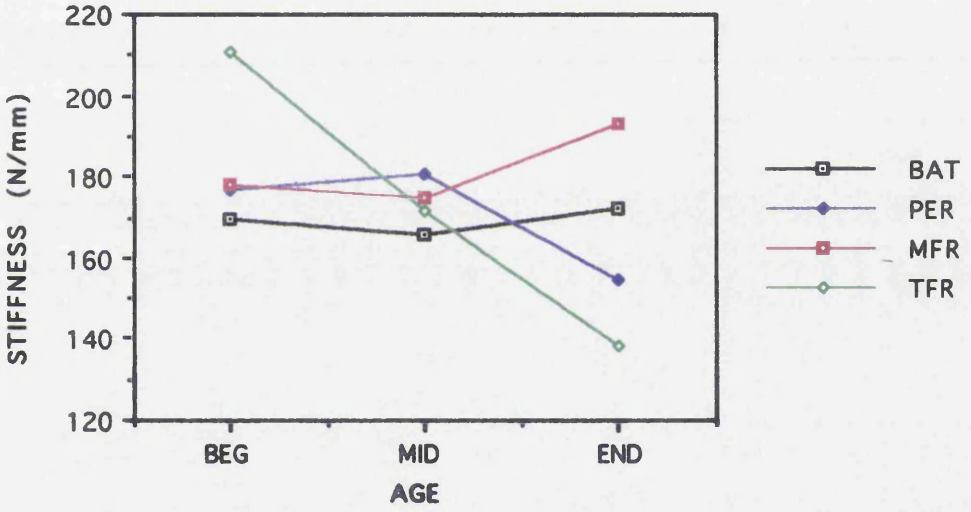
GRAPH 3. MEAN DEFORMATION ± S.D



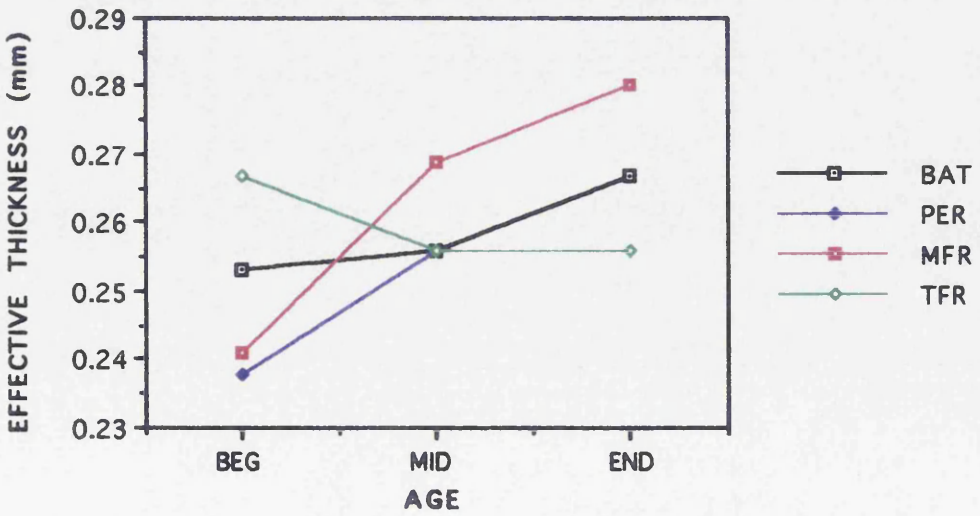
GRAPH 4. MEAN BREAKING STRENGTH ± S.D



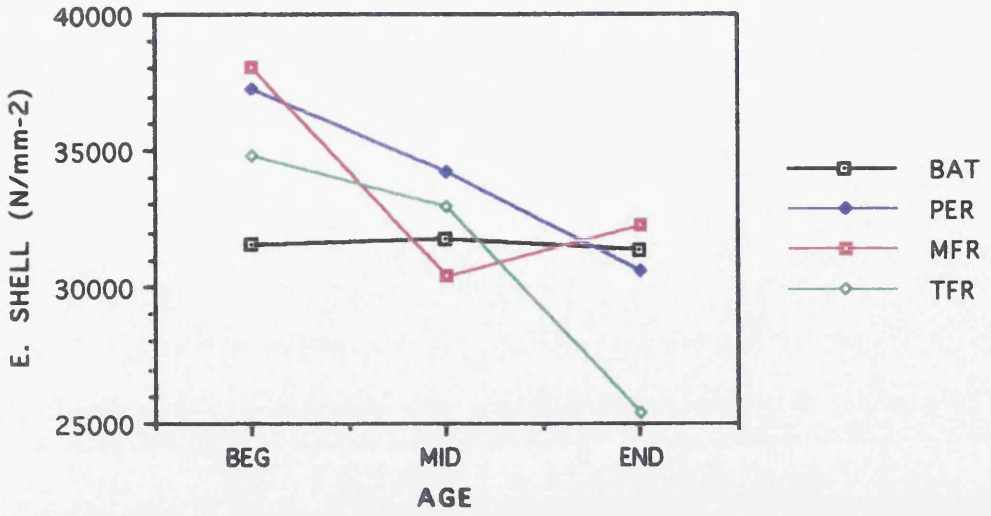
GRAPH 5. MEAN STIFFNESS ± S.D



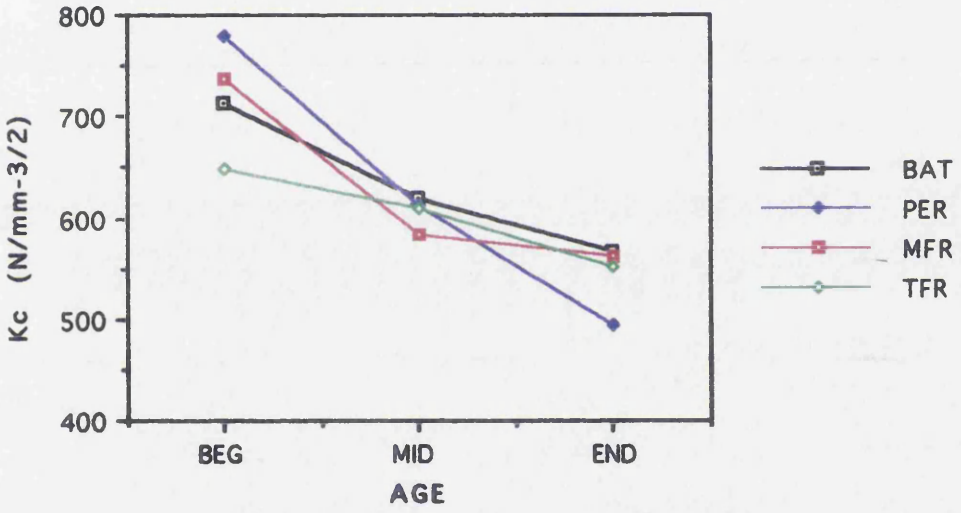
GRAPH 6. MEAN EFFECTIVE THICKNESS ± S.D



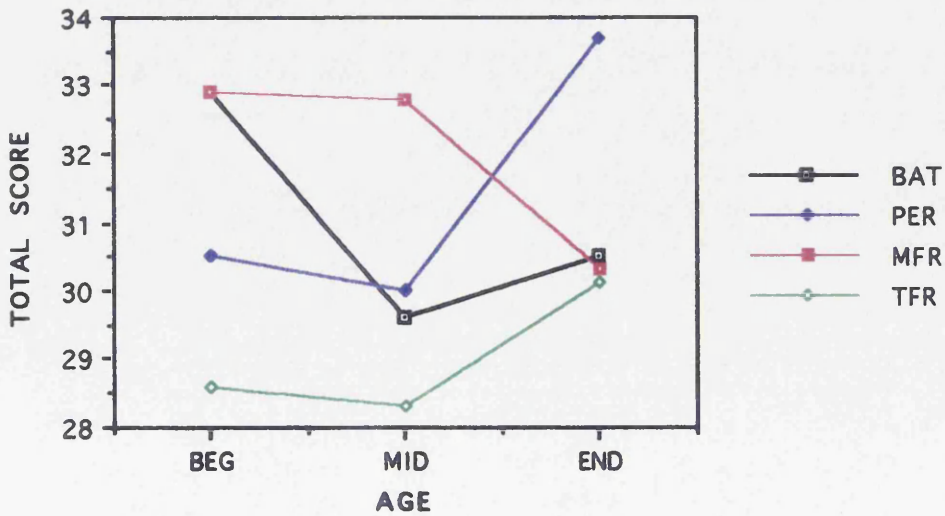
GRAPH 7. MEAN ELASTIC MODULUS ± S.D



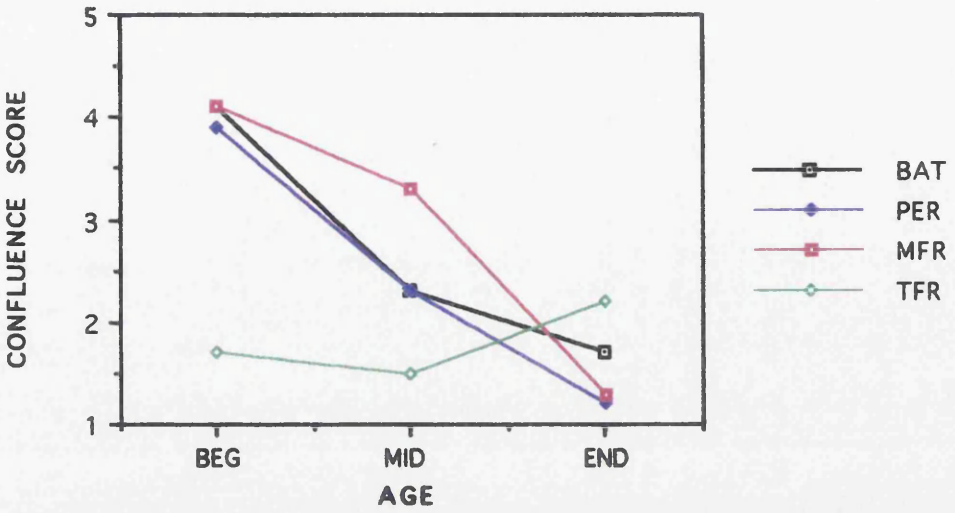
GRAPH 8. MEAN FRACTURE TOUGHNESS ± S.D



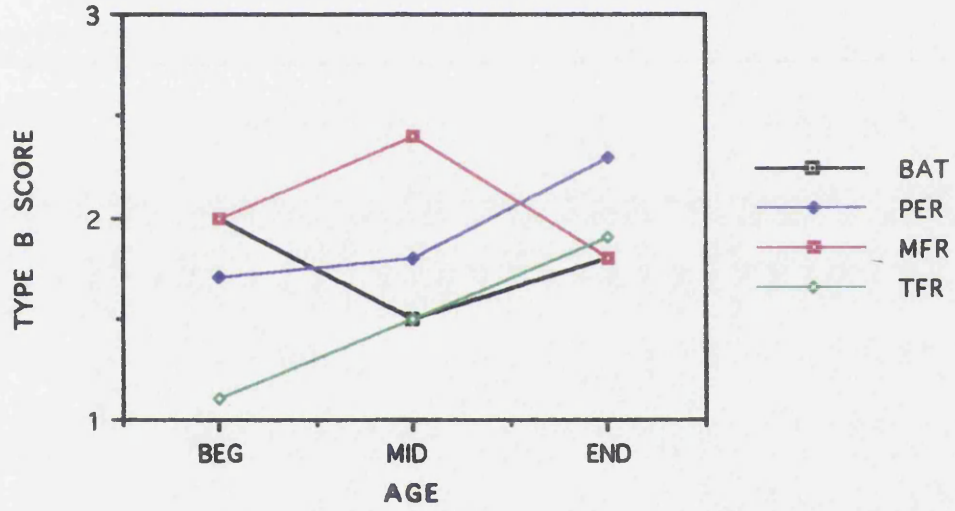
GRAPH 9. MEAN TOTAL SCORE ± S.D



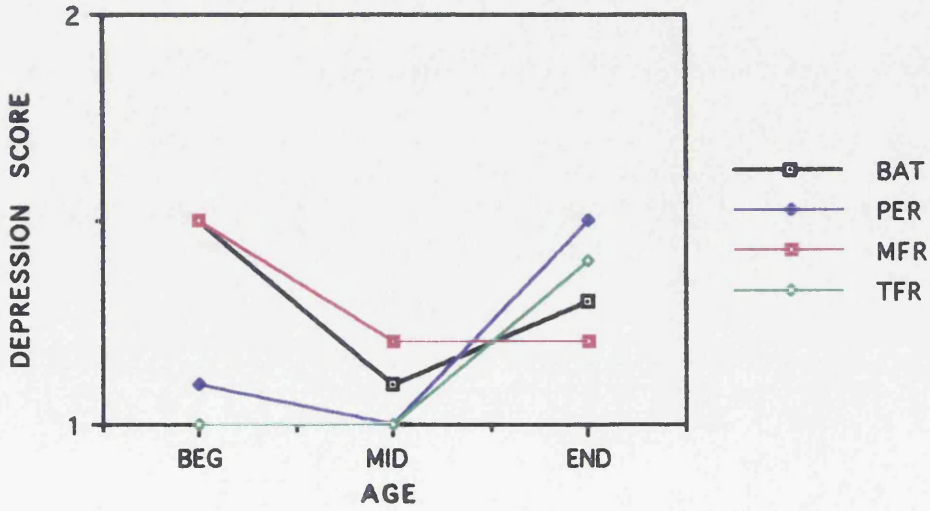
GRAPH 10. MEAN CONFLUENCE SCORE ± S.D



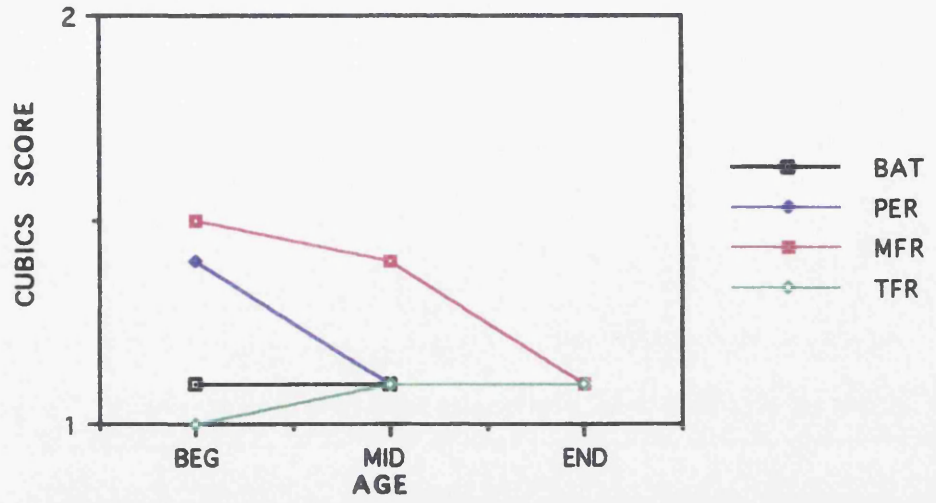
GRAPH 11. MEAN TYPE B SCORE ± S.D



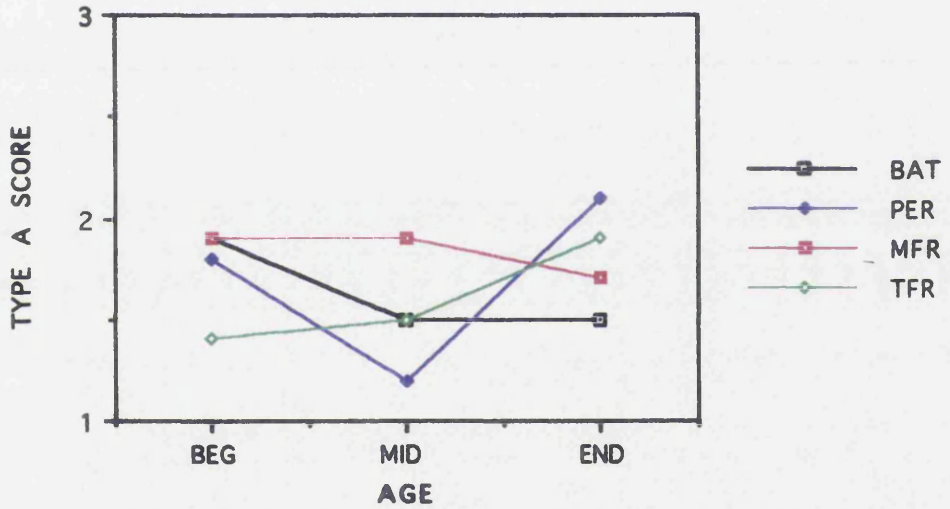
GRAPH 12. MEAN DEPRESSION SCORE ± S.D



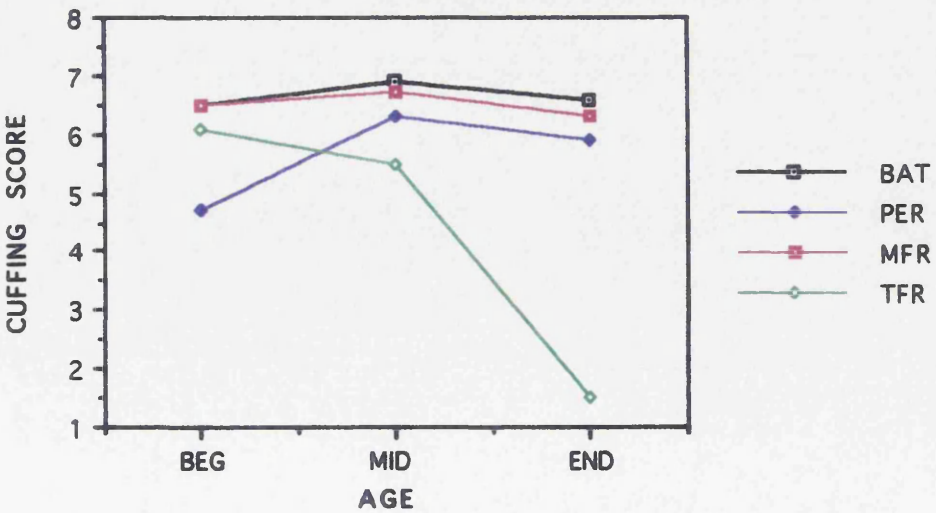
GRAPH 13. MEAN CUBICS SCORE ± S.D



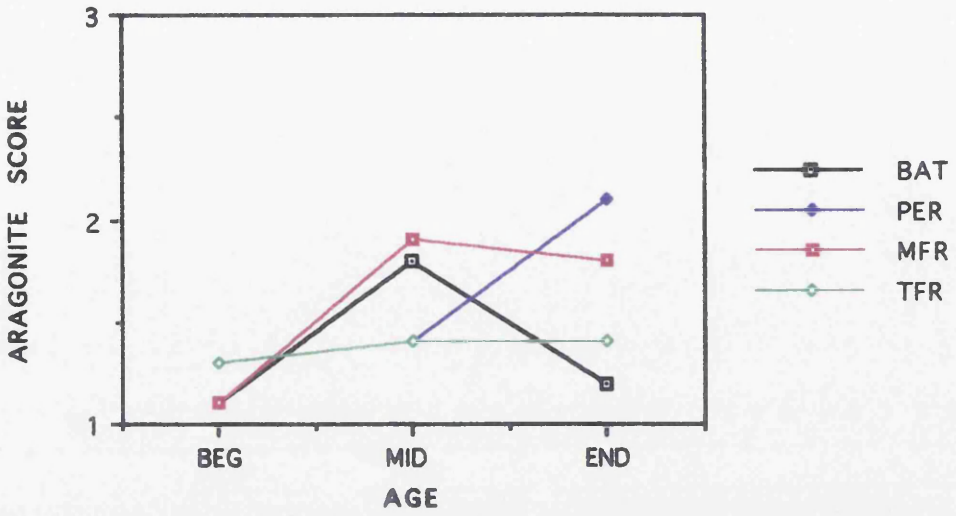
GRAPH 14. TYPE A SCORE ± S.D



GRAPH 15. MEAN CUFFING SCORE ± S.D



GRAPH 16. MEAN ARAGONITE SCORE ± S.D



VARIANT	INCIDENCE AND ASSOCIATED SCORES				
CONFLUENCE	NONE/ISOL. 1	ISOL/MOD. 3	MOD/EXT. 7		
CAPS	GOOD 1	AVERAGE 3	BELOW AV. 5	POOR 7	
FUSION	EARLY 1	MAINLY EARLY 3	50:50 5	MAINLY LATE 7	LATE 10
ALIGNMENT	RANDOM 1	MOD.ORDERED 3	ORDERED 5		
TYPE B	NONE/ISOL. 1	ISOL/MOD. 3	MOD/EXT. 7		
DEPRESSION	NONE 1	LIMITED 2	MOD. 4	DETRIMENTAL 6	
EROSION	NONE 1	LIMITED 3	MOD. 5	DETRIMENTAL 8	
ARAGONITE	NONE/ISOL. 1	ISOL/MOD. 3	MOD./EXT. 7		
TYPE A	NONE/ISOL. 1	ISOL/MOD. 3	MOD./EXT. 7		
CUBIC	NONE/ISOL. 1	ISOL/MOD. 3	MOD./EXT. 7		
CUFFING	NONE/ISOL. 7	ISOL/MOD. 3	MOD./EXT. 1		
CH. MEMB.	NONE/ISOL. 1	ISOL/MOD. 4	MOD./EXT. 7		

Figure 19. Ultrastructural score sheet. NB. the higher the score the less desirable the variant in terms of shell performance.

CHAPTER 3 - THE PROVISION OF A PERCH FOR BATTERY HOUSED HENS AND ITS EFFECT ON SHELL QUALITY AND STRUCTURE.

3. THE PROVISION OF A PERCH FOR BATTERY HOUSED HENS AND ITS EFFECT ON SHELL QUALITY AND STRUCTURE.

3.1. INTRODUCTION.

Currently, over 40 million laying hens are kept in battery cages in the UK. Although much criticised by the animal rights lobby, many of the welfare needs of the hen are met in this system. For example, the battery cage provides free access to food and water, protection from predators, parasites and disease. The small colony size also minimises aggressive interaction and vices. Moreover, the results of the previous chapter indicate that the eggs from the battery flock frequently performed better than or equal to many of their alternatively produced counterparts in terms of traditional, material and ultrastructural shell quality. Performance was also more readily influenced by environmental control. Nevertheless, the behavioural restrictions imposed by the barren environment provided by the battery cage are now scientifically acknowledged as being detrimental to bird welfare (**1.4.2.**, **1.5.3**) and must therefore be addressed in practical terms.

It is the opinion of the present author that the future of commercial egg production will depend upon a realistic compromise between the conventional battery cage and the welfare improvements potentially available within some alternative systems. For this reason, it was decided to pursue this investigation in terms of modified cage design.

It is the general consensus of opinion that provision of a nest, litter, dust bathing facilities etc. all present problems to the industry in terms of added cost, reduced efficiency, cage and egg hygiene and may also disrupt the automated processes which are integral to the ease with which the battery flock can be managed. The provision of a perch, however, is a relatively straightforward method of enhancing the cage environment, that can be easily installed into existing cages without compromising either hygiene or automation (Slaughter, *pers comm*).

In the natural environment, hens are known to roost at night on branches in bushes or trees (McBride *et al.*, 1969; cited by Baxter, 1994; Duncan *et al.*, 1978; Wood Gush *et al.*, 1978). This has been shown to be an important means of protection from ground predators (Wood Gush and Duncan, 1976). Modern strains of laying hen still express perching and roosting behaviour (Duncan *et al.*, 1978; Wood Gush *et al.*, 1978), which they cannot perform in the conventional battery cage. Indeed, Appleby and Hughes (1991) showed that the inability to perch can result in frustration and displacement behaviour. In a review of welfare problems of laying hens housed in battery cages, Baxter (1994) stated that the provision of a perch would improve the bird's overall welfare status.

Deleterious effects of providing perches have also been reported, such as the increased occurrence of keel deformation or bumblefoot, with these incidences usually being attributed to perches being of the wrong shape or in the wrong position in the cage (Duncan *et al.*, 1992; Engstrom and Schaller, 1993; cited by Baxter, 1994).

Appleby *et al.*, (1992) and Duncan *et al.*, (1992) reported that a perch should be long enough for all hens to use it simultaneously otherwise the same individuals would be excluded, particularly at night. Slaughter *(pers comm)* has confirmed that in 5 bird cages provided with perches the same individual is excluded from perching and that aggressive interactions are common between birds wishing to perch. More recently, Bain and Fraser (1993) undertook a pilot study to determine the effect on shell quality of providing birds stocked 5 to a cage with a perch where only 4 birds could perch at any one time. Little difference was observed in terms of traditional measures of quality. The presence of a perch did, however, result in significantly increased levels of ultrastructural variants some of which had been previously linked to stress under experimental conditions, Solomon *et al.*, (1987) and Watt (1989). Based on these findings, Bain and Fraser (1993) put forward the hypothesis that a stress situation may be established when not all the birds can freely utilise the perch and that this is reflected in quantifiable changes in shell ultrastructure.

In the current study, two trials were undertaken to further test this hypothesis. The first, was designed to compare two different stocking densities in cages fitted with perches (5 and 4 birds/cage; 450 cm^2 and 600 cm^2/bird) at three different sample periods during lay. This was carried out in order to establish whether enabling all birds free access to a perch would significantly influence shell structure and quality. As of January 1995 the legal stocking rate for birds housed in battery cages was set at 450 cm^2/bird, with a proposal that it be increased to 600 cm^2/bird at a future date (Hoelgaard, 1992; Elson, *pers comm*). With this proposed legislation in mind, it was decided to undertake a second (concurrent) trial using 4 birds per cage (600 cm^2/bird), with and without the provision of a perch, in order to assess whether it was the perch and not the reduced stocking density that was exerting an effect on shell structure and quality. Contact with the only British egg producer using perches in cages (at the time) established a means of continuing the investigation on a commercial scale.

3.2. MATERIALS AND METHODS.

30 eggs were sampled at random from 4 flocks (see Table 5) of commercial brown egg laying hens aged 28 weeks (period 1), 46 weeks (period 2) and 72 weeks (period 3). All flocks were housed on the same farm. Rearing and management procedures were identical in each case.

Table 5. Stocking density and perch availability for trials 1 and 2.

Trial 1.	Stocking density	Perch
Flock A.	5 birds (450 cm^2/bird)	Yes
Flock B.	4 birds (600 cm^2/bird)	Yes
Trial 2.		
Flock C.	4 birds (600 cm^2/bird)	Yes
Flock D.	4 birds (600cm^2/bird)	No

In trial 1, flocks A and B (see Table 5) were housed in conventional battery cages provided with a hardwood rectangular perch at a height of 5.5 cm situated towards the back of the cage. In flock A the birds were stocked 5 to a cage (450 cm^2/bird) and in flock B they were stocked 4 to a cage (600cm^2/bird). In trial 2, flocks C and D (see Table 5) were stocked 4 to a cage (600cm^2/bird), but in this case only the birds from flock C were provided with a perch.

All eggs collected were subjected to quality assessment viz. traditional measurements (**2.2.1.**, with the exception of nondestructive deformation), SEM assessment of effective thickness profiles (**2.3.2.**), ultrastructural assessment (**2.3.3.**) and evaluation of material properties (**2.3.4.**). These data were subsequently analysed using a two way analysis of variance.

Flocks A and B were molted aged 56 weeks and flocks C and D were molted aged 61 weeks (between the periods 2 and 3). This was a management decision outwith the original experimental protocol.

3.3. RESULTS.

3.3.1. TRIAL 1: FLOCKS A AND B.

3.3.1.1. TRADITIONAL ASSESSMENT OF SHELL QUALITY.

Mean values ± standard deviations are presented in Table 6. Table 7 summarises the results of the statistical analysis.

None of the traditional parameters measured showed a statistically significant difference between flocks A and B.

A statistically significant increase, however, was demonstrated within each respective flock from period 1 to period 3 (post molt) in terms of egg size, weight (Graph 17), length and breadth, shape index (Graph 18), breaking strength (Graph 19) and stiffness (Graph 20).

3.3.1.2. SEM ASSESSMENT OF SHELL QUALITY.

[i] Thickness profiles. No significant difference was demonstrated between flocks A and B in terms of thickness profiles. A statistically significant increase in terms of total thickness was, however, demonstrated from periods 1-3 (post molt) for both flocks (see Tables 6-7 and Graph 21), with no such difference being apparent for effective and mammillary thickness measurements.

[ii] Ultrastructural assessment of the mammillary layer.

Mean values ± standard deviations are presented in Table 8. Significant differences between and within each flock are presented in Table 9.

Flock A displayed a higher total ultrastructural score than flock B throughout the period of study (Graph 22), particularly during period 3 (post molt) and with reference to specific structural variants, the following are highlighted: alignment, fusion, cuffing and cap quality (see Tables 8-9 and Graphs 23-26).

A statistically significant difference was also demonstrated within each flock from period 1 to period 3 (post molt) in terms of the following specific structural variants viz. alignment, fusion, cuffing, cap quality, type A's (Graph 27), confluence (Graph 28) and changed membrane (Graph 29). These differences, however, were not reflected in the total ultrastructural score values associated with periods 1-3 (see Tables 8-9 and Graph 22).

3.3.1.3. MATERIAL ASSESSMENT OF SHELL QUALITY.

Mean values ± standard deviations are presented in Table 6.

No significant difference between flocks A and B was demonstrated in terms of fracture toughness or elastic modulus. Statistically significant differences were apparent within each flock. For example, at 72 weeks (post molt) fracture toughness values increased in flock B, while both flocks displayed a significant increase in elastic modulus values at this time (see Tables 6-7 and Graphs 30-31).

3.3.2. TRIAL 2: FLOCKS C AND D.

3.3.2.1. TRADITIONAL ASSESSMENT OF SHELL QUALITY.

Mean values ± standard deviations are presented in Table 10. Significant differences between and within the flocks are indicated in Table 11.

None of the traditional parameters measured showed a statistically significant difference between flocks C and D.

In contrast, all of the traditional measures (egg weight, breaking strength, and stiffness) showed a statistically significant increase from period 1 to period 3 (post molt) within each flock (see Tables 10-11 and Graphs 32-35).

3.3.3.2. SEM ASSESSMENT OF SHELL QUALITY.

[I] Thickness profiles. No significant difference was demonstrated between flocks C and D in terms of thickness profiles. A statistically significant increase was observed from period 1-3 (post molt) in terms of total, effective and mammillary thickness (see Tables 12-13 and Graph 35).

[II] Ultrastructural assessment of the mammillary layer.

Mean values ± standard deviations are presented in Table 12. Significant differences between and within flocks C and D are indicated in Table 13.

A statistically significant difference was demonstrated between flocks C and D in terms of total ultrastructural score, with flock D having the higher total ultrastructural score in periods 2 and 3 (Graph 36), specifically in terms of the scores associated with confluence, fusion and changed membrane (Graphs 37-39).

Within each flock significant differences were demonstrated with respect to the following specific structural variants: confluence, fusion, changed membrane, cubics (Graph 40), aragonite (Graph 41) and cap quality (Graph 42). Flock D followed the traditional pattern of increasing total ultrastructural score from the beginning to the end of lay, while flock C showed an improvement during period 3 (post molt), although none of these differences in themselves were statistically significant.

3.3.3.3. MATERIAL ASSESSMENT OF SHELL QUALITY.

Mean values ± standard deviations are presented in Table 10. No significant difference was found between flocks C and D in terms of their elastic modulus or fracture toughness. Significant differences were, however, apparent within both flocks for these measurements, most notably during periods 1-2 for fracture toughness and period 3 (post molt) for elastic modulus values (see Tables 10-11 and Graphs 43-44).

3.4. DISCUSSION.

3.4.1. DIFFERENCES BETWEEN FLOCKS.

Traditional measures of shell quality failed to reveal any significant differences between the flocks associated with trials 1 or 2 (see Tables 7 and 11). This is in agreement with the findings of Bain and Fraser (1993).

Similarly, no significant differences were observed in terms of thickness profiles or material properties ie. the elastic modulus and fracture toughness, in either trials 1 or 2 (see Tables 6-7 and 10-11 and Graphs 21, 30-31, 35, 43-44).

These results may lead the reader to believe that a uniformity of product quality exists between the four flocks. However, as previously discussed by Watt (1985, 1989), Bain (1990) and Nascimento (1992) such measurements are often insensitive to the subtle changes in ultrastructure which according to Solomon (1991), ultimately determine the way in which the shell will respond to the trauma of handling.

When ultrastructural criteria are considered, a somewhat different image emerges. The results of Trial 1 show that throughout the study period the eggs from flock A (5 birds+perch) had a significantly higher total ultrastructural score (summation of individual score values) than those from flock B (4 birds+perch) (see Tables 8-9 and Graph 22). As mentioned previously, this score is a summation of the ultrastructural characteristics of the eggshell. If these criteria are now taken into consideration, it would appear that the eggs produced by flock A were significantly inferior to those produced by flock B. These data may lend support to the hypothesis of Bain and Fraser (1993) that when not all birds can freely utilise the perch a stress situation may arise which is reflected in quantifiable changes in shell ultrastructure, as represented by the total ultrastructural score.

The individual ultrastructural variations within the shells of flock A, which together contribute to poor shell quality, are many and varied. Thus, the mammillary surface showed a more ordered alignment during periods 2 and 3 (Graph 23), the palisade columns fused later at all periods during the trial (Graph 24), the incidence of cuffing was less (Graph 25) and mammillary cap

quality was significantly poorer throughout lay (Graph 26). High levels of these structural defects often combine to encourage crack initiation and propagation, decrease effective thickness and introduce discontinuity between the mammillary surface and the shell membranes. At the end of lay (period 3), the incidence of type A bodies was significantly greater in flock A (Graph 27), although they were a more prominent feature in flock B during the beginning (period 1) and middle of lay (period 2). Type A bodies make no contact with the membrane fibres and like the higher incidence of poor quality mammillary caps, represent an area of weakness within the nucleation layer.

Stress, whether environmental or pharmacological, has a detrimental effect on shell structure (Watt, 1985, 1989). These changes are acknowledged to result from chemical and structural alterations in the epithelial cells lining the oviduct. Within the shell, Watt (1989) observed a higher incidence of aberrant crystal forms viz. type B bodies, aragonite, cubics and, as might have been anticipated, a deterioration in the quality of the mammillary caps.

In the present investigation, cap quality was significantly poorer in flock A during all periods and likewise at the beginning of lay, acknowledged to be a potentially stressful time (Solomon, *pers comm*), the incidence of type B bodies and cubics was greater, suggesting higher stress levels within this flock.

In trial 2, flock D (4 birds, no perch) showed a higher total ultrastructural score (and hence poorer ultrastructural quality) than flock C (4 birds with perch) throughout the period of study. These differences were most significant during periods 2 and 3 (see Table 12-13 and Graph 36). Thus, there is evidence that the provision of a perch is having a direct effect on bird welfare in terms of lowered stress levels (see Bain and Fraser, 1993) and that the results of trial 1 are not simply a reflection of reduced stocking density.

The structural variations accounting for the observed differences were not the same as previously recorded for flocks A and B. In terms of specific variants, the presence of confluence is considered beneficial (in the table egg) as it strengthens and maintains structural integrity within the shell (Solomon, 1993). During periods 1 and 2, eggs from flock C showed a significantly higher degree of confluence than those from flock D with this situation being

reversed in period 3, post molt (Graph 37). During periods 2 and 3 flock D demonstrated later fusion of the palisade columns (Graph 38) and hence reduced effective thickness. A higher incidence of the changed membrane phenomenon (which, as defined in the literature review, results from chemical changes in the isthmus region of the oviduct) was apparent in flock D during period 3 (Graph 39). According to Watt (1989), the occurrence of this feature is also stress related.

The ultrastructural variations observed in the eggshells of birds exposed to severe, sudden and specific stresses viz. a change in stocking density and adrenaline injection, were accompanied by overt changes in shell texture ie. the stress response manifested itself at all levels from the mammillary layer outwards (Watt, 1985, 1989). The latter author demonstrated a gradual return to normality with respect to shell texture, whilst acknowledging the persistence of intrashell defects for some time afterwards (up to 30 days). In the current study, stressors represent routine and continuous fluctuations experienced by the bird on a daily basis and no changes were observed at the shell surface. The results of the present thesis do, however, indicate that the provision of a perch improves the ultrastructural integrity of eggs produced within a given flock, if all birds have free access to that perch. Consideration of these data and the hypothesis put forward by Bain and Fraser (1993) relating stress incidence to shell ultrastructural quality, suggests that this improvement is the result of a reduction in overall stress levels within that flock. It hypothesised that these changes have come about as a direct result of a reduction in aggressive interactions between birds, allowing the formation of a more stable social order within the cage, hence leading to an improvement in terms of overall bird welfare.

3.4.2. DIFFERENCES WITHIN EACH FLOCK.

The effect of age on traditional measurements of shell quality has been well documented by Bain (1990), Nascimento (1992), and Solomon (1993). The results obtained in the present work are generally in agreement with the findings of these authors for periods 1 and 2.

Most laying flocks in the UK are slaughtered around 72 weeks of age due to a gradual decline in egg production, shell and internal quality associated with increasing bird age (Appleby *et al.*, 1992). In order to extend

this period the flock may be molted, rested and brought back into production. Molting improves both egg output (in terms of egg mass) and egg quality for approximately another 30 weeks (Appleby *et al.,* 1992) and this is normally achieved by depriving hens of food and water for 48 hours, causing laying to be interrupted until environmental conditions are returned to normal. However, the use of forced molting as a means of extending the productive life of laying hens is no longer common within the UK due to legislation which states that birds must have access to feed at least once every 24 hours and a regular water supply (MAFF, 1987). In the current study, molting was induced between periods 2 and 3 in all flocks by using a combination of short day length and feed restriction which met the stated legal requirements and was approved by a ministry veterinarian. The process was carried out in order to achieve an increase in egg size and hence a corresponding premium for the larger eggs produced (Slaughter, *pers comm*). This aim was achieved in flocks A, C and D, however, flock B did not conform to the anticipated pattern, as egg weight declined in this flock post molt (see Tables 6, 7, 10, 11 and Graphs 17 and 32).

Molting also significantly influenced many of the other measures of quality carried out in this study. For example, in both trials 1 and 2 there was a significant increase in breaking strength (Graphs 19 and 33) in all four flocks at period 3 with a concomitant increase in stiffness (Graphs 20 and 34). According to Bain (1990) shell breaking strength significantly declines at 72 weeks of age. Molting has therefore reversed this process of decline.

Roland (1981) has stated that eggshells become progressively thinner with increasing bird age, although this was not found to be the case in the results of the previous chapter where total thickness was found to increase from the beginning to the end of lay. Total thickness measurements did not exhibit a uniform pattern in either trial 1 or 2, although all showed an increase post molt. Hence flock A showed a decline in total thickness from period 1 to period 2 followed by an increase in period 3 (post molt), while flocks B and C showed an increase over the time periods examined. Flock D showed only a very slight increase in total thickness from period 1 to 2 followed by a significant increase in period 3 (see Tables 6-7, 12-13 and Graphs 21 and 35). Effective thickness values did not always mirror increases in total thickness, as in the case of flock B and C between periods 2 and 3. Thus, one must again question the validity of total thickness measurements when viewed in isolation

from the structural variations within the shell, as the increase in total thickness anticipated post molt (Slaughter, *pers comm*) was not always associated with an increase in effective thickness.

In all of the flocks, the effect of molting has been to prevent the increase in total ultrastructural score (decrease in shell quality) characteristically found in eggs from birds aged 72 weeks as reported by Bain (1990). In trial 1, flocks A (5 birds) and B (4 birds) showed little change in total ultrastructural score values with increasing bird age and also post molt. Thus, the slight improvement in ultrastructural shell quality occurring during the mid lay period as described by Solomon (*pers comm*) was not observed. In trial 2, flock C (4 birds plus perch) showed no change in total ultrastructural score from period 1 to 2, this again is contrary to the findings of the previous author. Post molt, however, the total ultrastructural score showed a significant decline in flock C indicating an improvement in shell ultrastructural quality. Flock D (4 birds, no perch) showed a trend towards increasing total ultrastructural score values throughout the period of study, again conflicting with the findings of Solomon (*pers comm*) for the mid lay period (see Tables 8-9, 12-13 and Graphs 22 and 36).

Molting also significantly influenced the material properties of the shell. According to Bain (1990) elastic modulus values decline towards the end of lay, resulting in more elastic shells. The effect of molting has been to reverse this and significantly increase elastic modulus values, thereby producing less flexible shells during period 3 (post molt) in all 4 flocks (see Tables 7, 11, and Graphs 30 and 43). Such shells may be more prone to damage during routine handling. According to Bain (1990) the elastic modulus of eggshells is considerably lower than the average published values for limestone and marble, both of which are of a similar chemical composition to the calcified shell. Tung *et al.,* (1968; cited by Bain, 1990) suggested that the presence of the organic matrix may lower the elastic modulus of the eggshell and in this respect elastic modulus values for eggshells are similar to that of bone. The current results reinforce the theory that the nature of the organic fraction of the shell can and does vary in an as yet unidentified manner.

The fracture toughness (Kc) relates to the rate of crack propagation within the shell and can be directly related to defects in shell ultrastructure (Bain, 1990). According to this author Kc values are typically lower at the end

of lay when shell ultrastructure is characteristically poor. In all flocks such a decline in fracture toughness at the end of lay (period 3) has been offset by the process of molting. It is interesting to note that in the flocks where there are 4 birds with access to a perch (B and C) fracture toughness has actually improved, although not significantly. These results are supported by the fact that total ultrastructural scores in groups B and C have also improved (see Tables 6-7, 10-11 and Graphs 31 and 44).

In her thesis entitled "Eggshell Strength: A Mechanical/Ultrastructural Evaluation" Bain (1990) was able to account for 72% of the shells response to load in terms of its inorganic organisation and at that time, made a statement to the fact that the missing 28% could be accounted for by the role of the organic matrix. The process of shell mineralization is protein led and any reduction in the volume or nature of the protein component will influence the pattern of subsequent calcium carbonate crystal growth. These processes occur at the molecular level, influencing the morphology of individual crystals. The resolution of this intimate relationship between the organic/inorganic fractions of the shell requires more advanced technology than that provided by hand callipers and the scanning electron microscope, although the latter has a part to play in resolving the complexity of matrix morphology and crystal structure.

The following chapters are therefore devoted to a study of the distribution, structure and function of the organic matrix of the eggshell and its molecular components.

		PERIOD 1		PERIOD 2		PERIOD 3	
CATEGORY	FLOCK	MEAN	± S.D	MEAN	± S.D	MEAN	± S.D
WEIGHT	A/5 birds	60.5	4.6	65.6	6.2	71.8	5.1
(g)	B/4 birds	62.4	4.2	68.6	4.6	66.5	5.9
LENGTH	A/5 birds	56.6	2.5	58.9	3.0	61.5	2.5
(mm)	B/4 birds	57.9	1.7	58.5	2.4	60.2	2.4
BREADTH	A/5 birds	44.1	3.7	44.4	1.6	45.7	1.0
(mm)	B/4 birds	44.3	1.1	44.9	2.0	45.3	1.4
SHAPE INDEX	A/5 birds	1.28	0.08	1.33	0.06	1.35	0.06
-	B/4 birds	1.31	0.05	1.31	0.07	1.33	0.05
STRENGTH	A/5 birds	26.0	3.7	30.4	5.1	33.2	11.3
(N)	B/4 birds	26.9	5.5	28.8	5.9	33.1	5.8
STIFFNESS	A/5 birds	109	12	136	23	194	69
(N/mm)	B/4 birds	112	15	150	30	199	47
THICKNESS	A/5 birds	0.318	0.025	0.312	0.022	0.327	0.024
(mm)	B/4 birds	0.313	0.022	0.320	0.019	0.325	0.028
T. eff	A/5 birds	0.247	0.024	0.244	0.022	0.259	0.024
(mm)	B/4 birds	0.247	0.029	0.255	0.025	0.256	0.025
T. mam	A/5 birds	0.071	0.015	0.068	0.012	0.068	0.017
(mm)	B/4 birds	0.066	0.016	0.066	0.010	0.069	0.008
Kc	A/5 birds	542	102	650	90	628	203
(Nmm-3/2)	B/4 birds	559	136	561	102	644	114
E. shell	A/5 birds	22517	4704	29231	6274	37378	9487
(Nmm-2)	B/4 birds	23559	6415	29251	6274	39612	9438

Table 6. Trial 1: traditional measures, thickness profiles and material properties. Means ± standard deviations.

CATEGORY	FACTOR	P VALUE	SIGNIFICANCE
WEIGHT	AGE	0.000	***
(g)	SYSTEM	0.792	N.S
	INTERACTION	0.015	*
LENGTH	AGE	0.000	***
(mm)	SYSTEM	0.722	N.S
	INTERACTION	0.000	***
BREADTH	AGE	0.000	***
(mm)	SYSTEM	0.629	N.S
	INTERACTION	0.266	N.S
SHAPE INDEX	AGE	0.000	***
-	SYSTEM	0.560	N.S
	INTERACTION	0.040	*
STRENGTH	AGE/MOLT	0.000	***
(N)	BET. FLOCKS	0.646	N.S
	INTERACTION	0.423	N.S
STIFFNESS	AGE/MOLT	0.000	***
(N/mm)	BET. FLOCKS	0.094	N.S
	INTERACTION	0.688	N.S
THICKNESS	AGE/MOLT	0.034	*
(mm)	BET. FLOCKS	0.876	N.S
	INTERACTION	0.307	N.S
T. eff	AGE/MOLT	0.065	N.S
(mm)	BET. FLOCKS	0.439	N.S
	INTERACTION	0.283	N.S
T. mam	AGE/MOLT	0.657	N.S
(mm)	BET. FLOCKS	0.078	N.S
	INTERACTION	0.413	N.S
Kc	AGE/MOLT	0.002	**
(Nmm-3/2)	BET. FLOCKS	0.331	N.S
	INTERACTION	0.039	*
E. shell	AGE/MOLT	0.000	***
(Nmm-2)	BET. FLOCKS	0.108	N.S
	INTERACTION	0.773	N.S

Table 7. Trial 1: traditional measures, thickness profiles and material properties. P values and significance levels.
$P \leq 0.001$ = ***, $P \leq 0.01$= **, $P \leq 0.1$= *, N.S = non significant.

		PERIOD 1		PERIOD 2		PERIOD 3	
CATEGORY	FLOCK	MEAN	± S.D	MEAN	± S.D	MEAN	± S.D
CONFLUENCE	A/5 birds	3.1	2.4	2.2	1.6	1.5	0.7
	B/4 birds	3.9	2.6	2.7	2.0	1.4	0.8
FUSION	A/5 birds	6.6	1.8	7.2	1.6	8.5	1.6
	B/4 birds	6.0	1.2	6.8	1.4	6.9	1.5
CUFFING	A/5 birds	5.2	2.1	6.7	1.0	6.3	1.7
	B/4 birds	4.2	1.9	6.1	1.8	5.6	2.2
ALIGNMENT	A/5 birds	3.5	1.0	3.0	0.5	3.1	0.5
	B/4 birds	3.5	0.9	2.5	0.9	2.9	0.7
TYPE B	A/5 birds	1.9	1.6	1.5	1.9	1.5	0.9
	B/4 birds	1.5	1.3	1.8	1.3	1.3	0.7
DEPRESSIONS	A/5 birds	1.1	0.6	1.2	0.6	1.4	1.2
	B/4 birds	1.4	0.9	1.1	0.6	1.3	0.8
EROSIONS	A/5 birds	1.0	0.0	1.0	0.0	1.0	0.0
	B/4 birds	1.0	0.0	1.0	0.0	1.0	0.0
CUBICS	A/5 birds	1.5	0.9	1.3	0.8	1.1	0.4
	B/4 birds	1.1	0.5	1.1	0.4	1.2	0.6
ARAGONITE	A/5 birds	1.4	1.2	1.4	0.8	1.1	0.5
	B/4 birds	1.4	0.8	1.5	0.9	1.2	0.8
CAP QUALITY	A/5 birds	2.5	1.6	2.5	1.1	3.7	1.2
	B/4 birds	2.0	1.3	2.2	1.1	2.6	1.0
TYPE A'S	A/5 birds	1.1	0.5	1.7	1.0	2.0	1.5
	B/4 birds	1.5	1.3	1.9	1.0	1.3	0.8
CH. MEMBRANE	A/5 birds	2.1	1.7	1.5	1.3	1.4	1.0
	B/4 birds	2.2	1.5	1.2	0.8	1.8	1.3
TOTAL SCORE	A/5 birds	31.0	7.1	31.4	3.8	32.5	4.4
	B/4 birds	29.6	6.0	30.0	4.4	28.6	4.0

Table 8. Trial 1: ultrastructural score, means ± standard deviations.

CATEGORY	FACTOR	P VALUE	SIGNIFICANCE
CONFLUENCE	AGE/MOLT	0.000	***
	BET. FLOCKS	0.103	N.S
	INTERACTION	0.427	N.S
FUSION	AGE/MOLT	0.000	***
	BET. FLOCKS	0.000	***
	INTERACTION	0.075	N.S
CUFFING	AGE/MOLT	0.000	***
	BET. FLOCKS	0.007	**
	INTERACTION	0.817	N.S
ALIGNMENT	AGE/MOLT	0.000	***
	BET. FLOCKS	0.041	*
	INTERACTION	0.266	N.S
TYPE B	AGE/MOLT	0.260	N.S
	BET. FLOCKS	0.522	N.S
	INTERACTION	0.275	N.S
DEPRESSIONS	AGE/MOLT	0.524	N.S
	BET. FLOCKS	0.926	N.S
	INTERACTION	0.412	N.S
EROSIONS	AGE/MOLT	-	N.S
	BET. FLOCKS	-	N.S
	INTERACTION	-	N.S
CUBICS	AGE/MOLT	0.320	N.S
	BET. FLOCKS	0.072	N.S
	INTERACTION	0.078	N.S
ARAGONITE	AGE/MOLT	0.314	N.S
	BET. FLOCKS	0.388	N.S
	INTERACTION	0.811	N.S
CAP QUALITY	AGE/MOLT	0.000	***
	BET. FLOCKS	0.001	***
	INTERACTION	0.211	N.S
TYPE A'S	AGE/MOLT	0.027	*
	BET. FLOCKS	0.688	N.S
	INTERACTION	0.023	*
CH. MEMBRANE	AGE/MOLT	0.003	**
	BET. FLOCKS	0.727	N.S
	INTERACTION	0.324	N.S
TOTAL SCORE	AGE/MOLT	0.421	N.S
	BET. FLOCKS	0.004	**
	INTERACTION	0.000	***

Table 9. Trial 1: ultrastructural score P values and significance levels.
$P \leq 0.001$ = ***, $P \leq 0.01$= **, $P \leq 0.1$= *, N.S = non significant.

		PERIOD 1		PERIOD 2		PERIOD 3	
CATEGORY	FLOCK	MEAN	± S.D	MEAN	± S.D	MEAN	± S.D
WEIGHT	C/+PERCH	61.8	4.0	66.3	4.0	70.2	5.4
(g)	D/- PERCH	61.4	4.7	66.5	4.4	71.3	6.1
LENGTH	C/+PERCH	55.9	1.9	58.6	1.6	59.8	2.3
(mm)	D/- PERCH	55.8	2.1	59.2	3.4	59.9	1.9
BREADTH	C/+PERCH	44.1	1.7	44.6	1.0	45.5	1.4
(mm)	D/- PERCH	43.8	1.0	44.7	1.2	44.5	1.3
SHAPE INDEX	C/+PERCH	1.27	0.07	1.31	0.04	1.31	0.05
-	D/- PERCH	1.32	0.04	1.33	0.08	1.32	0.04
STRENGTH	C/+PERCH	34.5	6.7	31.4	4.2	34.0	4.7
(N)	D/- PERCH	33.7	5.9	30.8	5.1	34.1	6.1
STIFFNESS	C/+PERCH	152	32	153	24	183	23
(N/mm)	D/- PERCH	162	20	131	25	198	44
THICKNESS	C/+PERCH	0.309	0.022	0.321	0.020	0.329	0.023
(mm)	D/- PERCH	0.315	0.020	0.316	0.028	0.339	0.020
T. eff	C/+PERCH	0.241	0.020	0.255	0.021	0.257	0.022
(mm)	D/- PERCH	0.246	0.018	0.246	0.027	0.267	0.023
T. mam	C/+PERCH	0.067	0.011	0.066	0.008	0.072	0.010
(mm)	D/- PERCH	0.069	0.080	0.068	0.010	0.073	0.008
Kc	C/+PERCH	736	136	618	90	653	96
(Nmm-3/2)	D/- PERCH	705	118	636	105	627	144
E. shell	C/+PERCH	31423	4425	29805	4950	35940	6293
(Nmm-2)	D/- PERCH	32634	4198	32633	4040	36935	8887

Table 10. Trial 2: traditional measures, thickness profiles and material properties. Means ± standard deviations.

CATEGORY	FACTOR	P VALUE	SIGNIFICANCE
WEIGHT	AGE	0.000	***
(g)	SYSTEM	0.857	N.S
	INTERACTION	0.523	N.S
LENGTH	AGE	0.000	***
(mm)	SYSTEM	0.568	N.S
	INTERACTION	0.604	N.S
BREADTH	AGE	0.000	***
(mm)	SYSTEM	0.776	N.S
	INTERACTION	0.758	N.S
SHAPE INDEX	AGE	0.000	***
-	SYSTEM	0.574	N.S
	INTERACTION	0.863	N.S
STRENGTH	AGE	0.004	**
(N)	SYSTEM	0.607	N.S
	INTERACTION	0.870	N.S
STIFFNESS	AGE	0.000	***
(N/mm)	SYSTEM	0.737	N.S
	INTERACTION	0.001	***
THICKNESS	AGE	0.000	***
(mm)	SYSTEM	0.103	N.S
	INTERACTION	0.127	N.S
T. eff	AGE	0.000	***
(mm)	SYSTEM	0.424	N.S
	INTERACTION	0.127	N.S
T. mam	AGE	0.004	**
(mm)	SYSTEM	0.057	N.S
	INTERACTION	0.987	***
Kc	AGE	0.000	***
(Nmm-3/2)	SYSTEM	0.350	N.S
	INTERACTION	0.621	N.S
E. shell	AGE	0.000	***
(Nmm -2)	SYSTEM	0.617	N.S
	INTERACTION	0.103	N.S

Table 11. Trial 2 : traditional measures, thickness profiles and material properties. P values and significance levels.
$P \leq 0.001$ = ***, $P \leq 0.01$= **, $P \leq 0.1$= *, N.S = non significant.

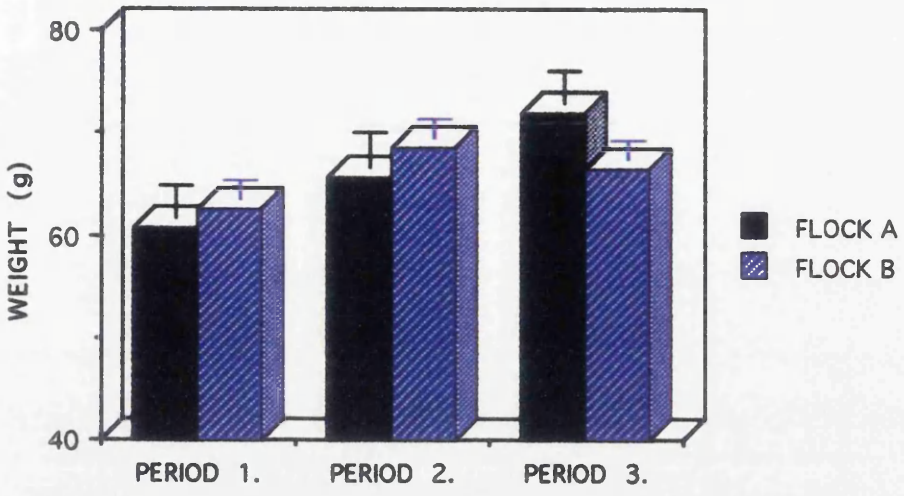
		PERIOD 1		PERIOD 2		PERIOD 3	
CATEGORY	FLOCK	MEAN	± S.D	MEAN	± S.D	MEAN	± S.D
CONFLUENCE	C/+PERCH	4.7	2.3	2.5	1.6	1.5	0.9
	D/-PERCH	3.6	2.1	1.9	1.0	3.3	2.1
FUSION	C/+PERCH	5.5	1.2	6.8	1.6	6.1	1.0
	D/-PERCH	5.7	3.0	8.4	1.7	7.3	1.5
CUFFING	C/+PERCH	6.1	1.7	5.9	1.8	6.2	3.0
	D/-PERCH	6.4	1.5	6.2	1.6	6.6	1.5
ALIGNMENT	C/+PERCH	2.9	0.9	3.0	0.5	2.7	0.9
	D/-PERCH	2.7	0.9	3.1	0.6	2.9	0.6
TYPE B	C/+PERCH	1.3	1.1	1.7	1.0	1.1	0.0
	D/-PERCH	1.5	1.6	1.5	1.3	1.4	1.2
DEPRESSIONS	C/+PERCH	1.1	0.3	1.1	0.3	1.1	0.3
	D/-PERCH	1.5	0.9	1.1	0.3	1.4	1.3
EROSIONS	C/+PERCH	1.0	0.0	1.0	0.0	1.0	0.0
	D/-PERCH	1.2	0.7	1.1	0.1	1.0	0.2
CUBICS	C/+PERCH	1.3	0.7	1.3	0.7	1.0	0.0
	D/-PERCH	1.1	0.5	1.1	0.4	1.0	0.2
ARAGONITE	C/+PERCH	2.1	1.4	1.3	0.8	1.5	0.9
	D/-PERCH	2.0	1.0	2.0	1.4	1.1	0.5
CAP QUALITY	C/+PERCH	2.1	1.1	2.9	1.1	2.1	1.1
	D/-PERCH	2.8	1.1	3.4	1.2	2.6	1.1
TYPE A'S	C/+PERCH	1.3	0.8	1.9	1.0	1.3	0.7
	D/-PERCH	1.5	0.9	1.6	0.9	1.6	0.9
CH. MEMBRANE	C/+PERCH	1.2	0.8	1.2	0.8	2.6	1.5
	D/-PERCH	1.2	0.8	1.3	0.9	3.4	2.0
TOTAL SCORE	C/+PERCH	30.5	3.5	30.5	4.1	28.1	3.5
	D/-PERCH	31.3	5.5	32.5	4.5	33.7	4.5

Table 12. Trial 2: ultrastructural score means ± standard deviations.

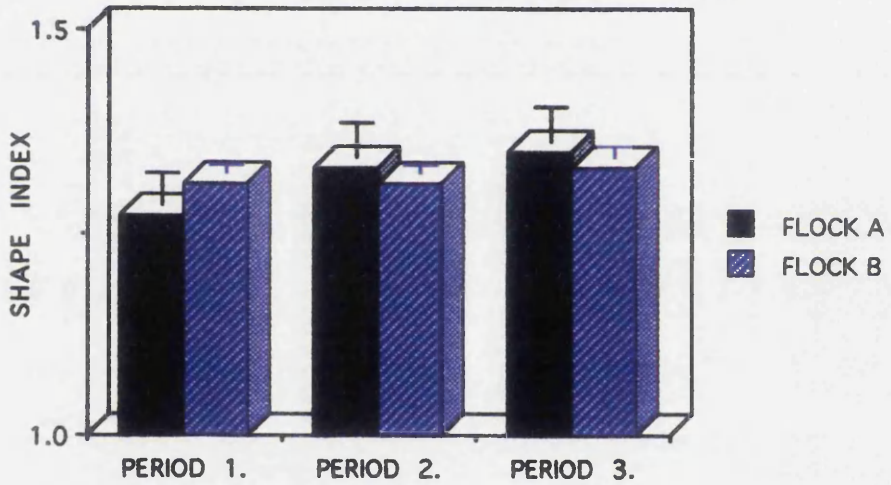
CATEGORY	FACTOR	P VALUE	SIGNIFICANCE
CONFLUENCE	AGE/MOLT	0.000	***
	BET. FLOCKS	0.004	**
	INTERACTION	0.001	***
FUSION	AGE/MOLT	0.000	***
	BET. FLOCKS	0.000	***
	INTERACTION	0.000	***
CUFFING	AGE/MOLT	0.497	N.S
	BET. FLOCKS	0.647	N.S
	INTERACTION	0.383	N.S
ALIGNMENT	AGE/MOLT	0.158	N.S
	BET. FLOCKS	0.111	N.S
	INTERACTION	0.767	N.S
TYPE B	AGE/MOLT	0.284	N.S
	BET. FLOCKS	0.795	N.S
	INTERACTION	0.293	N.S
DEPRESSIONS	AGE/MOLT	0.288	N.S
	BET. FLOCKS	0.415	N.S
	INTERACTION	0.023	*
EROSIONS	AGE/MOLT	0.551	N.S
	BET. FLOCKS	0.668	N.S
	INTERACTION	0.141	N.S
CUBICS	AGE/MOLT	0.049	*
	BET. FLOCKS	0.753	N.S
	INTERACTION	0.156	N.S
ARAGONITE	AGE/MOLT	0.001	***
	BET. FLOCKS	0.471	N.S
	INTERACTION	0.020	*
CAP QUALITY	AGE/MOLT	0.000	***
	BET. FLOCKS	0.600	N.S
	INTERACTION	0.008	**
TYPE A'S	AGE/MOLT	0.072	N.S
	BET. FLOCKS	0.864	N.S
	INTERACTION	0.143	N.S
CH. MEMBRANE	AGE/MOLT	0.000	***
	BET. FLOCKS	0.008	**
	INTERACTION	0.148	N.S
TOTAL SCORE	AGE/MOLT	0.670	N.S
	BET. FLOCKS	0.000	***
	INTERACTION	0.000	***

Table 13. Trial 2: ultrastructural score P values and significance levels. $P \leq 0.001$ = ***, $P \leq 0.01$= **, $P \leq 0.1$= *, N.S = non significant.

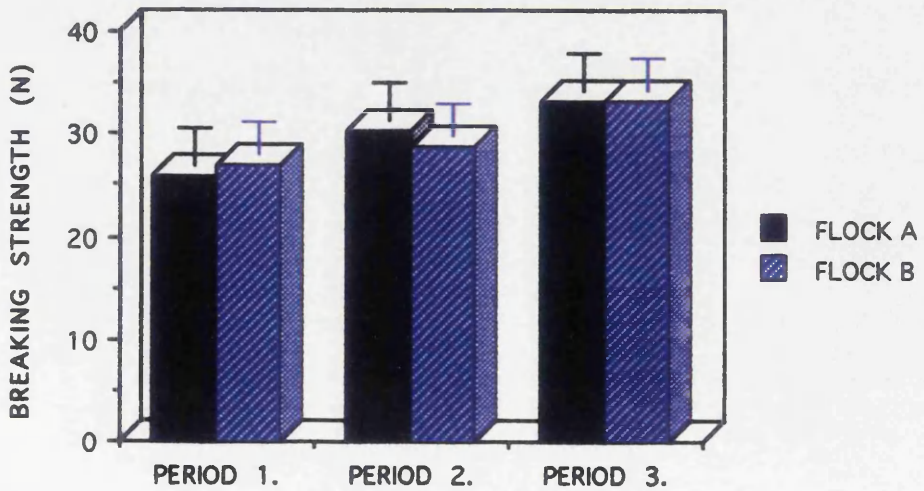
GRAPH 17. MEAN EGG WEIGHT ± S.D



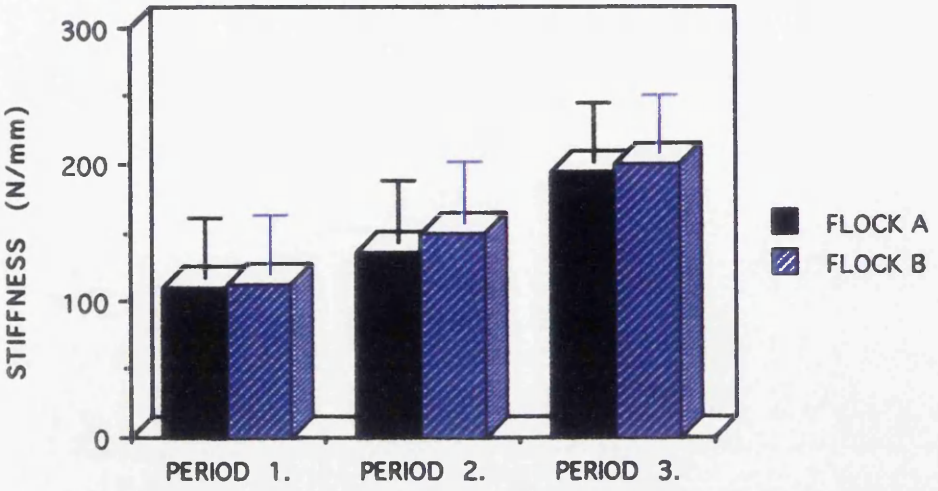
GRAPH 18. MEAN SHAPE INDEX ± S.D



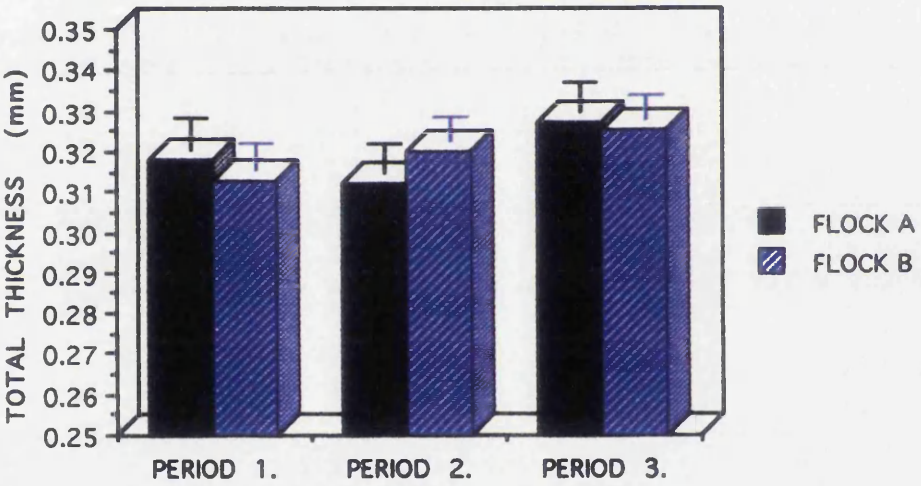
GRAPH 19. MEAN BREAKING STRENGTH ± S.D



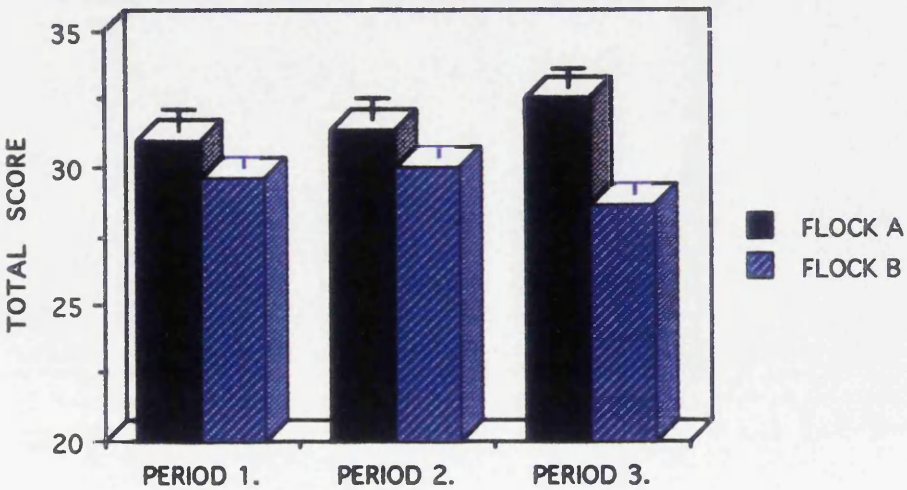
GRAPH 20. MEAN STIFFNESS ± S.D



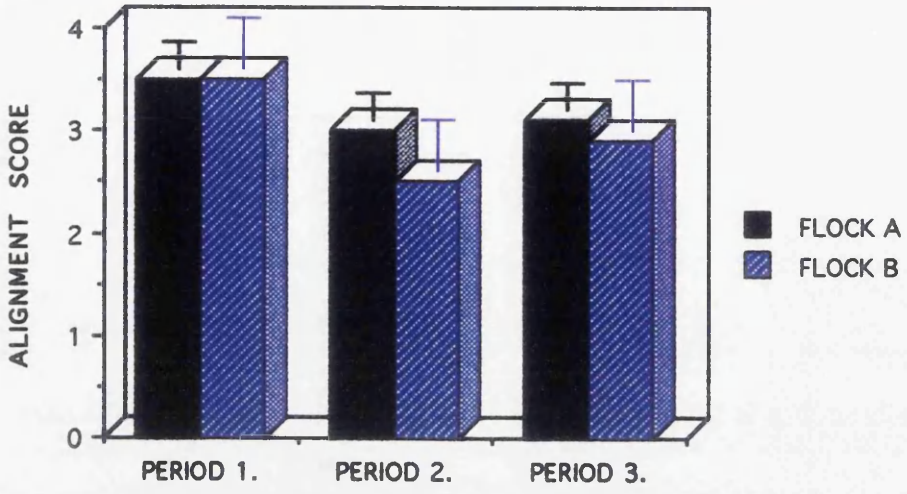
GRAPH 21. MEAN TOTAL THICKNESS ± S.D



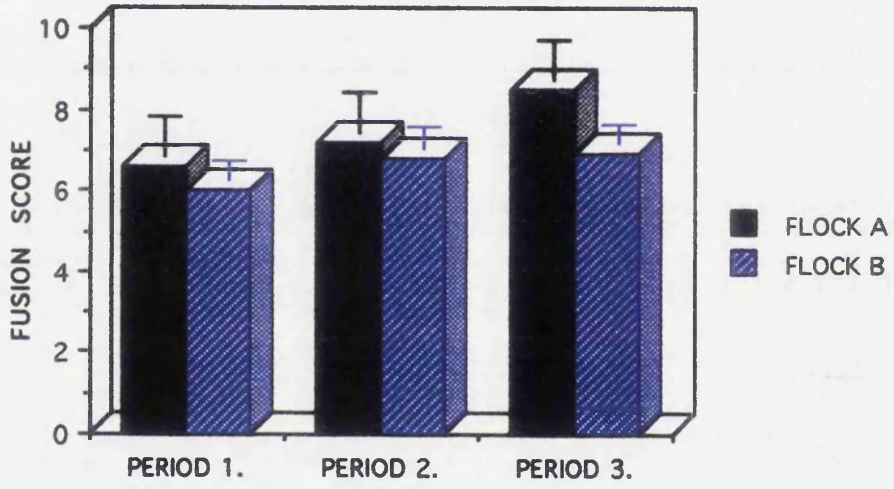
GRAPH 22. MEAN TOTAL SCORE ± S.D



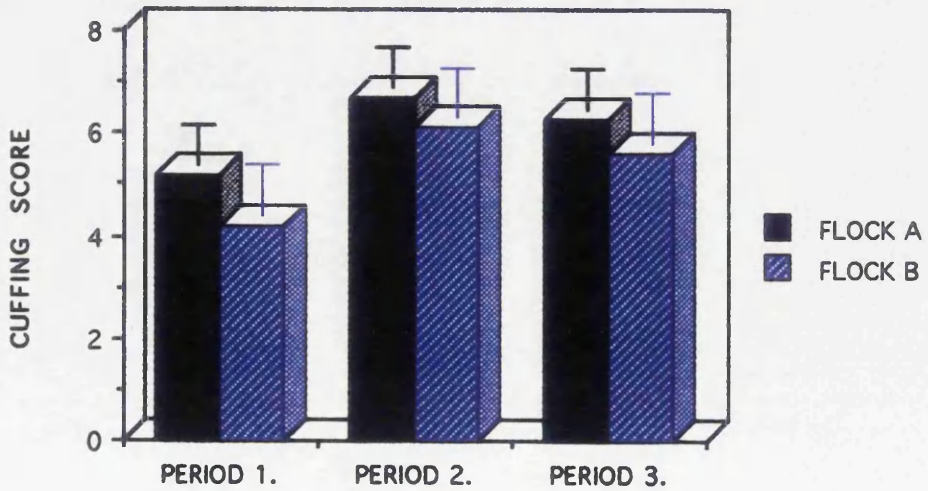
GRAPH 23. MEAN ALIGNMENT SCORE ± S.D



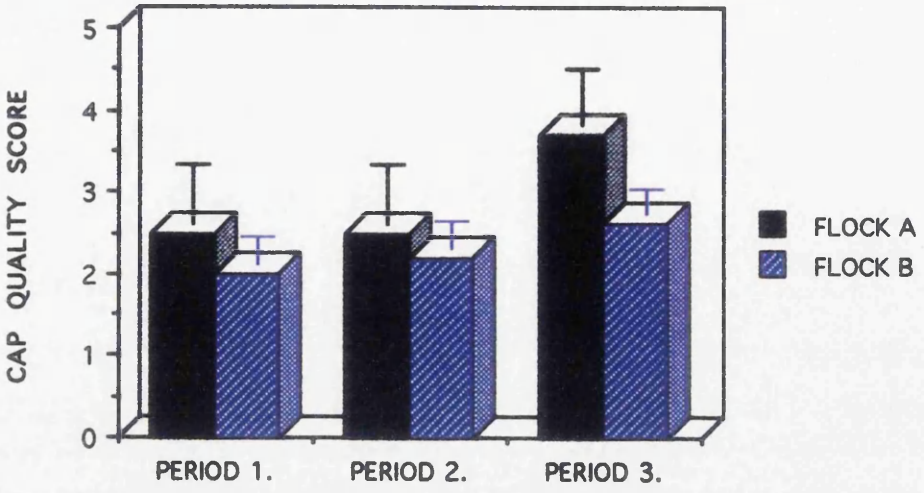
GRAPH 24. MEAN FUSION SCORE ± S.D



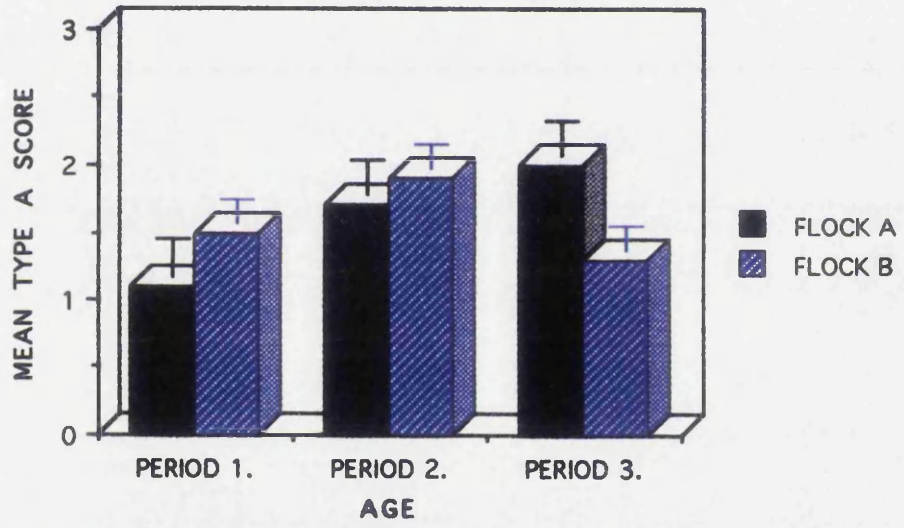
GRAPH 25. MEAN CUFFING SCORE ± S.D



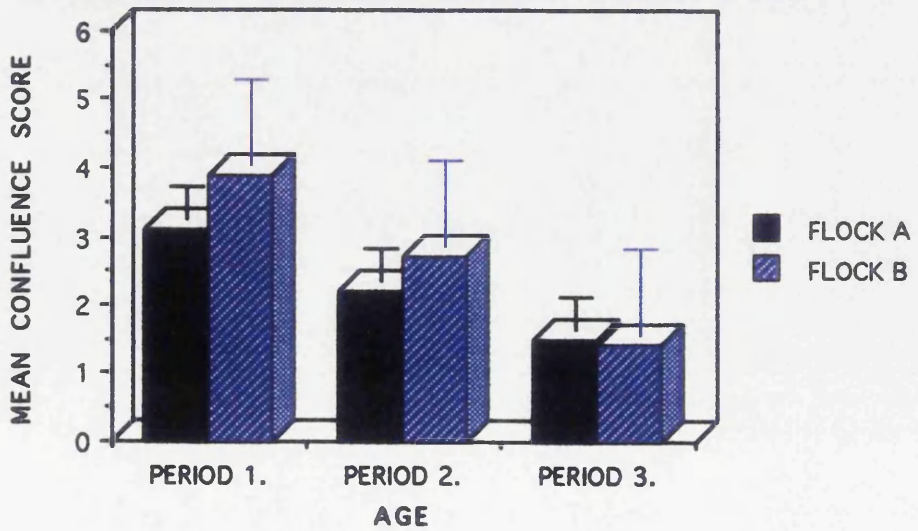
GRAPH 26. MEAN CAP QUALITY SCORE ± S.D



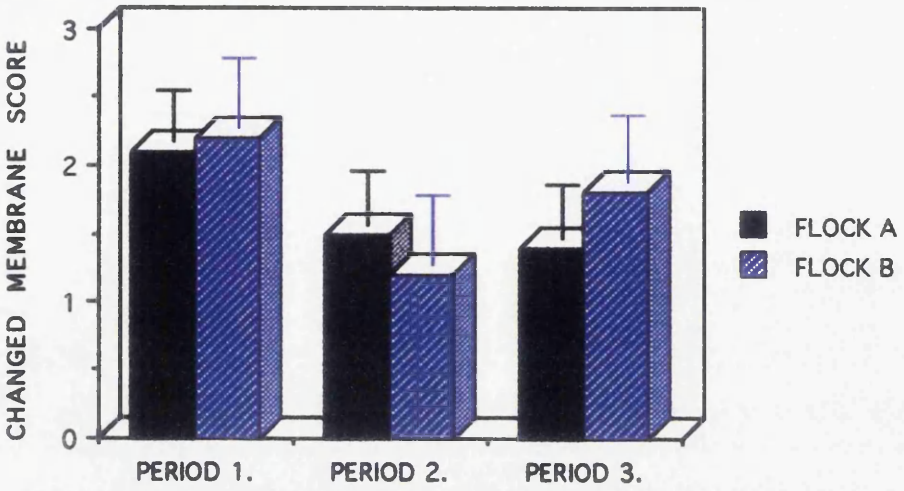
GRAPH 27. MEAN TYPE A SCORE ± S.D



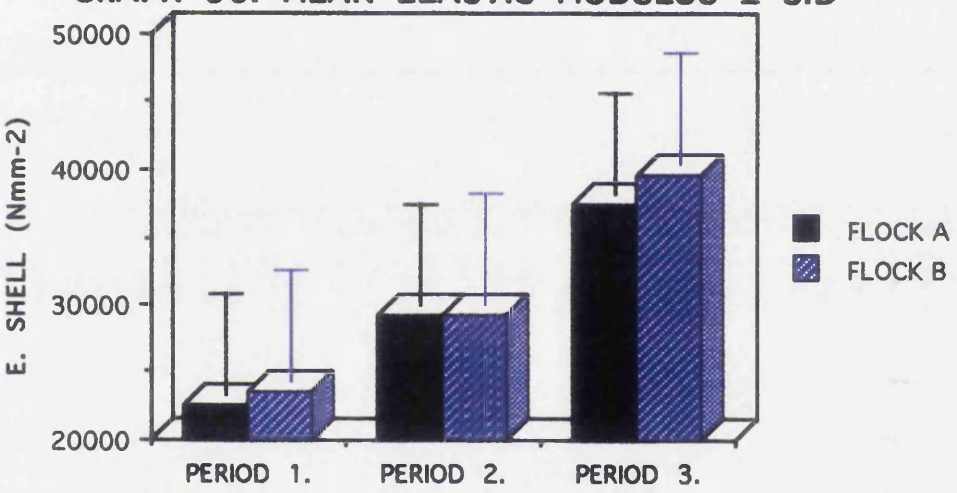
GRAPH 28. MEAN CONFLUENCE SCORE ± S.D



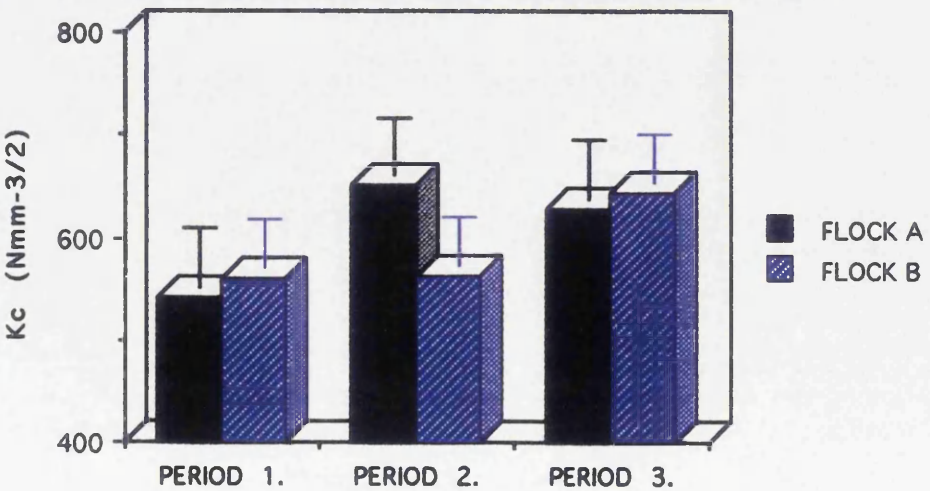
GRAPH 29. MEAN CH. MEMBRANE SCORE ± S.D



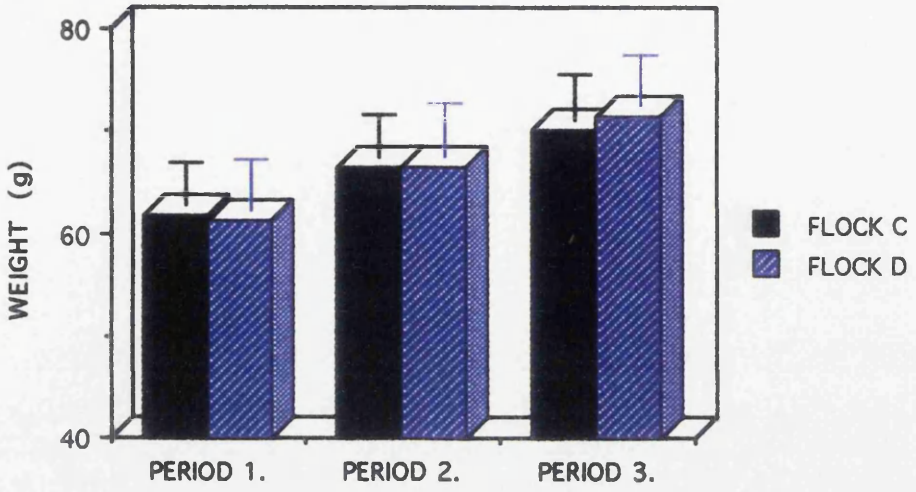
GRAPH 30. MEAN ELASTIC MODULUS ± S.D



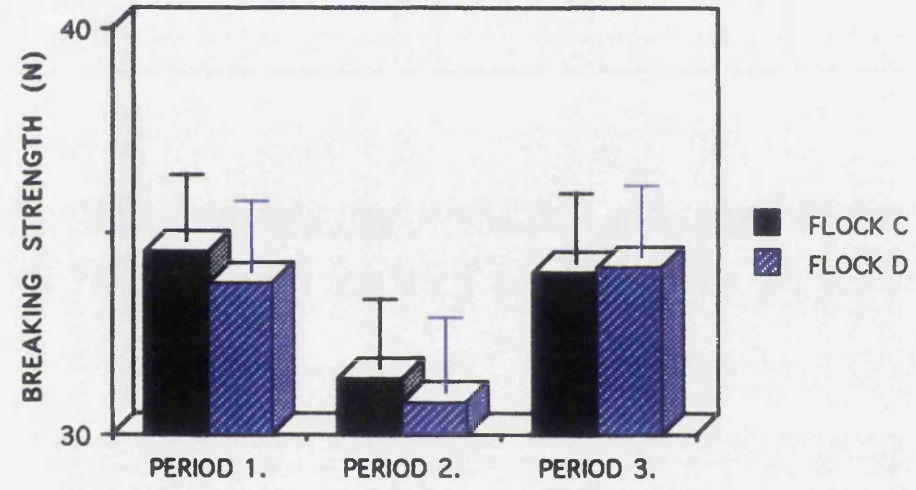
GRAPH 31. MEAN FRACTURE TOUGHNESS ± S.D



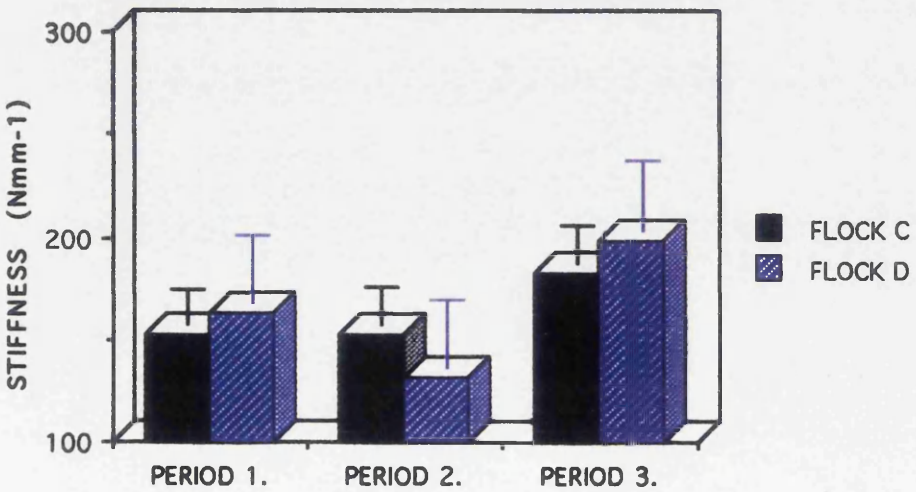
GRAPH 32. MEAN EGG WEIGHT ± S.D



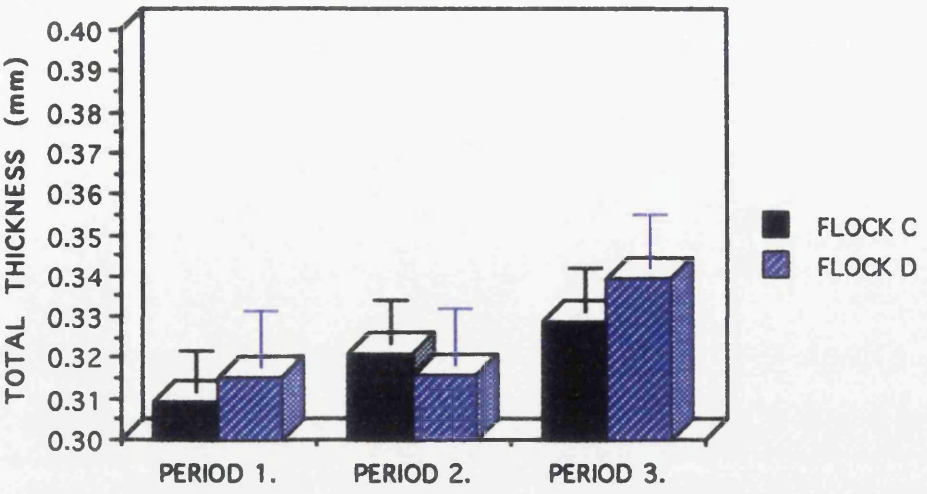
GRAPH 33. MEAN BREAKING STRENGTH ± S.D



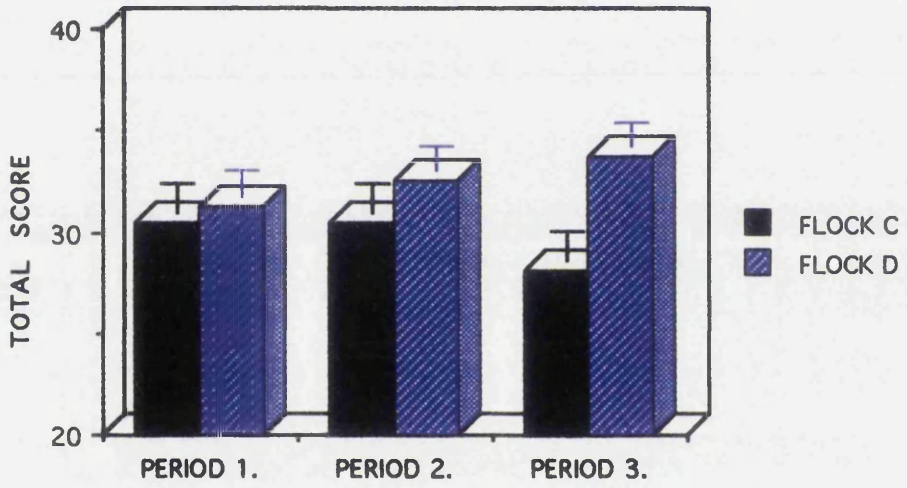
GRAPH 34. MEAN STIFFNESS ± S.D



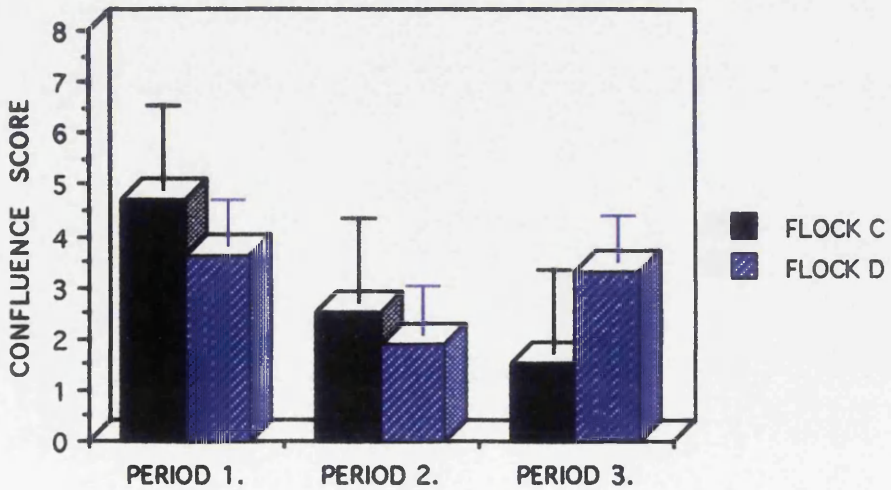
GRAPH 35. MEAN TOTAL THICKNESS ± S.D



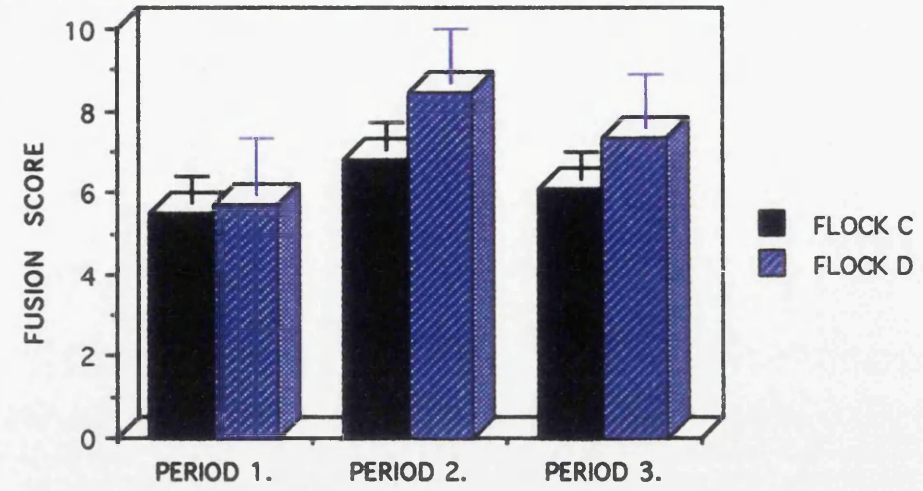
GRAPH 36. MEAN TOTAL SCORE ± S.D



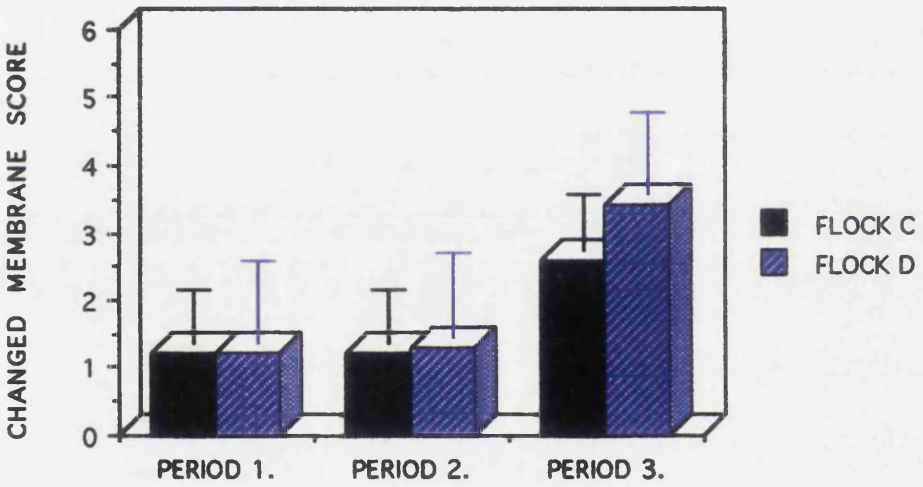
GRAPH 37. MEAN CONFLUENCE SCORE ± S.D



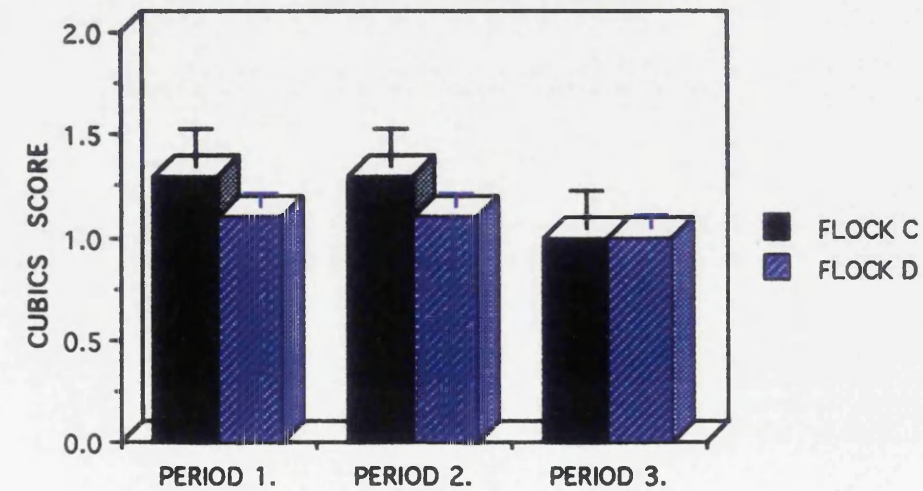
GRAPH 38. MEAN FUSION SCORE ± S.D



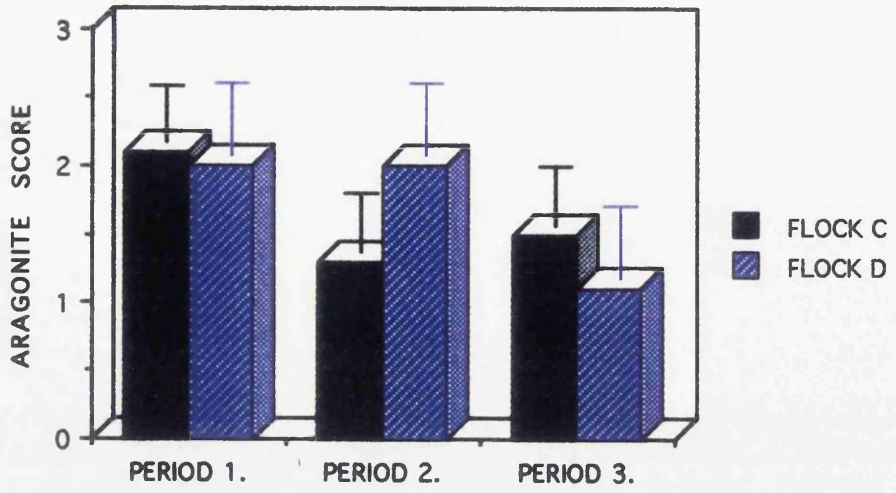
GRAPH 39. MEAN CH. MEMBRANE SCORE ± S.D



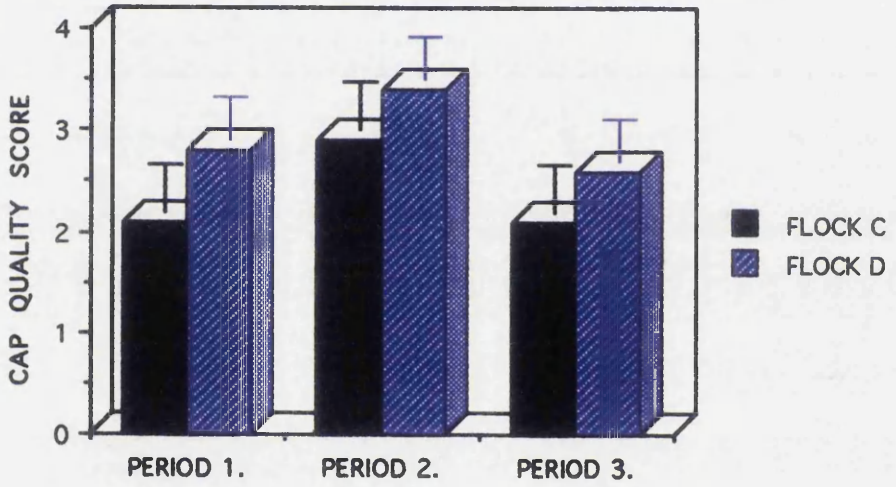
GRAPH 40. MEAN CUBICS SCORE ± S.D



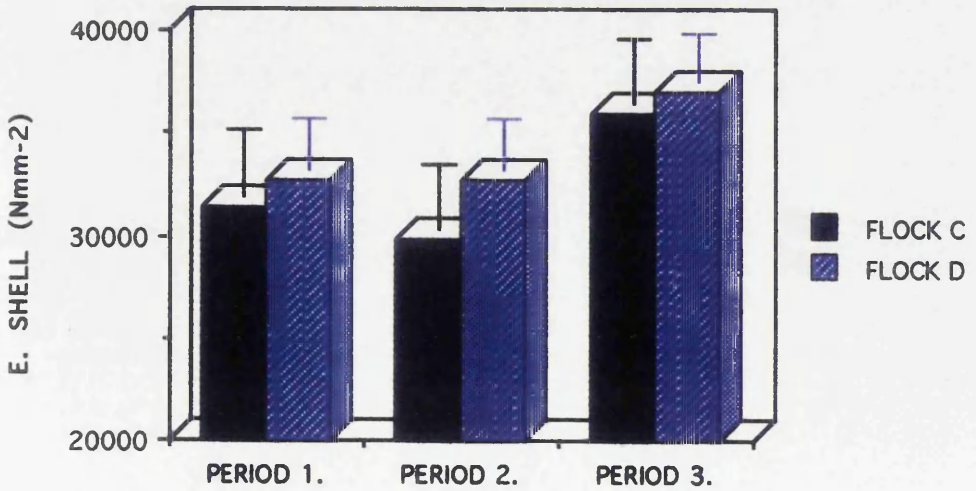
GRAPH 41. MEAN ARAGONITE SCORE ± S.D



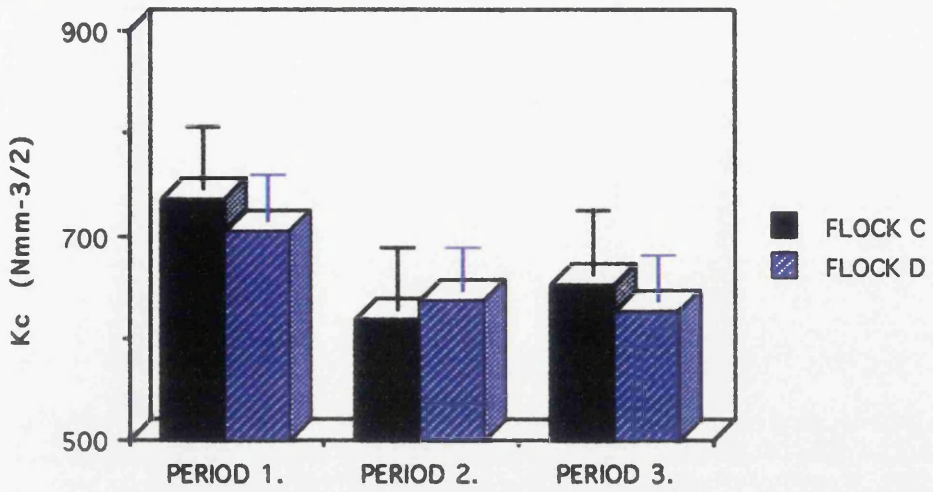
GRAPH 42. MEAN CAP QUALITY SCORE ± S.D



GRAPH 43. MEAN ELASTIC MODULUS ± S.D



GRAPH 44. MEAN FRACTURE TOUGHNESS ± S.D



CHAPTER 4 - THE EFFECT OF BIRD AGE ON MATRIX MORPHOLOGY AND DISTRIBUTION WITHIN THE EGGSHELL.

4. THE EFFECT OF BIRD AGE ON MATRIX MORPHOLOGY AND DISTRIBUTION WITHIN THE EGGSHELL.

4.1. INTRODUCTION.

The eggshell of the domestic fowl has been the focus of much research over the years, with the bulk of the literature to date concentrating on the calcified portion of the shell (Solomon, 1993). While the composition, structure and morphology of the inorganic phase is well described, the nature of the organic matrix and its associated vesicles is less well understood (Krampitz, 1993; Arias and Fernandez, *pers comm*).

It has been suggested that the organic matrix of the eggshell may be involved in the regulation of various stages of crystal growth (Addadi and Weiner, 1992; Hincke *et al.,* 1992, 1993; Gautron *et al.,* 1993,1995 in press; Hincke, 1995). This hypothesis is investigated further in chapter **6**.

An alternative explanation is provided by Silyn-Roberts and Sharp (1986). These authors suggest that the matrix proteins may influence the material and mechanical properties of the crystalline shell by the formation of a strengthening fibrous network, similar to the steel in reinforced concrete. Using computer modelling techniques Bain (1990) described the material strength of an eggshell in terms of two properties, namely its elastic modulus (E. shell) and its fracture toughness (Kc). This author demonstrated, as in the case of other calcified tissues such as bone, that the material strength of the eggshell is greater than can be predicted from its two main components (calcium and carbonate ions). Also, there appears to be considerable variation between individual eggs which cannot be explained in terms of the inorganic fraction alone. The emerging view is of a multiple role for the matrix proteins (Hincke *et al.,* 1993; Hincke, 1995).

The current study involves an investigation into the morphology and distribution of organic matrix and matrix vesicles in the palisade layer of eggs from battery housed hens, using light microscopy, transmission electron microscopy and image analysis techniques. Although not observed in the present thesis, the deterioration in shell quality towards the end of lay is well documented (Bain, 1990; Solomon,1991; Nascimento, 1992). Matrix profiles were compared using eggs from three different time periods during lay, in

order to assess whether matrix characteristics change with increasing bird age. The study also involves an examination of the nature and distribution of the organic material within the vertical crystal and cuticular layers, as the data available to date regarding this matter are inconsistent (Simons, 1971; Baird *et al.,* 1975; Arias *et al.*, 1992, 1993; Fink, 1993; Sparks, 1994; Arias and Fernandez, 1993, 1995).

4.2. MATERIALS AND METHODS.

Nine eggshells were selected at random from the beginning (24 weeks), middle (46 weeks) and end of lay (72 weeks) from the commercial battery system samples used in chapter **2**. Three $1cm^2$ sections were cut from each shell using a dental drill with a small rotary blade attached. The shell membranes were subsequently removed by plasma etching (**2.3.3.** and Reid, 1983). Each sample was then decalcified using a solution of 20% EDTA (pH 6.9-7.0) in 2% paraformaldehyde and 0.5% gluteraldehyde in phosphate buffer for 3-4 days at room temperature, the solution being changed daily. The phosphate buffer was prepared by dissolving 6.32g of $Na_2HPO_4.2H_20$ in 1 litre of water (solution A). A second solution (solution B) was prepared by dissolving 4.83g of KH_2PO_4 in 1 litre of water. $95cm^3$ of solution A was then mixed with $5cm^3$ solution B to give the required buffer.

After the calcium carbonate fraction of the shell had completely dissolved, the fragile organic matrix remained. This was then prepared for transmission electron microscopy (TEM) as follows.

Each matrix sample was carefully transferred into a small glass bottle and placed in Karnovsky's fixative for three hours. The fixative was then drained and replaced with 0.1M Sorenson's buffer for 1 hour.

The samples were post fixed in osmium tetroxide for an hour and washed with distilled water three times. Following this, they were dehydrated using a graded series of acetones and transferred to propylene oxide followed by 50/50 propylene oxide/Emix resin. This was drained and replaced with Emix resin for four hours. The samples were then cut into strips using a razor blade and flat embedded in Emix resin. Polymerisation was carried out over night at $60^{\circ}C$.

The samples were subsequently trimmed using an LKB pyramitome and thick sections (1μm) cut for light microscopy. This enabled orientation to be established for TEM. Light micrographs of each thick section (see Figure 20) were taken using a Leitz Laborlux microscope with a camera attached.

Using a glass knife an LKB ultramicrotome 3 was used to cut thin sections (40Å) which were mounted onto copper grids and stained with Reynold's Uranyl acetate/Lead citrate. The sections were then examined in a Jeol 100C X2 Transmission Microscope at an accelerating voltage of 80Kv.

For comparative purposes photomicrographs were taken of selected areas corresponding to outer palisade, mid palisade and inner palisade in each sample (see Figure 20) at a magnification of x20000.

Micrographs of the vertical crystal layer and cuticle (when present) were also obtained at various magnifications.

4.2.1. IMAGE ANALYSIS.

Each photomicrograph was transferred onto computer disc by means of an Applescan SCSI scanner. The percentage matrix present in each scanned image was then established using a commercially available image analysis package. In addition to this, the number of matrix vesicles present in each micrograph was assessed. The resulting data were then analysed using a two way analysis of variance, to establish any significant differences in % matrix and vesicle numbers in the selected regions of the palisade layer during the time periods under investigation.

4.3. RESULTS AND DISCUSSION.

4.3.1. LIGHT MICROSCOPY.

Using light microscopy, Romanoff and Romanoff (1949) described three zones in the palisade layer after protein staining of the eggshell and described a fairly even distribution of matrix within each layer. According to these authors matrix material was most concentrated in the inner layer and least abundant in the outer. Terepka (1963) demonstrated zones of relative EDTA resistance in radial sections of shell and postulated that the outer palisade had a higher organic matter content, thus disagreeing with the findings of Romanoff and Romanoff (1949). In the current study, light microscopy indicates that the organic matrix is least concentrated at the inner palisade and is most concentrated at the outer palisade layer. These results are in agreement with Terepka (1963) who also described the organic matrix as having a fine, fibrous and well ordered orientation with a herring bone like appearance. This can be clearly seen in Figure 20 especially at the level of the mid palisade (M). The inner palisade (I) revealed a fibrous appearance with a transition towards a more compact matrix structure occurring towards the mid (M) and outer (O) layers of the palisade (see Figure 20). At the mammillary level (MA) the matrix appears to be concentrated in the cores, this supporting the findings of a light microscopy study by Simons (1971).

4.3.2. TEM AND IMAGE ANALYSIS.

Mean % matrix values and vesicle numbers are shown in Table 14. P values and levels of significance are indicated in Table 15.

According to Simons (1971) the organic matrix of the outer part of the shell, including that of the vertical crystal layer, is more compact than the matrix from the inner palisade layer. In general terms, the matrix morphology of the palisade layer described in the current study agrees with this description and is illustrated in Figures 21-27. The herring bone appearance described by Terepka (1963) was not apparent, possibly due to the higher magnification of the electron micrographs. A more detailed account of the inner, mid and outer palisade at times corresponding to the beginning, middle and end of lay now follows.

[i] Beginning of lay. (Table 14, Figures 21-23 and Graphs 45 and 46). According to the % matrix values there was an even distribution of matrix within all three regions of the shell. However, the morphological appearance of the matrix material within these regions was in fact, very different. The outer palisade was largely vesicular in nature, whilst the inner region was mostly fibrous.

[ii] Middle of lay. (Table 14, Figures 24-26 and Graphs 45 and 46). The % matrix associated with the outer and mid regions was less than that observed in similar areas during the beginning of lay, whilst the inner region showed little change in % matrix. In general, there was an increase in the presence of fibrous material present. The % matrix associated with the inner palisade was unchanged but was considered more vesicular in form at this time.

[iii] End of lay. (Table 14, Figure 27 and Graphs 45 and 46). The % matrix associated with the inner palisade was significantly increased. This was due both to an increase in fibrillar material and vesicle numbers. The % matrix present in the mid and outer regions of the shell was similar to that observed at the beginning of lay, with the matrix also becoming more vesicular in nature. These results are similar to the findings of Romanoff and Romanoff (1949) and suggest that the eggshells used in their studies came from the end of lay period.

In chapter **2**, a decrease in fracture toughness values at the end of lay suggests an increase in the incidence of defects in the eggshells under investigation. This was not associated with a concomitant increase in total ultrastructural score. However, elastic modulus values were similar throughout, indicating that the elasticity of the material had not changed with increasing bird age. These observations notwithstanding, the morphology of the matrix, particularly from the inner palisade at the end of lay, was distinctly different from that at the beginning. It is therefore hypothesised that this reorganisation of matrix material is somehow responsible for the observed changes in fracture toughness. These results then support the hypothesis of Silyn-Roberts and Sharp (1986) that the organic matrix of the shell influences the material and mechanical properties of the shell. Such changes in matrix morphology may account, in part, for other discrepancies in the calculated material strength of eggshells as identified by Bain (1990).

An essential prerequisite for controlled mineralization is spatial localisation (Mann, 1986) and it is suggested that matrix vesicles fulfil such a role in the formation of the eggshell. In addition, it is known, in lower plants and animals, that membrane bound vesicles regulate physiochemical factors through selectivity in biochemical processes by the operation of "ion pumps." They are therefore capable of maintaining and modifying particular chemical environments (Mann, 1986, 1988; Simkiss, 1986; Williams, 1989). It may well also be the case that matrix vesicles are able to expand and contract, both responding to and influencing the chemical and physical conditions within the mineralizing environment (Williams, 1989). Organic matrix molecules would obviously be influenced by this interaction, ultimately affecting the mineralization process. It seems likely that the vesicles associated with the various layers of the eggshell are involved in such mechanisms, accounting in part, for the morphological variation in the organic matrix *per se* observed within the regions of the palisade layer as indicated. It is also probable that a degree of influence over the chemical composition of uterine fluid and the extra vesicular mileau is exerted through the activity of organs such as the kidney, which are in effect macro-pumps for certain ionic species (Thompson, 1968; Williams, 1989).

4.3.3. THE VERTICAL CRYSTAL LAYER.

According to Arias *et al.*, (1993) the organic matrix of the avian eggshell is distributed between the cone layer and the base of the vertical crystal layer (VCL), with the absence of an organic matrix in the latter accounting for the vertical orientation of the crystals. The results of the current study, however, indicate an apparent change in matrix morphology at the level of the VCL, contrary to the findings of Arias *et al.*, (1993). As indicated by Figure 20 shell matrix varies in its configuration from the inner layer outwards. Thus, at the innermost surface of the eggshell the matrix fibres are perpendicularly orientated, becoming progressively more vertical in nature towards the outer surface in the region of the vertical crystal layer (see Figures 20 and 28). Simons (1971) described the decalcified vertical crystal layer as being rich in organic matter of a predominantly fibrillar nature. This may be the vertically orientated matrix identified in the present study.

All eggs, irrespective of their final colour, contain a complement of pigment. The porphyrins responsible for the pigmentation of the egg of the domestic fowl occur primarily in the cuticle, yet pigment can be detected in the mineralized shell, immediately opposed to the cuticle (Baird *et al*, 1975; Sparks, 1985, 1994). To what degree the structure of the shell is influenced by the presence of pigment is, at this time, a matter of debate. However, it is not unreasonable to assume that the presence of porphyrins exert some influence on the orientation of the matrix molecules and/or calcite crystals at this level.

4.3.4. THE CUTICLE.

The cuticle has been described as an amorphous shell surface accessory material with water resistant and antibacterial properties, the birds final attempt to secure a healthy and stable environment for the developing embryo (Sparks, 1994). Electron microscopy has disproved its amorphous appearance (Cranstoun, 1992). It is in fact, as with all aspects of eggshell structure, a complex entity.

The results of the current study show the presence of a two layered cuticle (see Figure 28 and 29). The inner layer is composed of a matrix like material containing vesicles of variable size (see Figures 28-30) each containing a core (C) and mantle (M) (see Figure 29). This layer is henceforth referred to as the vesicular cuticle (VC). The core material is of variable electron density while the mantle is consistently more electron dense and contains distinctly granular material (see Figure 29). The outer layer of cuticle is much more compact and homogeneous in nature and does not appear to contain any matrix vesicles, this layer is henceforth referred to as the nonvesicular cuticle (NVC).

In terms of structure, the cuticle has been variously described as: granular, homogeneous, porous, layered with air inclusions, and vesicular in nature (Simons, 1971; Sparks, 1994). Simons (1971) described the cuticle in decalcified sections as being completely vesicular and did not describe a nonvesicular region as outlined in the present study. Two distinct layers were, however, distinguished by the latter author, with the upper strata of the cuticle containing smaller vesicles and being more compact in nature than the lower. Some of these vesicles appeared to be empty, whilst others contained

crystalline/granular material along the inner periphery similar to that described in the present study (see Figures 28-30). Fink *et al.,* (1993) have also described the presence of vesicle-like structures in the cuticle which appear to contain high concentrations of the phosphorus rich mineral hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$).

The role of phosphorus in the termination of shell formation is well documented but the exact mode of action remains unclear (Fink *et al.,* 1993; Nys, *pers comm*). Gautron and Nys (1993) and Nys (1995) have reported that whole uterine fluid inhibited the formation of calcium carbonate when sampled coincidentally with the completion of eggshell formation, although high concentrations of calcium and carbonate ions were still present in the milieu at this stage. Secretion of an inhibitor (such as phosphorus) in higher concentrations might end the process of shell formation, as established in other supersaturated biological fluids (Multinger *et al .,* 1983; cited by Gautron and Nys, 1993). The large effect of dialysed uterine fluid on the rate of calcium carbonate precipitation and the absence of effect of ultrafiltrate, however, suggest that the inhibition is due to a specific macromolecule, rather than to inorganic phosphorus.

It is also known that several species of bacteria of dental and medical interest deposit hydroxyapatite when cultured in media containing calcium and phosphate. *Bacterionema matruchotti* has been studied extensively in an investigation of the mechanism of mineral deposition. It appears that deposition of hydroxyapatite may be initiated by lipoprotein and controlled by protein-lipid complexes within the organism (Ennver *et al.,* 1968, 1981; Boyan *et al.,* 1984; Boyan *et al.,* 1989; cited by Simkiss and Wheeler, 1989). Lipoprotein and protein-lipid complexes are also known to be major components of the eggshell cuticle (Sparks, 1994). In view of these findings, it is hypothesised that the vesicles associated with the cuticle contain a phosphorus macromolecule (possibly hydroxyapatite as suggested by Fink *et al.,* 1993), which is somehow implicated in the competitive termination of calcium carbonate precipitation and hence the end of eggshell formation. Although other researchers have indicted that the cuticle may be mineralized (Arias and Fernandez, *pers comm*), no specific crystalline structure has yet been identified. Unfortunately, such an exercise was outwith the remit of this thesis.

The cuticle is also known to contain higher levels of potassium and magnesium than other regions of the shell (Simons, 1971), although the role of these molecules in the process of shell termination remains unestablished. It is known, however, that amorphous calcium phosphate changes autocatalyticaly into hydroxyapatite if it is not stabilised by Mg^{2+} and ATP within cellular processes (Simkiss and Wilbur, 1989). Once stabilised amorphous calcium phosphate is reported to be able to cross intracellular membranes and act as a precursor for the more stable hydroxyapatite. It may be that, in the case of the cuticle, amorphous calcium phosphate binds with Mg^{2+} in a manner similar to that described and is able to pass through the vesicle mantle and accumulate in the cores. Such a hypothesis ties in well with the proposed physiochemical role for matrix vesicles as outlined in section **4.3.2.**.

In molluscan shells, an organic matrix also plays a principal role in the normal mineralization of calcium carbonate. In certain species of mollusc the organic matrix is deposited as a substrate prior to crystal nucleation, with the resulting mineral being embedded in the matrix (Crenshaw, 1982). This has been described as the "compartment hypothesis" of biomineralizite formation, Krampitz and Grasser (1988). In view of the evidence provided by TEM (see Figures 28 and 29) it is hypothesised that the secretions produced by the SGP during the final stages of shell formation include a layer of organic matrix material, different in nature to that found in the mammillary and palisade layers, which is laid down prior to final mineralization in a manner similar to that occurring in the molluscan shell. Between this substrate is deposited the final cuticular layer. As the organic matrix is already present the morphology of this final layer is free to assume a different framework. The organic material may migrate between the cuticular vesicles and, on maturation of the cuticle, set to its final form, this supporting the role of spatial localisation for matrix vesicles (see **4.3.2.**). Close inspection of the cuticular surface adds to this hypothesis as vesicles can be seen situated between surface cracks, Simons (1971) and Sparks (1994). The vesiculate nature of the cuticle would therefore appear well suited to the function of mammillary pore cap and/or plug as described by Solomon (1991), Nascimento (1992), Cranstoun (1992) and Sparks (1994).

In the following chapter, the next stage of the investigation into the nature of the matrix moves from the morphological aspect to a study of the individual protein components, which together constitute the organic fraction of the eggshell of the domestic fowl.

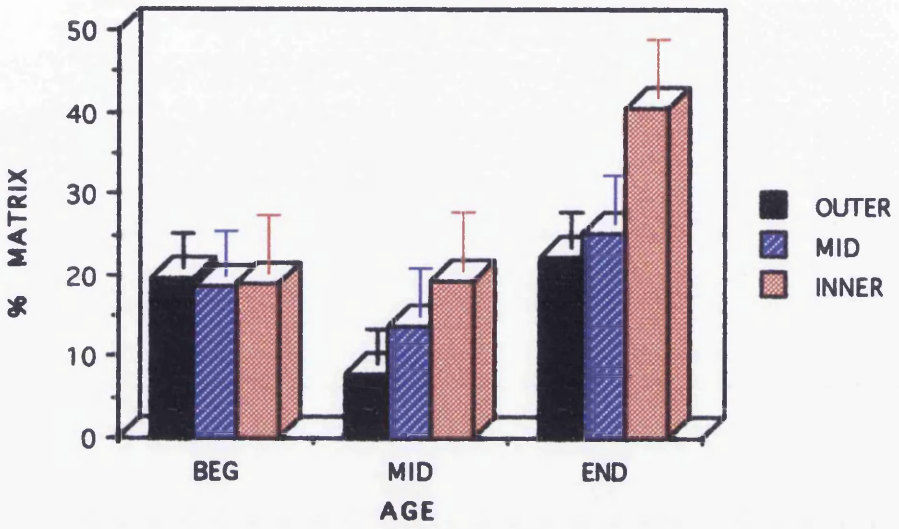
BEG. LAY	MEAN	±S.D
OUTER % MATRIX	19.56	5.34
OUTER VESICLE NOS.	107.22	26.35
MIDDLE % MATRIX	18.44	4.16
MIDDLE VESICLE NOS.	77.78	19.22
INNER % MATRIX	18.89	6.05
INNER VESICLE NOS.	11.67	7.50
MID. LAY	MEAN	±S.D
OUTER % MATRIX	7.68	1.47
OUTER VESICLE NOS.	89.33	46.71
MIDDLE % MATRIX	13.69	5.52
MIDDLE VESICLE NOS.	87.22	33.27
INNER % MATRIX	19.14	5.55
INNER VESICLE NOS.	56.11	26.43
END LAY	MEAN	±S.D
OUTER % MATRIX	22.10	5.93
OUTER VESICLE NOS.	117.78	71.38
MIDDLE % MATRIX	25.22	6.79
MIDDLE VESICLE NOS.	108.89	22.05
INNER % MATRIX	40.63	19.67
INNER VESICLE NOS.	81.11	44.57

Table 14. % matrix values and vesicle nos. ± S.D.

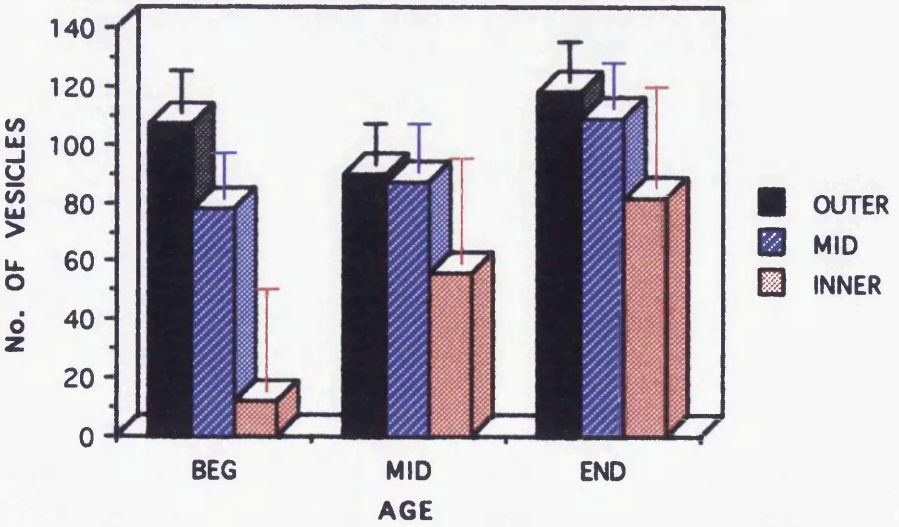
FACTOR	P VALUE	SIGNIFICANCE
% MATRIX	0.000	***
AGE	0.000	***
INTERACTION	0.010	**
VESICLE NOS.	0.000	***
AGE	0.002	**
INTERACTION	0.103	N.S

Table 15. % matrix and vesicle nos. P values and significance levels.
$P \leq 0.001$ = ***, $P \leq 0.01$= **, $P \leq 0.1$= *, N.S = non significant.

GRAPH 45. MEAN % MATRIX ± S.D



GRAPH 46. MEAN No. VESICLES ± S.D



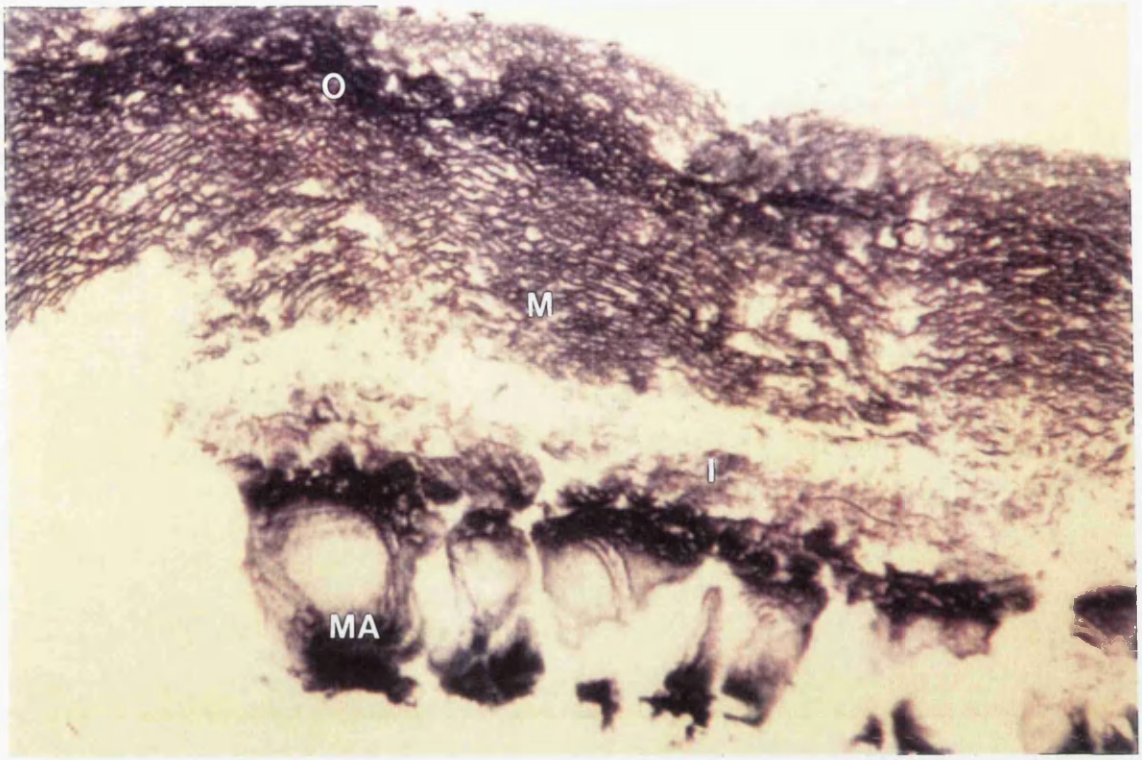


Figure 20: Transverse section of decalcified shell from early lay showing mammillary cores (MA), inner palisade (I), mid palisade (M) and outer palisade (O) regions. Cuticle has been removed with EDTA in this case (x 90).

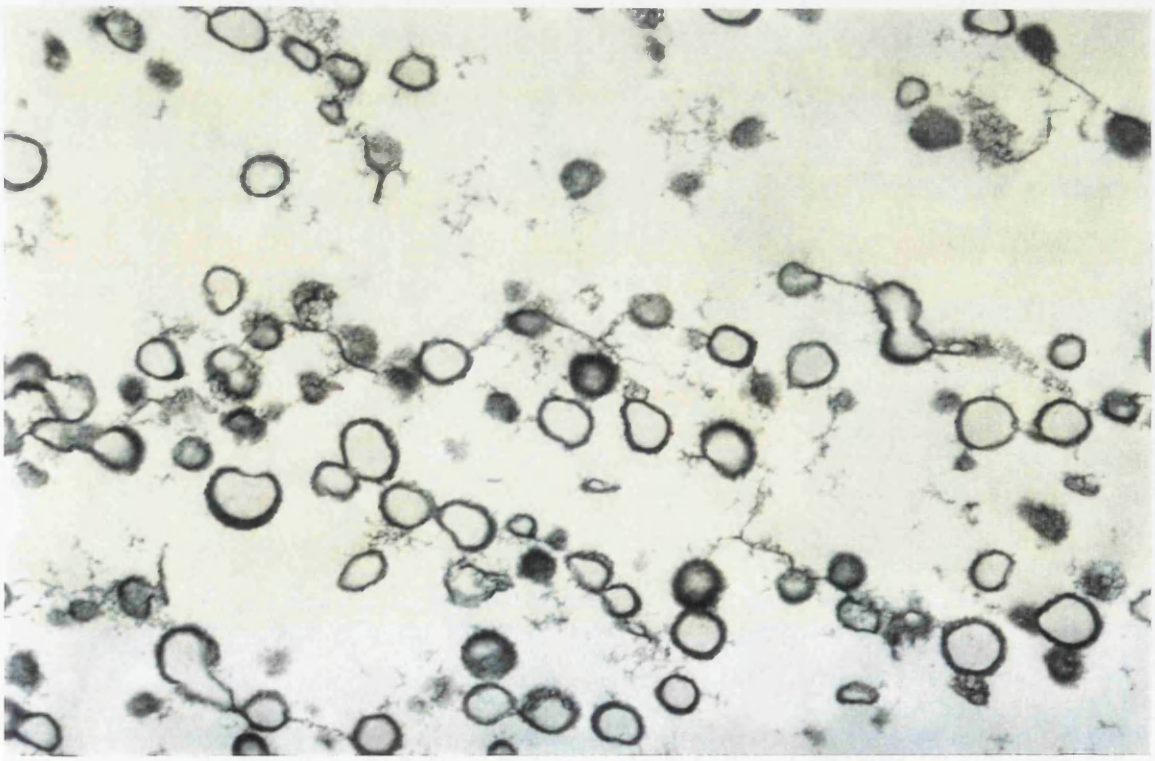


Figure 21: Vesicular matrix from the outer palisade region of a shell from the beginning of lay (x20000).

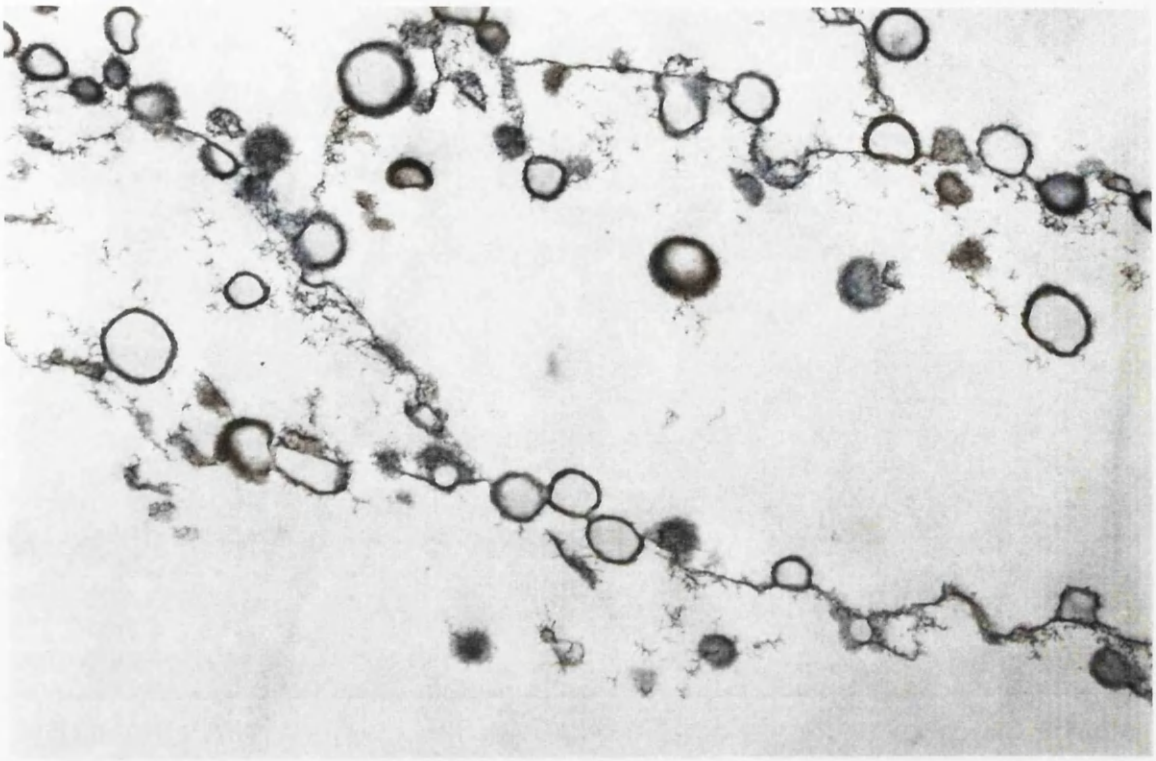


Figure 22: A reduction in the number of matrix vesicles was observed in the mid palisade region sample at the beginning of lay (x20000).

Figure 23: The matrix from the inner palisade region at the beginning of lay was more fibrous in nature with fewer vesicles being apparent (x20000).

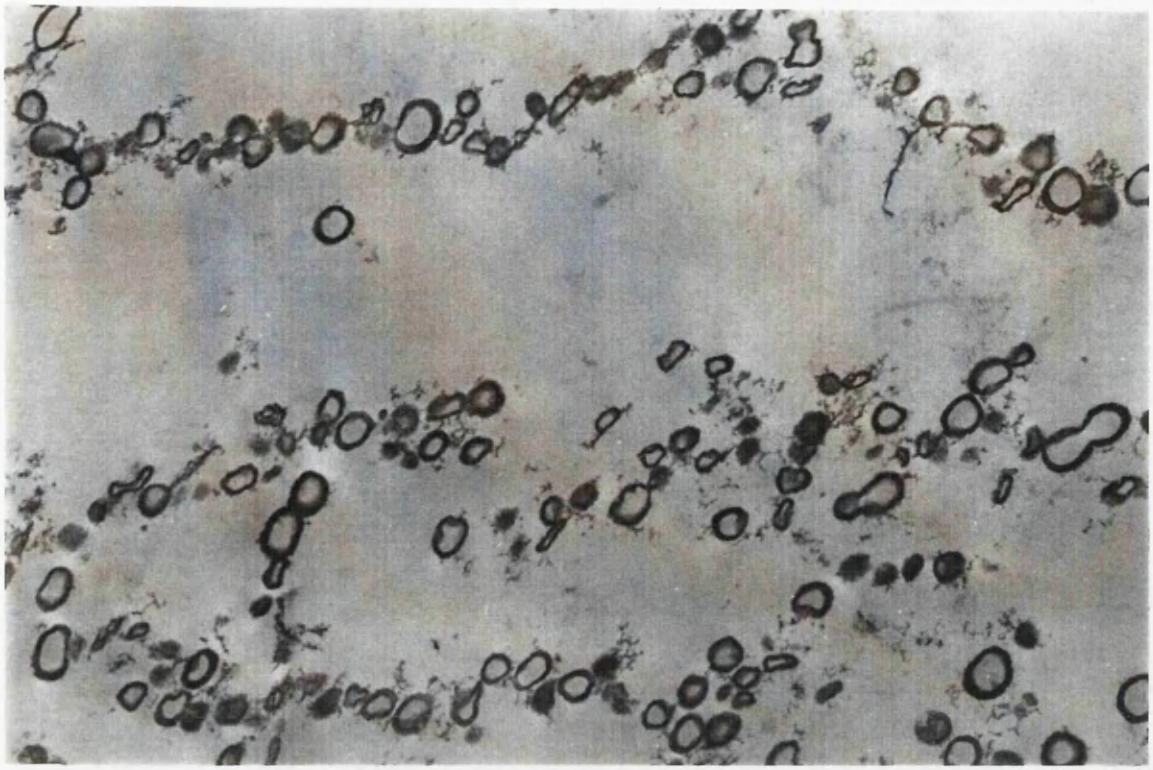


Figure 24: The % matrix in the outer palisade region decreased significantly during mid lay. It was mostly vesicular in nature (x20000).

Figure 25: A change towards a more fibrous nature was observed in the mid palisade region during mid lay (x20000).

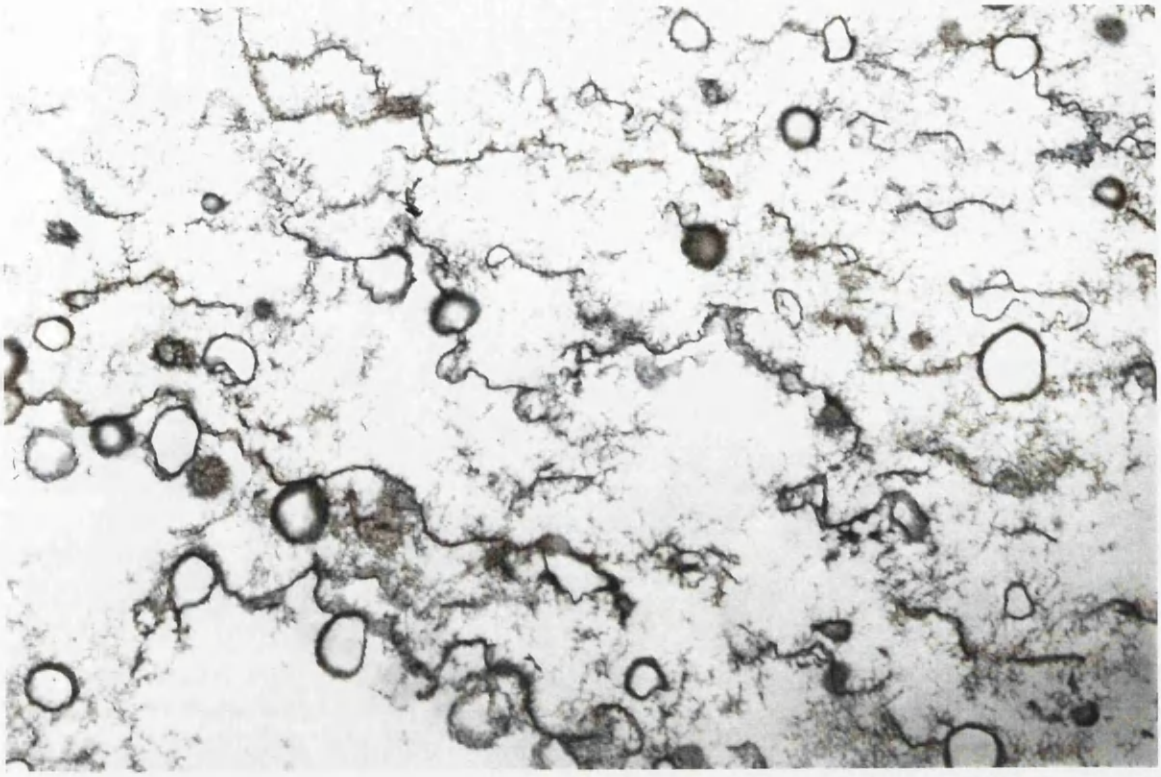


Figure 26: The inner palisade region shows an increase in fibrous material and vesicle numbers during mid lay (x20000).

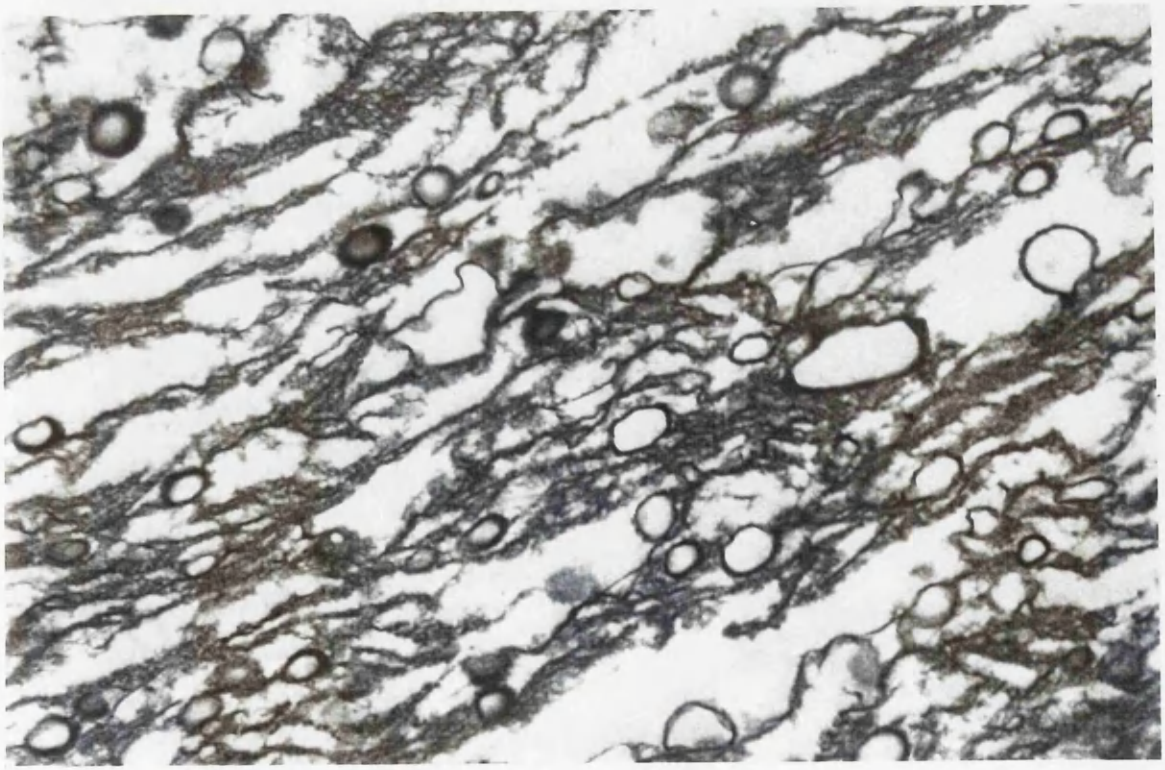


Figure 27: The % matrix was greatest at the end of lay, significantly so in the inner palisade region (x20000).

Figure 28: Micrograph showing vertical orientation of matrix in the vertical crystal layer (VCL), large vesicles associated with the vesicular cuticle (VC) and the more compact outer nonvesicular cuticle (NVC) (x20000).

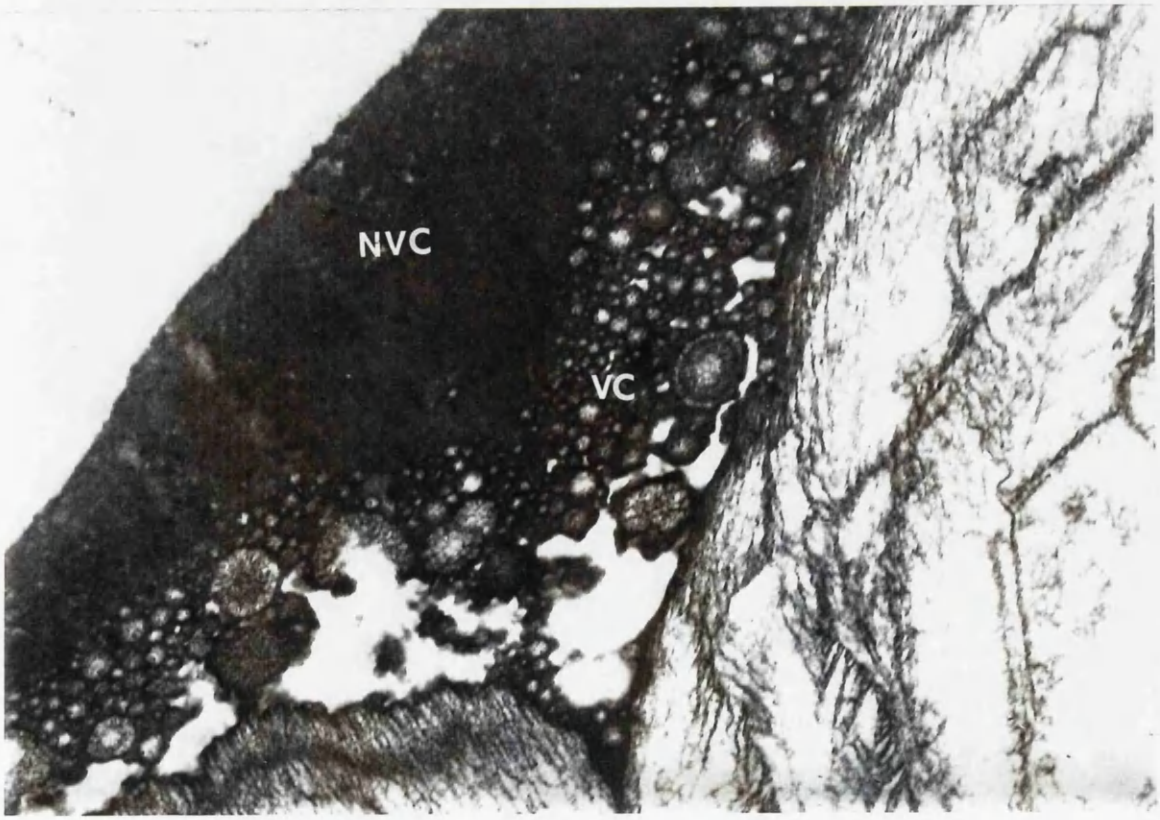


Figure 29: Cuticle showing two distinct layers, the VC and NVC. The inner VC shows large vesicles containing granular material which appear to be absent in the outer NVC (x40000).

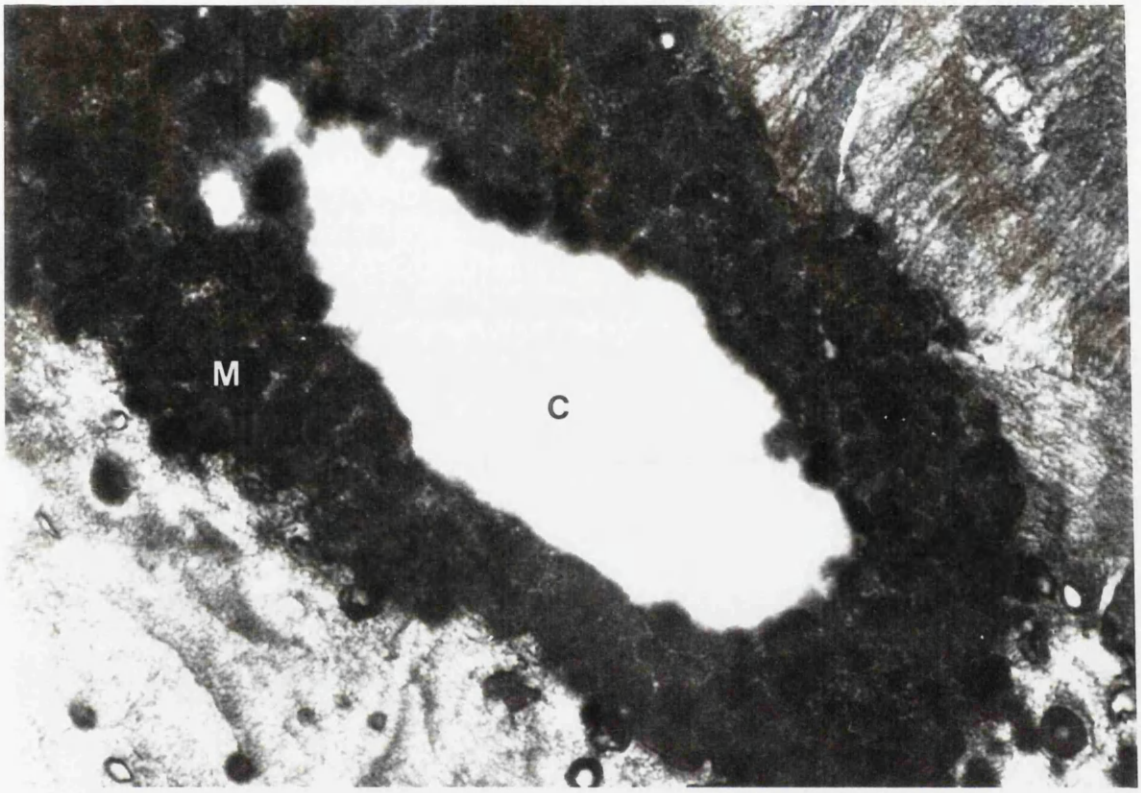


Figure 30: High power micrograph highlighting the variable electron dense core (C) and crystalline appearance of the electron dense mantle (M) (x200000).

CHAPTER 5 - DISSOLUTION AND EXTRACTION OF ORGANIC MATRIX PROTEINS FROM GOOD AND POOR QUALITY EGGSHELLS USING SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

5. DISSOLUTION AND EXTRACTION OF ORGANIC MATRIX PROTEINS FROM GOOD AND POOR QUALITY EGGSHELLS USING SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

5.1. INTRODUCTION.

As outlined in section **1.2.4.**, the organic matrix consists of two components, namely the soluble and insoluble fractions. During the process of biomineralization soluble matrix proteins may become overgrown by mineral and trapped in the crystal. This would influence the strength as hypothesised by Silyn-Roberts and Sharp (1986) and is borne out by the results of the previous chapter. The insoluble matrix may form a structural framework which is subsequently covered by a layer of the more reactive soluble matrix. This "sandwich theory" of soluble and insoluble matrix components was originally suggested by Degens (1976; cited by Simkiss and Wilbur, 1989) and has subsequently been supported by ultrastructural and biochemical studies (Simkiss and Wilbur, 1989). By virtue of their very nature, the soluble matrix proteins have attracted most attention from researchers to date. In the fully calcified shell, soluble matrix proteins are primarily intramineral, whilst the insoluble proteins are mostly extramineral (Gautron *et al.*, 1995 in press).

The isolation and identification of soluble matrix proteins governing the pattern of mineralization of the avian eggshell using SDS-PAGE has been extensively investigated by Gautron (1994) and Gautron *et al.*, (1995 in press). These authors identified three main protein bands in the soluble matrix corresponding to 80, 43 and 17kDa. Using a similar methodology, Hincke *et al.*, (1992) resolved a comparable soluble matrix protein profile from the mineralized layers of the eggshell and also demonstrated the presence of a 43kDa protein in the insoluble matrix fraction. Hincke *et al.*, (1993) isolated and purified the 17kDa protein naming it ovocleidin-17 (OC-17) [ovum, Latin-egg; kleidoun, Greek, to lock in. See **1.2.4.**]. Hincke *et al.*, (1995) have subsequently described the amino acid sequence of this matrix protein.

The dissociation of the shell membranes from the calcified shell by manual stripping, as employed by Gautron (1994), has been shown by scanning electron microscopy to be ineffective (Reid, 1983). Likewise, their dissociation by acid or caustic etching, as described by Hincke *et al.,* (1992) is open to question. Indeed, Reid (1983) showed that acid etching erodes the mammillary layer to various degrees depending on the length of exposure to the acid medium. In a series of micrographs the latter author demonstrated a pattern approximating to that observed in the hatched egg, with complete removal of the mammillary caps. As a result of these observations, one must question whether the proteins extracted by Gautron (1994) and Gautron *et al.,* (1995 in press) were solely derived from the shell matrix. One must also question whether the methodology utilised by Hincke *et al.,* (1992) inadvertently removed some of the matrix proteins along with the shell membranes? In addition, the results of Hincke *et al.,* (1992), Gautron (1994) and Gautron *et al.,* (1995 in press) relate to eggshells of unknown provenance and differences in the matrix protein profiles of eggshells varying in quality were not investigated.

The aim of the current study was therefore to establish protein profiles for soluble and insoluble matrix extracted from eggshells which had first been subjected to the process of plasma etching in order to ensure complete removal of the shell membranes, without interference to the underlying mammillary structure. A comparison of the profiles of eggshells classified as good and poor quality, according to total ultrastructural score, is then described in order to establish whether or not ultrastructural quality reflects the nature and incidence of matrix proteins located within the shell.

5.2. MATERIALS AND METHODS.

The shells used in this study were selected from the commercial battery system described in chapter **2** and classified, with reference to total ultrastructural score, as good or poor quality. One group was plasma etched see (**2.3.3.** and Reid, 1983) and the other left with membranes attached (nonetched). The resulting samples were identified as follows:

GM Good quality + membranes (nonetched)
GE Good quality etched
PM Poor quality + membranes (nonetched)
PE Poor quality etched

The shells were ground into a coarse powder by hand, then placed in a SPEX 6700 freezer mill with liquid nitrogen. The low temperature prevents denaturation of the proteins present and allows for very fine grinding, thereby increasing the exposed surface area.

In selecting samples for analysis, the test required a total of 10 shells per sample. It must be remembered that total ultrastructural score reflects a summation of a variety of structural variants. In these analyses the eggs were pooled, so there was no way of knowing which faults predominated in the poor quality shells. The results herein are therefore interpreted with reference to the inclusion of a broad spectrum of ultrastructural variation in the shells classified as poor quality.

5.2.1. EXTRACTION OF SOLUBLE EXTRAMINERAL PROTEINS USING GUANIDINE HCl (GuHCl).

The following procedure is summarised in Figure 31. Each sample was placed in a beaker with 100 ml of a solution containing 4 M GuHCl with protease inhibitors (P.I.) at a temperature of 4°C and continually stirred with a magnetic stirrer for 24 hours. The final composition of the solution (GuHCl + P. I.) was as follows:

Guanidine HCl	4 M
Tris base	50 mM
Benzamidin-HCl	2.5mM
γ-amino-n-caproïc acid	50 mM
N-ethylmaleimid	0.5mM
Phenyl methyl sulphonyl fluoride	1mM

The pH was adjusted to 7.4 with HCl.

The samples were then centrifuged for 10 minutes at 4000xg. The supernatant was placed in a glass bottle and stored in a deep freeze while the pellet was again placed in 100 ml of fresh GuHCl and the process repeated. The resulting supernatant was added to the original giving a total volume of 200 ml. This solution formed the dilute soluble extramineral fraction (see Figure 31). In theory, this fraction contains soluble proteins from any remaining shell membranes and the cuticle, and should not contain intracrystalline matrix proteins (Gautron, 1994).

5.2.2. EXTRACTION OF THE SOLUBLE INTRAMINERAL PROTEINS USING EDTA.

The pellet resulting from the previous process was placed in a beaker with 300 ml of the following solution.

P.I.	as before
EDTA	0.5 M
Tris base	50 mM

The pH was adjusted to 7.4 with HCl.

This mixture was stored at 4°C and stirred continuously for 4 hours. It was subsequently placed in a Spectrapor 7 dialysis bag (molecular weight cut off 3.5kDa) and immersed in 4 litres of the EDTA solution until complete demineralization had occurred (21 days at 4°C). The EDTA solution was replenished periodically during this time.

The intramineral proteins now exposed were solubilised using GuHCl. Each dialysis bag was placed in 4 litres GuHCl solution at 4°C for 4 days with one change of solution. The contents of each bag were then emptied and centrifuged for 10 minutes at 2500xg. The supernatant was separated from the pellet and both placed in a deep freeze. The resulting pellet consisted of the insoluble 1 fraction of the eggshell matrix while the supernatant was referred to as the dilute soluble intramineral fraction of the eggshell matrix (see Figure 31).

5.2.3. REMOVAL OF SALTS FROM THE DILUTE SOLUBLE EXTRA AND INTRAMINERAL FRACTIONS.

The dilute soluble extramineral (**5.2.1.**) and intramineral fractions (**5.2.2.**) were placed in a dialysis bag (as before) and immersed in the following solution for 24 hours at 4°C.

P.I. + Sodium acetate 0.5M, Tris 50mM, the pH being adjusted to 7.4 with HCl.

After this time each bag was immersed in demineralized water for 1 week, with the water being changed on a regular basis.

5.2.4. CONCENTRATION OF THE DILUTE SOLUBLE EXTRA AND INTRAMINERAL FRACTIONS.

[i] **Extramineral.** The sample was placed in a 200 ml Amicon ultrafiltration cell fitted with an ultrafiltration membrane (cut off 5kDa) and filtered using a compressor at 75 PSI, until only 20 ml remained. The ultrafiltrate was subsequently discarded. 100 ml demineralized water was then added and the process repeated. The final solution being henceforth referred to as the soluble extramineral fraction (see Figure 31).

[ii] **Intramineral.** Ultrafiltration was carried out as above after which time the intramineral fractions were centrifuged at 2000xg for 10 minutes. The resulting pellet constituted the insoluble 2 fraction, while the supernatant contained the remaining soluble intramineral fraction (see Figure 31).

5.2.5. SPECTROPHOTOMETRIC ANALYSIS.

The amount of protein in each fraction was measured by spectrophotometric analysis using Coomassie blue protein makers (after Bradford, 1976).

A calibration curve was prepared with ovalbumin (in place of bovine serum albumin) as standard (Titres used=0, 2, 5.5, 7.5, 10, 15, 20, 25, 30, 50 µg/ml). The optical density of the standard was compared to the unknown samples and the protein content of each fraction determined. This was carried out to ensure that enough protein was present in each fraction to allow for gel electrophoresis to take place and to allow the determination of a standard amount of protein (40µg) for each migration.

5.2.6. GEL ELECTROPHORESIS.

SDS-PAGE slab gels were prepared according to the original procedure of Laemmli (1970) and adapted according to Hames and Rickwood (1981), as described by Gautron (1994). The protein migration was run on a Protean II, Bio-Rad discontinuous system using a dissociating buffer.

[i] Preparation of the slab gels.

The final concentration of the resolving gel was as follows:

0.1 %	SDS
0.05 %	TEMED*
0.075%	Ammonium persulphate
0.375 M	Tris-HCl pH 8.8

Variable acrylamide and bis-acrylamide concentrations (5 to 17.5% and 0.13 to 0.47% respectively).

* Biorad, N, N, N', N' tetramethylethylynediamine, commercial solution.

The final concentration of the stacking gel was as follows:

3.75%	Acrylamide
0.1%	Bis-acrylamide
0.1%	SDS
0.0125 %	Tris HCl pH 6.8
0.075%	Ammonium persulphate
0.075 %	TEMED

Stacking and resolving gels were prepared by mixing stock solutions in different proportions, as reported in Table 16 (after Hames and Rickwood, 1981). First the water, bis-acrylamide solution and buffer were mixed. The mixtures were then degassed for 1-5 minutes using a water pump and the correct volume of SDS, ammonium persulphate and TEMED solution added to the medium. The preparations were then quickly poured between the gel plates.

The resolving linear gradient gel was obtained by mixing two gel preparations. The light solution (5% acrylamide) was poured into the reservoir chamber of the gradient former, followed by the heavy solution (17.5% acrylamide). The valve stem was quickly opened and the linear gradient gel poured between plates with 0.75 mm spacers. After total polymerisation (~I hour) the remaining space between the gel plates was filled with stacking gel mixture. The comb was inserted immediately into the stacking gel and removed after polymerisation to expose the sample wells, which were then rinsed with reservoir buffer (0.025M Tris, 0.192M glycine, pH 8.3).

[ii] Sample preparation, loading and electrophoresis.

Samples and molecular weight markers were dissolved in a solution composed of Tris-HCl 0.0625M (pH 6.8), 2% SDS, 0.5% ß-mercaptoethanol, 5% glycerol and 0.375% bromophenol blue then heated in a boiling water bath for five minutes, prior to electrophoresis.

The lower and upper reservoirs of the electrophoresis apparatus were filled with reservoir buffer. The samples were then carefully loaded into the wells using a micropipette and the electrophoresis was run overnight with a constant 15 mA current.

[iii] Gel staining.

Gels were fixed for 1 hour in 25% propanol-2. They were then stained with a Coomassie blue solution (0.1% Coomassie Blue R-250, water:methanol:glacial acetic acid: 60:30:10 by volume) and de-stained in water:methanol:glacial acetic acid: 70:25:5 by volume.

5.3. RESULTS.

5.3.1. NONETCHED SHELLS.

The protein profiles of the nonetched shells are presented in Figure 32.

The soluble intramineral fractions from good and poor nonetched shells showed almost identical profiles with bands appearing at 17, 21, 24, 35, 43, 66 and 80kDa.

The soluble extramineral fractions from good and poor nonetched shells showed different profiles. Whilst both showed bands at 43 and 80kDa, the good shells also displayed bands around 17 and 35kDa. The latter appear to be absent in the poor quality shells.

The insoluble 1 fraction from the good and poor nonetched shells showed near identical profiles with bands at 17, 21, 24, 31, 43 (strong) 66, 80 and 120kDa (strong).

There was insufficient protein present in the insoluble 2 fraction to allow for electrophoresis.

5.3.2. ETCHED SHELLS.

The protein profiles of the etched shells are presented in Figure 33.

The soluble intramineral fractions from the good and poor etched shells showed similar profiles, with a strong band at 17kDa and much weaker bands at 21, 24, 35, 43, 66 and 80kDa.

The soluble extramineral fractions of good and poor etched shells both showed bands at 15, 17 and 19kDa, with no other significant bands being present.

The insoluble 1 fractions from the good and poor etched shells showed similar profiles with bands at 17, 21, 24, 31, 43 and 120kDa, while the good and poor shells from the insoluble 2 fraction both revealed strong banding at 17kDa with much lighter banding at 21, 24, 31, 43, 66 and 80kDa.

5.4. DISCUSSION.

The soluble extramineral fractions from good and poor quality nonetched shells (Figure 32) showed a difference in terms of their profiles. Bands present at 17kDa (likely to be OC-17) and 35kDa were absent in the poor quality shell profiles. The corresponding fractions extracted from etched shells (Figure 33), however, had identical profiles to one another, indicating perhaps that the former difference may be membrane associated.

The eggshells used in this study were selected on the basis of total ultrastructural score, with poor shells exhibiting a higher incidence of variants at the mammillary level than good quality shells. As stated in section **5.2.**, it was not possible to identify which variants were prevalent in the poor quality shells. It is therefore hypothesised that the nature of the faults in the etched and unetched poor quality shells was not the same (and that removal of the membranes prior to dissolution resulted in a more accurate representation of the proteins which constitute the extramineral fraction of the organic matrix).

The soluble extramineral profile from the poor quality nonetched shells (lacking bands at 17 and 35kDa) suggests that in these samples the process of nucleation has been sporadic, with abnormal bonding occurring between the membrane fibres and the mammillary caps. The most likely explanation for this phenomenon in ultrastructural terms is changed membrane, which occurs as the result of chemical changes within the isthmus leading to the formation of a sulphur rich membrane layer (see **1.2.5.**). This adheres tightly to the mammillary caps (Solomon, 1993). It is therefore suggested that the presence of such changed membrane has prevented the complete dissolution of the extramineral fractions in the nonetched poor quality shells. Alternatively, it may have resulted in fracturing at the cap/cone interface during manual stripping, causing the latter to be lost as described by Reid (1983), prior to solubilisation.

The presence of a 17kDa protein (OC-17) in the soluble extramineral profiles of etched poor quality shell indicates a preponderance of faults associated with the inter-mammillary spaces ie. aragonite and cubics. These faults would not interfere with the bonding between mammillary caps and membrane fibres, allowing for their complete dissociation and identification of matrix material following gel electrophoresis.

The fact that the 35, 43, and 80kDa proteins in the good quality soluble extramineral fraction are removed by plasma etching indicates that these proteins may also be mainly membrane associated. Also, no influence was apparent regarding the low molecular weight proteins. Thus, the process of plasma etching does not appear to be exerting an influence on the mammillary cores, this being in agreement with the findings of Reid (1983).

Hincke *et al.,* (1993) reported the presence a 43kDa protein in the insoluble fraction of eggshell preparations. Gautron (1994) studied only the soluble fraction and also demonstrated the presence of a protein of approximately 43kDa molecular weight. The results of the current study indicate the presence of a 43kDa protein in both the soluble and insoluble fractions. Hincke (1995) has identified the 43kDa protein as ovalbumin.

The presence of a 17kDa protein (OC-17) in both the soluble extramineral etched and nonetched shells (with the exception of poor quality nonetched) suggests that this protein is associated with both the membranes and mammillary cores. It may also be associated with proteins of the palisade layer, not directly included in the mineral phase, which contribute to the extramineral fraction (Gautron, *pers comm*).

The results of the current thesis clearly show the presence of a 17kDa protein in all but the poor quality extramineral nonetched shell migration (Figure 32). These findings are not in agreement with Gautron (1994) who has shown that OC-17 is present only in the intramineral fraction of the eggshell matrix extract. Gautron *et al.,* (1995 in press) have described the presence of a prominent band of 15kDa confined to the extramineral fraction which also appears in the current etched extramineral migrations.

The intramineral fractions from all the samples showed almost identical profiles. However, with the exception of the 17kDa band (OC-17), the profiles from the etched samples are much weaker than those from their nonetched counterparts. As the protein content in each well was uniform (40μg) this suggests that the amount of the relative proteins present in the etched samples, apart from OC-17, was considerably less. Such an interpretation is somewhat subjective and an accurate quantitative assessment of the various protein concentrations was outwith the remit of this dissertation. Nevertheless the results obtained do indicate that either the membranes and intramineral

matrix share a common molecular composition or alternatively, that small amounts of membrane associated proteins have in some way remained associated with the intramineral matrix, despite the process of plasma etching. In either case, it must be that the profiles obtained from the plasma etched samples give a more accurate indication of the intramineral fraction of the shells under investigation than was demonstrated by either Hincke *et al.*, (1992) or Gautron (1994).

The nonetched insoluble 1 and etched insoluble 1 and 2 fractions all show very similar profiles. Differences do occur in terms of the larger molecular proteins, probably due to an incomplete dissociation of the supramolecular matrix aggregate. Many of the bands present can also be found in the corresponding intramineral fractions indicating a substantial degree of similarity in composition between the two and perhaps, as outlined earlier, the shell membranes.

The results obtained in this chapter indicate that one must question the the definition given by Gautron (1994), in which soluble extramineral proteins are reported to consist of membrane and cuticular components only. Indeed, Gautron and Nys (*pers comm*) have recently suggested that they may also include proteins associated with the palisade layer, not closely associated with the mineral phase. The current research (and the results described in chapter **7**) suggests that the soluble extramineral fraction of the eggshell matrix also contains proteins associated with the organic cores which remain *in situ* after plasma etching, as well as matrix subunits such as OC-17.

Stock Solution	Stacking gel (ammonium persulphate as catalyst)	Stacking gel (riboflavin as catalyst)	Final acrylamide concentration in resolving gel(%)[a]							Reservoir buffer[b]
			20.0	17.5	15.0	12.5	10.0	7.5	5.0	
Acrylamide-bisacrylamide (30:0:8)	2.5	2.5	20.0	17.5	15.0	12.5	10.0	7.5	5.0	-
Stacking gel buffer stock[c]	5.0	5.0	-	-	-	-	-	-	-	-
Resolving gel buffer stock[d]	-	-	3.75	3.75	3.75	3.75	3.75	3.75	3.75	-
Reservoir buffer stock[e]	-	-	-	-	-	-	-	-	-	100
10% SDS	0.2	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	-
1.5% ammonium persulphate	1.0	-	1.5	1.5	1.5	1.5	1.5	1.5	1.5	-
0.004% riboflavin	-	2.5	-	-	-	-	-	-	-	
Water	11.3	9.8	4.45	6.95	9.45	11.95	14.45	16.95	19.45	900
TEMED	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	-

Final concentration of buffers: stacking gel; 0.125M Tris-HCl, pH 6.8
resolving gel; 0.375M Tris-HCl, pH 8.8
reservoir buffer; 0.025M Tris, 0.192M glycine, pH 8.3

a The columns represent volumes (ml) of the various reagents required to make 30 ml of gel mixture.

b Volumes (ml) of reagents required to make 1 litre of reservoir buffer.

c Stacking gel buffer stock: 0.5M Tris-HCl (pH 6.8); 6.0g Tris is dissolved in 40ml water, titrated to pH 6.8 with 1M HCl (~48ml), and brought to 100ml final volume with water. The solution is filtered through Whatman No.1 filter paper and stored at 4oC.

d Resolving gel buffer stock: 3.0M Tris-HCl (pH 8.8); 36.3g Tris and 48.0ml 1M HCl are mixed and brought to 100ml final volume with water. This buffer is then filtered through Whatman No.1 filter paper and stored at 4°C.

e Reservoir buffer stock: 0.25M Tris, 1.92M glycine, 1% SDS (pH 8.3); 30.3g Tris, 144.0g glycine, and 10.0g SDS are dissolved in and made to 1 litre with water. The solution is stored at 4°C.

Table 16. Method for Gel preparation using the SDS-discontinuous buffer system

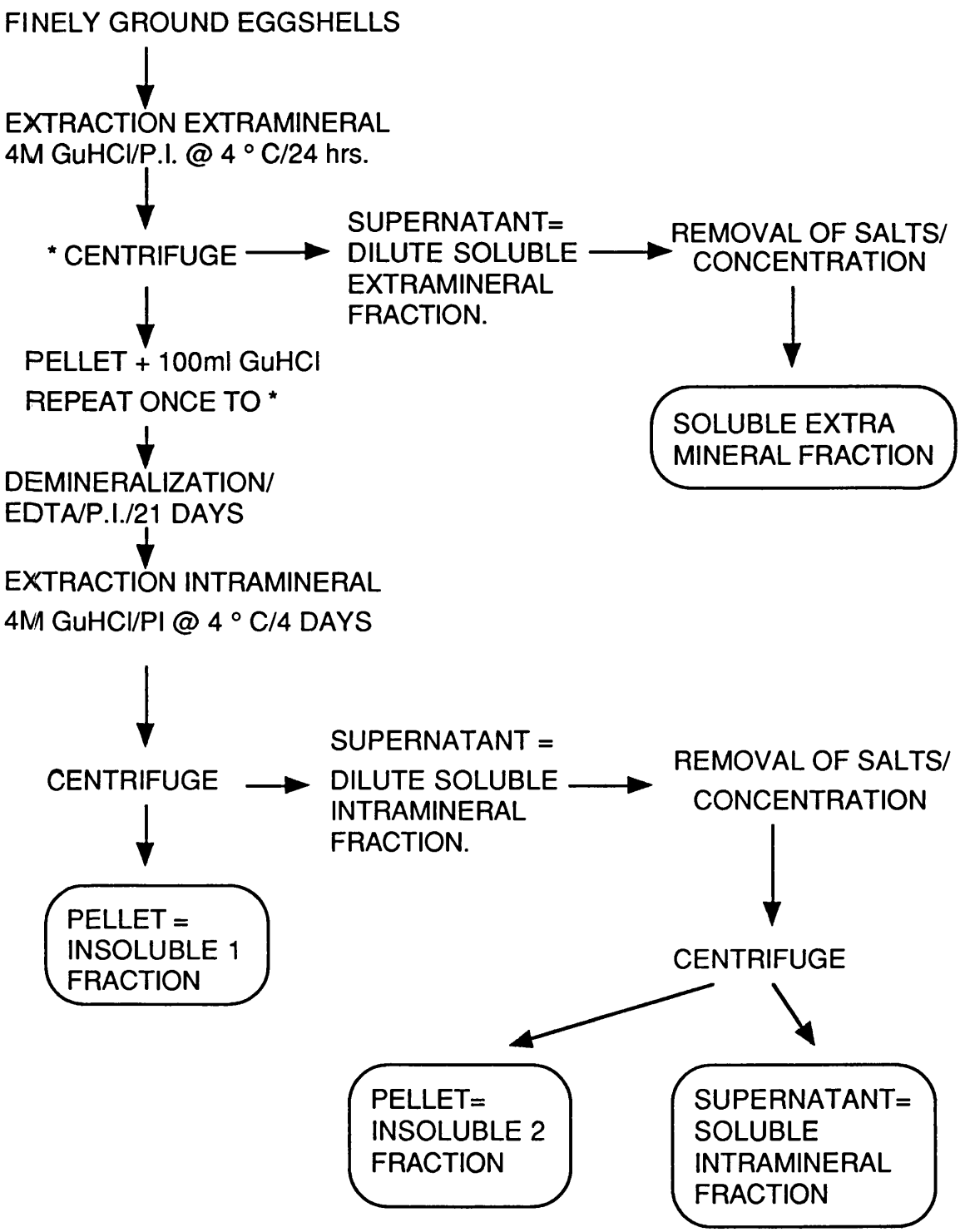


Figure 31. Schematic representation of the dissolution and extraction of organic matrix proteins.

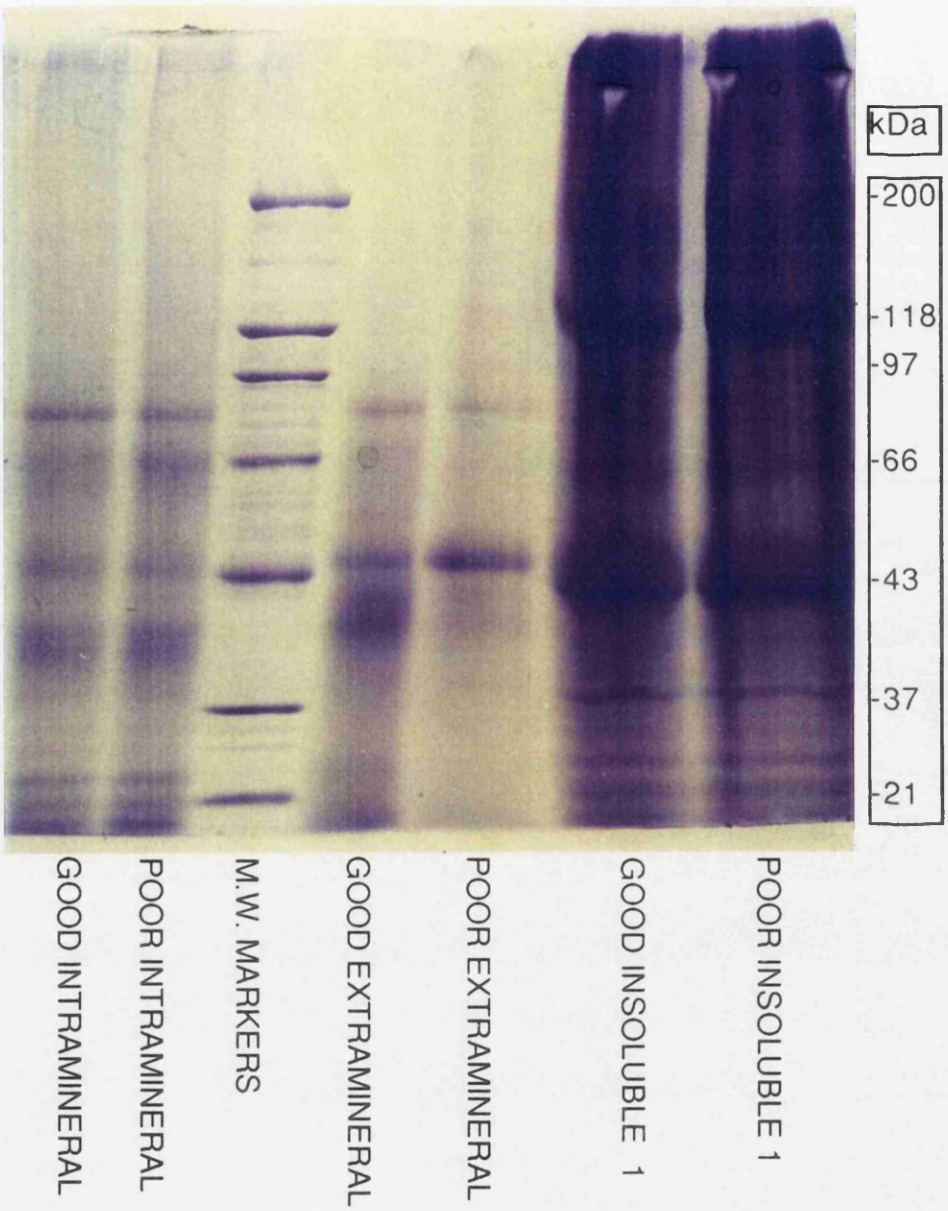


Figure 32. Gradient gel electrophoresis migration plate showing soluble intramineral, extramineral and insoluble 1 matrix profiles from good and poor quality nonetched shells.

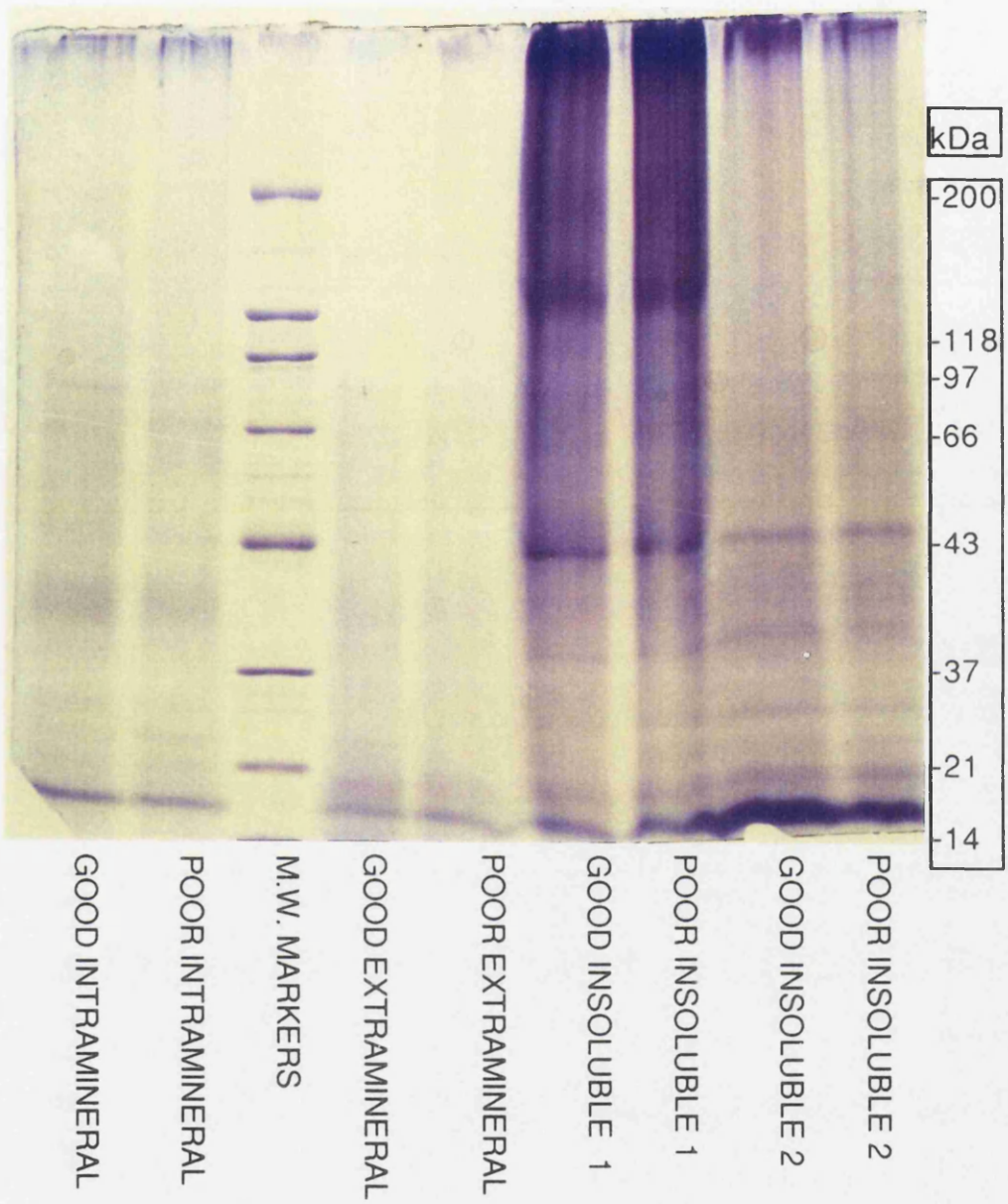


Figure 33. Gradient gel electrophoresis migration plate showing soluble intramineral, extramineral and insoluble 1 matrix profiles from good and poor quality plasma etched shells.

CHAPTER 6 - THE INFLUENCE OF ORGANIC MATRIX PROTEINS FROM GOOD AND POOR QUALITY EGGSHELLS ON THE PRECIPITATION AND MORPHOLOGY OF CALCIUM CARBONATE CRYSTALS *IN VITRO*.

6. THE INFLUENCE OF ORGANIC MATRIX PROTEINS FROM GOOD AND POOR QUALITY EGGSHELLS ON THE PRECIPITATION AND MORPHOLOGY OF CALCIUM CARBONATE CRYSTALS *IN VITRO*.

6.1. INTRODUCTION.

The eggshell of the domestic fowl (*Gallus domesticus*) is a natural biopolymer and mineral composite. The biomineralization process occurs very rapidly, with approximately 5g of calcium carbonate being deposited during a 24 hour ovulation cycle. Its organisation is unique, in that large (~200μm) columns of seemingly single crystal calcite are laid down perpendicular to the underlying eggshell membranes (Wu *et al.*, 1994). The eggshell has a complex, multi-compartmental, micro-structure in which a variety of organic matrix protein macromolecules are combined in several structural regions (Arias *et al.*, 1993; **1.2.4.**). These matrix proteins are thought to influence the material and mechanical properties of the eggshell by forming a strengthening fibrous framework, similar to the steel used in reinforced concrete (Silyn-Roberts and Sharp, 1986). The results outlined in chapter **4** lend support to this hypothesis.

In addition to this role, the organic matrix has been implicated as the causal agent of a number of the characteristic features occurring in calcitic biogenesis. The eggshell is formed by a mechanism in which calcium is complexed with carbonate ions and nucleation sites are initially provided by the shell membranes (Solomon, 1993). Unlike spontaneous crystal growth, it involves regulated nucleation and growth of an inorganic phase onto or within an organic matrix protein infrastructure. Nucleation, in general, represents an activation energy barrier to the spontaneous formation of a solid phase from a supersaturated solution. This kinetic boundary may be sufficient to offset the thermodynamic drive towards precipitation, resulting in the formation of a metastable solution. The solid phase will continue to grow only if the energy required to form the new interface is overcome by the formation of bonds in the bulk of the aggregate. In biological environments, the activation energy for nucleation can be reduced by lowering the interfacial energy and/or by increasing the degree of supersaturation. Interfacial energies can be lowered by the presence of organic surfaces at the nucleation site and by confining the mineralizing reaction to diffusion-limited sites, such as membrane bound

extracellular compartments (or intracellular vesicles), where selective ion transport to and from the mineralized zone can occur (Mann, 1986, 1988, 1990; Williams, 1989). The eggshell membranes and organic matrix meet such requirements. Concentration of inorganic ions causes an electrostatic accumulation and helps induce the supersaturation necessary for nucleation to occur. This is the basis of the ionotropic theory for mineral deposition (Mann, 1988, 1990).

Subsequent shell growth is also thought to be influenced by the presence of the organic matrix. Thus, matrix proteins may actively initiate crystal growth or alternatively, they may inhibit growth on certain crystal planes, facilitating ordered rather than spontaneous crystal formation (Addadi and Weiner, 1989, 1992; Wheeler and Sikes, 1989; Hincke *et al.,* 1992; Albeck *et al.,* 1993 and Gautron *et al.,* 1993). Such a process involves structural correspondence, or epitaxy, at the organic-inorganic interface. Epitaxy is defined as a mechanism by which two chemically and/or structurally different crystalline phases grow together. This suggests a one to one relationship between the functional groups of the matrix and the crystallographic lattice dimensions of a specific crystal phase in the overlying mineral phase (Weiner, 1984; Krampitz and Grasser, 1988; Mann, 1988, 1989, 1990; Addadi and Weiner, 1989, 1992; Addadi, 1993). These stereochemical requirements depend essentially on numerological relationships in one or two dimensions at the matrix-mineral interface, although crystal structure ultimately depends upon a network of interactions between single molecules or ions, repeated in three dimensions (Addadi and Weiner, 1989; Lowenstam and Weiner, 1989; Mann, 1993). Thus, in simple terms, complementarity at the nucleation ion clusters can be considered analogous to the case of enzyme substrates, co-factors and antigens, whilst matrix nucleation centres can be considered analogous to receptor sites in enzymes, genes and antibodies.

In summary, five functional properties have been attributed to the organic matrix to date viz. a reinforcing network, crystal nucleation, crystal orientation, control of crystal morphology and control of polymorphic phase.

Most early research into the role of matrix in biomineralization involved the study of material obtained from the shells of molluscs and bivalves. Thus, using soluble matrix proteins extracted from mollusc shell, Wheeler and Sikes (1984, 1989), Addadi and Weiner (1985) and Albeck *et al.,* (1993) demonstrated inhibition of both nucleation and growth of calcium carbonate crystals *in vitro.* These authors also demonstrated that crystals grown in the presence of matrix proteins had a morphology distinct from those grown without matrix.

Nys and Gautron (1987) demonstrated a similar inhibition using eggshell cuticle extract and Gautron and Nys (1993) report that dialysed uterine fluid (irrespective of the stage of shell formation) and total uterine fluid (from the end of shell formation) have the same effect. Arias *et al.,* (1992, 1993), Gautron *et al.,* (1993, 1995 in press) and Wu *et al.,* (1992, 1994) have also reported that soluble matrix proteins extracted from the eggshell of the domestic fowl delay the precipitation of calcium carbonate *in vitro* and influence crystal morphology, in a manner similar to that described by Wheeler and Sikes (1984, 1989), Addadi and Weiner (1985) and Albeck *et al.,* (1993). The current investigation was designed to develop the hypotheses of Arias *et al.,* (1993) and Gautron *et al.*, (1993, 1995 in press), by examining the influence of soluble matrix proteins, from shells classified as good or poor in ultrastructural terms, on *in vitro* calcium carbonate precipitation. It was hoped that biochemical characterisation of the molecular constituents of the organic matrices from these samples (see chapter **5**) could lead to the identification of a more specific inhibition process than outlined by previous researchers.

Wu *et al.,* (1994) also described the influence that the eggshell membranes have in controlling the nucleation process. These authors used the demineralized eggshell membranes as the platform upon which calcite crystallization was initiated, after which additional organic species were added to study how these materials might modulate the mineralization. The inner and outer eggshell membranes are composed primarily of a network of collagenous fibres encapsulated in a continuous mantle of proteoglycans and other macromolecules which can influence the biomineralization process (see **1.2.4.**). In view of these observations, it was considered pertinent to investigate the effect of membrane removal by means of plasma etching for both categories of shell, prior to dissolution of the soluble matrix proteins.

6.2. MATERIALS AND METHODS.

The matrix extracts used in this study were liophylised soluble extra and intramineral fractions obtained from etched and nonetched shells classed as good and poor quality in ultrastructural terms, as outlined in chapter **5**. Following rehydration with double deionised water the concentration of protein in µg/ml for each sample was determined using a spectrophotometric analysis using Coomassie blue protein markers (after Bradford, 1976). The results are presented in Table 17.

Table 17. Matrix sample identification and protein concentration.

Extramineral.		Protein content (µg/ml).
Good quality, nonetched	(GN)	1534
Good quality, etched	(GE)	810
Poor quality, nonetched	(PN)	702
Poor quality, etched	(PE)	650

Intramineral.		Protein content (µg/ml).
Good quality, nonetched	(GN)	1346
Good quality, etched	(GE)	920
Poor quality, nonetched	(PN)	1795
Poor quality, etched	(PE)	534

6.2.1. *IN VITRO* CRYSTALLIZATION.

Calcium chloride-hydrate was placed in an oven at 100°C over night (2-3 hours minimum) to remove any adsorbed water. A 7.5mM solution of calcium chloride was then prepared.

Small glass coverslips were cleaned with a mixture of potassium dichromate and sulphuric acid. Following this, they were rinsed with double deionised water, alcohol and then blotted dry.

Each coverslip was subsequently placed in a polystyrene Corning cell well and 0.75ml calcium chloride solution added. Increasing amounts of matrix extract were added to consecutive wells (0µl, 25µl, 50µl, 75 µl, 100 µl)

then made up to the same volume (0.85ml) using double deionised water. In the controls only double deionised water was used. The concentration of protein present in each well in µg/ml could then be calculated, the results are presented in Table 18.

Table 18. Matrix concentration in each sample well (µg/ml).

	Volume of extramineral extract (µl).				
Conc. (µg/µl)	**0**	**25**	**50**	**75**	**100**
GN	0	45	90	135	180
GE	0	24	48	71	95
PN	0	20	41	61	82
PE	0	16	32	48	64

	Volume of intramineral extract (µl).				
Conc. (µg/µl)	**0**	**25**	**50**	**75**	**100**
GN	0	40	80	120	160
GE	0	27	54	81	108
PN	0	53	106	159	212
PE	0	13	26	39	52

The wells were subsequently covered with aluminium foil, each chamber pierced 2 or 3 times with a needle, then placed in a dessicator saturated with ammonium carbonate vapour for 2-4 days until precipitation of calcium carbonate had occurred onto the coverslips. Each coverslip was then washed gently with double deionised water, left to air dry, mounted onto a stub using conductive silver paint and sputter coated with gold/palladium for 4 minutes prior to viewing with a Philips 501B scanning electron microscope at 15Kv. The experiment was replicated twice.

6.3. RESULTS AND DISCUSSION.

The crystals obtained from the control wells were all perfect single rhombohedra with sharp angles and smooth sides, typical of freely growing calcite (see Figure 34). These observations support the findings of Wheeler and Sikes (1984, 1989), Addadi and Weiner, (1985), Albeck *et al.*, (1993), Arias *et al.*, (1993) and Gautron *et al.*, (1993, 1995 in press).

In the current research, both the etched and nonetched extramineral matrix proteins influenced the precipitation and morphology of calcium carbonate crystals formed *in vitro*. The effect was much more pronounced in nonetched shells and in both cases was dose dependent (see figures 35-38). These results are contrary to the findings of Gautron *et al.*, (1993, 1995 in press) who, after manual removal of the shell membranes, found soluble extramineral matrix proteins had no effect on crystal formation *in vitro*. According to Gautron (1994), the soluble extramineral fraction consists of membrane and cuticular components only, although Gautron and Nys (*pers comm*) have since suggested that it may also contain molecules associated with the palisade layer, not closely associated with the mineral phase. Evidence lending support to this hypothesis has been provided by the results obtained in chapter **5**, which indicate that the extramineral fraction does contain proteins, such as OC-17, which occur in association with the organic cores (see **5.4.**).

Existing knowledge as regards the precise biochemical composition of the eggshell membranes is incomplete (see **1.2.4.**). However, their collagenous nature has been confirmed and the proteoglycan keratan sulphate identified as a component of the outer membrane (Arias *et al.*, 1992, 1993; Arias and Fernandez, 1993, 1995). Collagen molecules are known to be intrinsic to biomineralization processes in many organisms (Cusack, *pers comm*) and proteoglycans are calcium binding polyionic molecules which can modulate phosphate and carbonate precipitation *in vitro* (Wu *et al.*, 1992, 1994; Arias and Fernandez, 1995). It does not therefore seem unreasonable that the extramineral fraction of unetched shells exerts an influence on crystal morphology and size, or indeed that complete removal of the membranes by means of plasma etching, reduces this influence as described. Also, the fact that cuticular extract is known to influence crystallization (Nys and Gautron, 1987) suggests that extramineral proteins behave in a similar manner.

In the previous chapter, SDS-PAGE (**5.3.1.**) showed that nonetched shells revealed extramineral protein bands at 43 and 80kDa. These were not observed in their etched counterparts and it may be that these higher molecular weight proteins are responsible for the observed variations in crystal morphology. Higher molecular weight organic matrix proteins would undoubtedly contain more associated anionic functional groups, which are known to exert a major influence on crystallization as a result of their calcium binding properties.

No identifiable difference was observed regarding the effect on crystal formation of extramineral proteins from good and poor quality shells, although SDS-PAGE revealed the absence of 17 and 35kDa proteins in the poor quality nonetched shells. Good and poor quality etched shells had identical low molecular weight protein profiles. This supports the theory that the higher molecular weight proteins are those exerting most effect (see section **5.3.**).

The presence of soluble intramineral matrix proteins in the experimental mileau also influenced both crystal size and morphology, irrespective of whether they were extracted from good or poor shells. This effect was again dose dependent (see Figures 39-42). These results are in agreement with the findings of Gautron *et al.,* (1993, 1995 in press). Moreover, the soluble intramineral matrix derived from good quality shells (Figures 44-45) appeared to have more of an effect on crystal morphology than that from poor shells (41-43) and the removal of membranes by plasma etching prior to extraction seemed to reduce the level of influence observed (Figures 41-45).

Gautron (1994) has described the intramineral fraction as corresponding to matrix constituents of the biocrystalline layer, with all remnants of membrane and cuticle having been removed. Constituents of the mammillary cores are also likely to be present, as they are embedded within the shell (Solomon, *pers comm*). These cores accommodate the initial nucleation sites for calcium and carbonate ions (Solomon, 1993) and contain molecules such as keratan sulphate. The palisade layer is also known to contain keratan and dermatan sulphate (Arias *et al.,* 1992, 1993; Arias and Fernandez, 1993, 1995). These molecules are known to exert an influence on calcium carbonate crystal formation *in vitro* Arias *et al.,* (1992, 1993), Wu *et al.,* (1992, 1994) and Gautron *et al.,* (1993, 1995 in press). In section **5.3.**,

matrix proteins ranging from 15-120kDa were identified. It may be that these proteins are subunits of keratan and dermatan sulphate, which have molecular weights of between 4000-19000kDa and 15000-40000kDa respectively, depending on the length of the repeating polysaccharide chain (Beeley, 1985). Some, or indeed all, of these molecules are perhaps exerting an influence on the mineralization process to varying degrees.

The results obtained in chapter **5** show that, after removal of membranes by plasma etching, the differences in ultrastructural quality were not reflected in terms of matrix protein profiles following SDS-PAGE. The findings of the current chapter, however, clearly demonstrate that the soluble intramineral matrix protein from good quality shells had more of an effect on crystal morphology than that obtained from poor quality shells. This suggests a difference in intramineral matrix composition between good and poor quality shells not identified by SDS-PAGE. As stated in the literature review, many modifications of matrix subunit proteins are possible (Krampitz, 1993). In view of this it is hypothesised that slight modifications in matrix structure, perhaps affecting shape, ionic charge and polarization are responsible for the features observed in the current study. This may be the result of changes (such as pH) in the oviducal environment during shell formation as detailed by Watt (1985, 1989). Consequently, these changes are seen to be influencing the precipitation and morphology of calcium carbonate crystals *in vitro.* These data then, support the hypotheses that matrix proteins influence the nucleation, orientation, morphology and polymorphic phase of calcium carbonate crystals during the formation of the eggshell as indicated by Hincke *et al.,* (1992) and Gautron *et al.,* (1993, 1995 in press).

The results obtained in the current investigation indicate that both the soluble extra and intramineral proteins exert a similar, dose dependent, influence on the morphology and growth of calcium carbonate crystals *in vitro*, this being contrary to the findings of Gautron *et al.,* (1993, 1995 in press) who demonstrated an effect for the intramineral fraction only. The specific structural features of the macromolecules responsible, their exact location within the shell and their precise mode/s of action remain to be elucidated. It was possible, however, using rabbit anti-OC-17 antiserum, to investigate the localisation of this molecule in selected eggshells using immunohistological technology, as outlined in the following chapter.

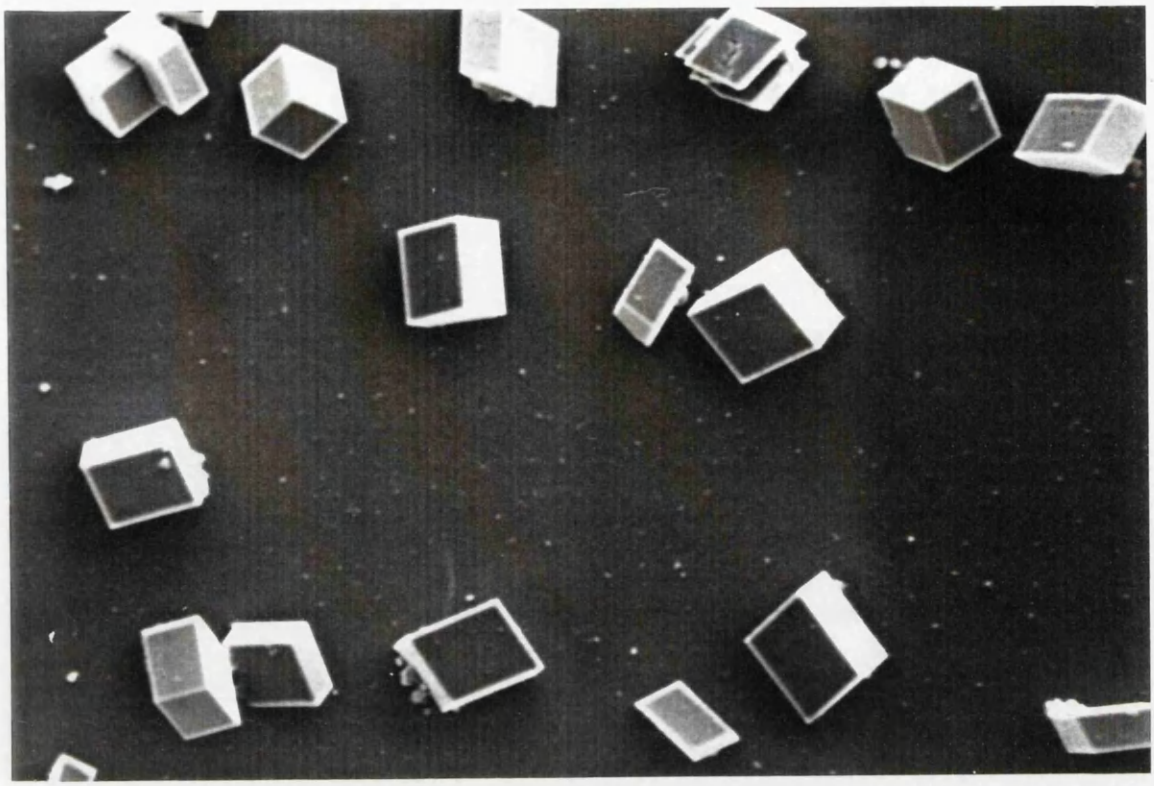


Figure 34: SEM micrograph showing rhombohedral crystals typical of freely growing calcite, taken from *in vitro* control (x720).

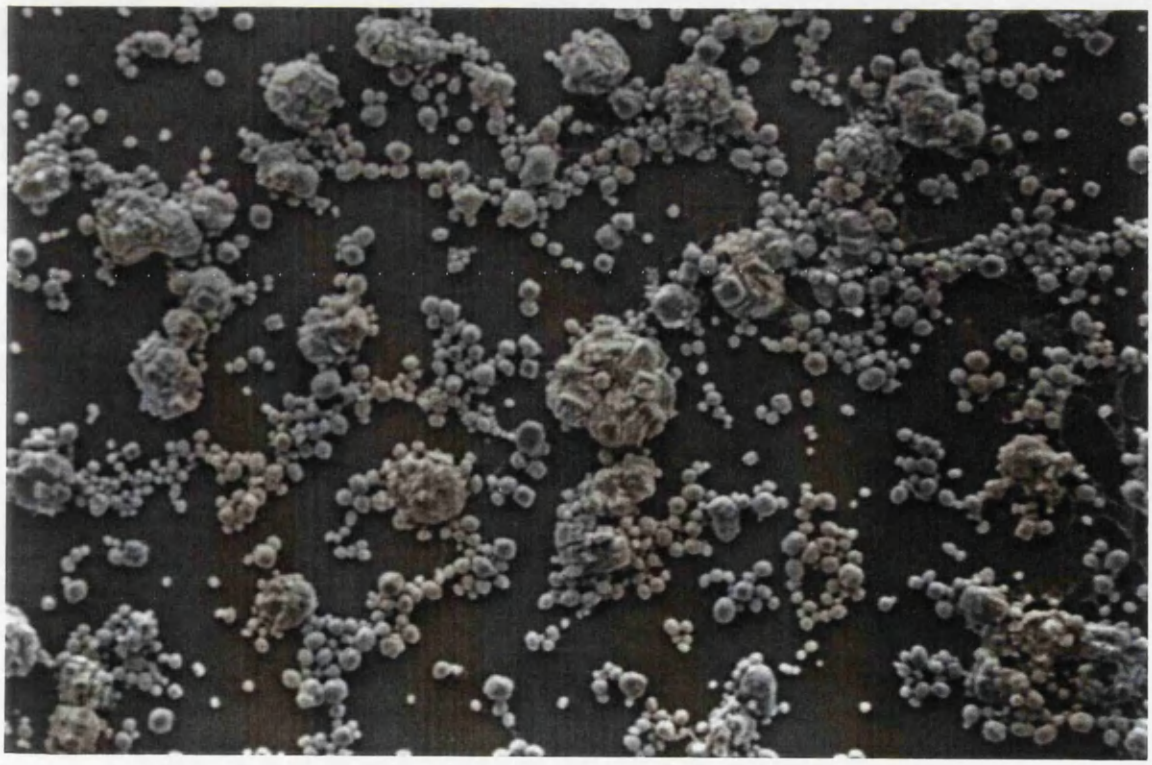


Figure 35: Addition of 20µg/ml soluble extramineral protein (nonetched) results in a definite change to crystal morphology. Crystals are clumped, vary in size and are more rounded than controls (x360).

Figure 36: The addition of 82μg/ml soluble extramineral protein (nonetched) results in more uniform, smaller crystals which appear to show a degree of orientation. Some amorphous material is also present (x360).

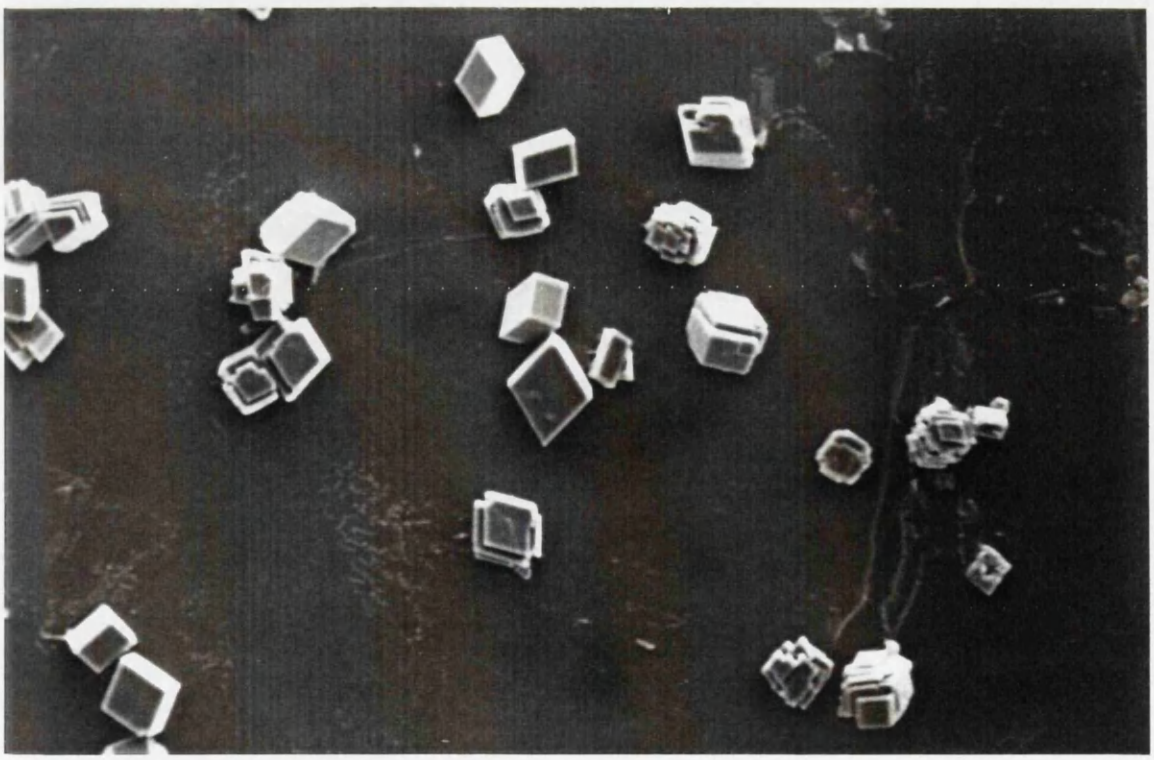


Figure 37: Calcium carbonate crystals formed in the presence of 45μg/ml soluble extramineral (etched) protein. Only a slight change from the control (Figure 34) is apparent with straight edges and angles remaining (x720).

Figure 38: The addition of 180µg/ml soluble extramineral (etched) protein results in a more pronounced change in morphology than observed in Figure 37 (x1440).

Figure 39: The presence of 13µg/ml soluble intramineral protein from poor quality, etched shells shows clumping of the crystals formed. They nevertheless have retained their rhombohedral appearance (x720).

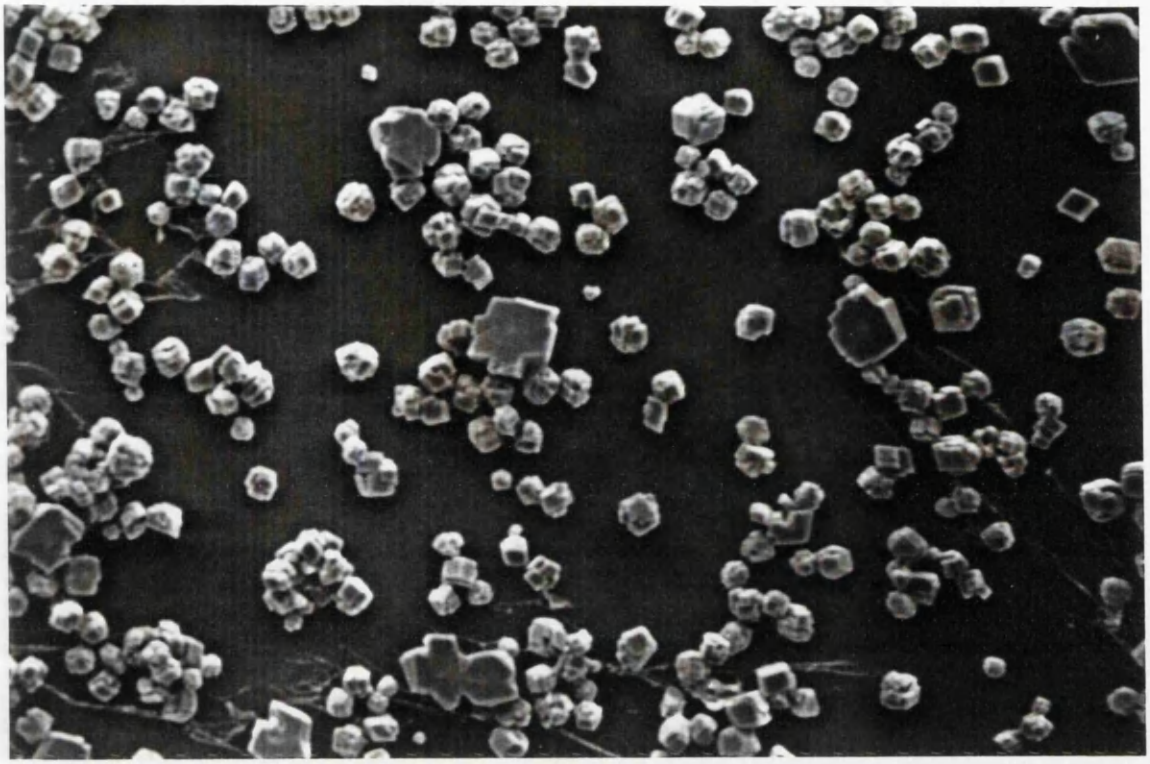


Figure 40: Addition of 26µg/ml soluble intramineral protein from poor quality, etched shells results in some disruption to crystal morphology. Crystals of various sizes are present and edges are still angular (x720).

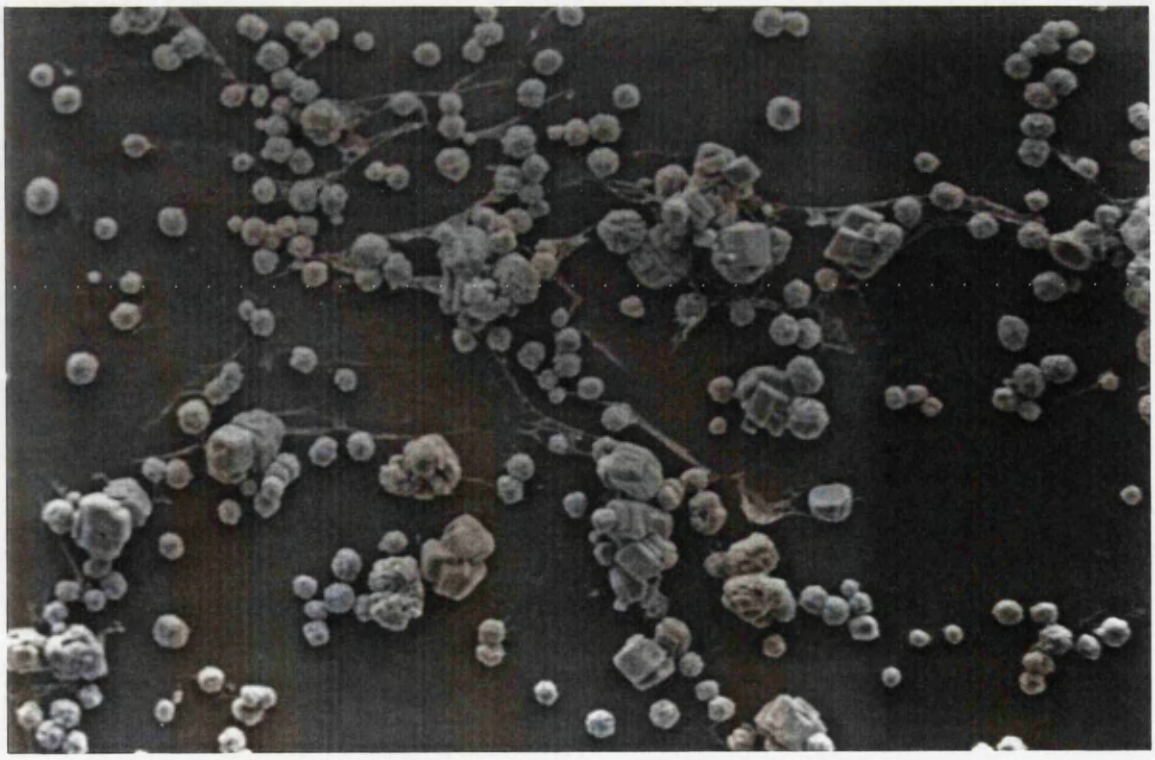


Figure 41: 39µg/ml soluble intramineral protein from poor quality, etched shells causes more severe disruption. A mixture of large angular and smaller spherical crystals are present (x720).

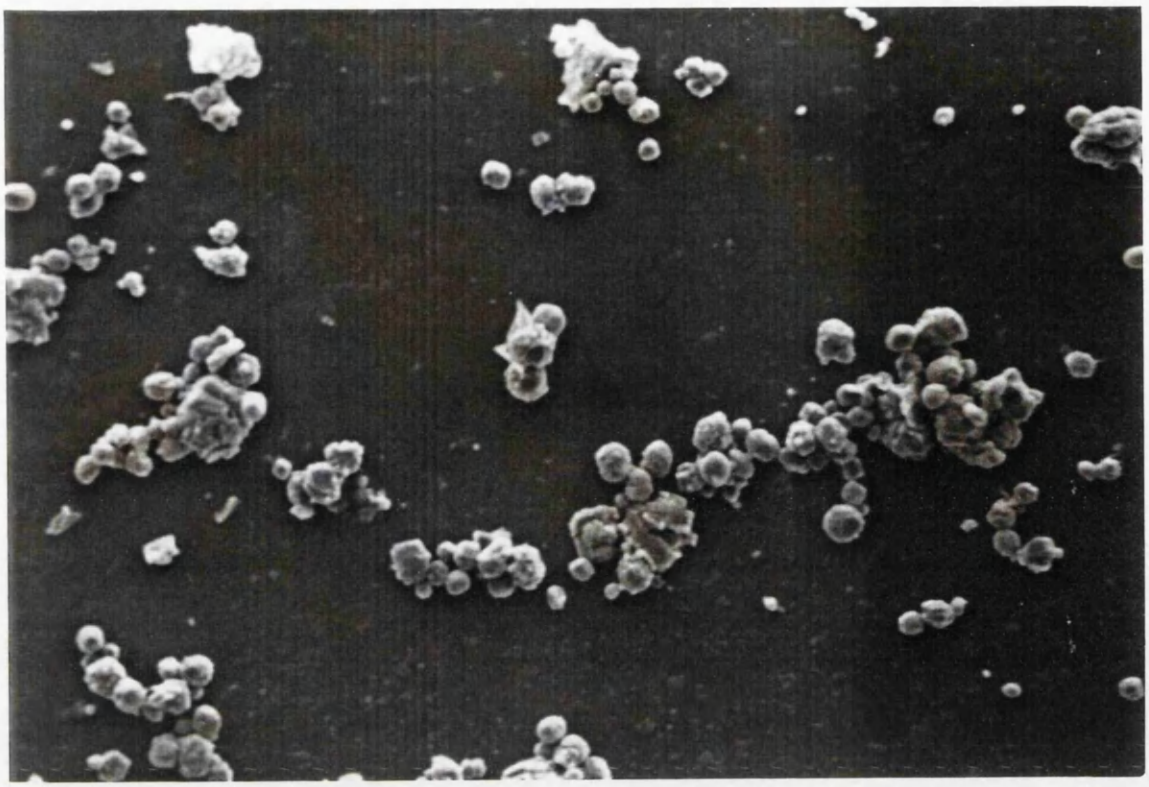


Figure 42: 52µg/ml soluble intramineral protein from poor quality, etched shells results in most change to morphology indicating a dose dependent rate of change. Crystals are spherical and clumped. (x720).

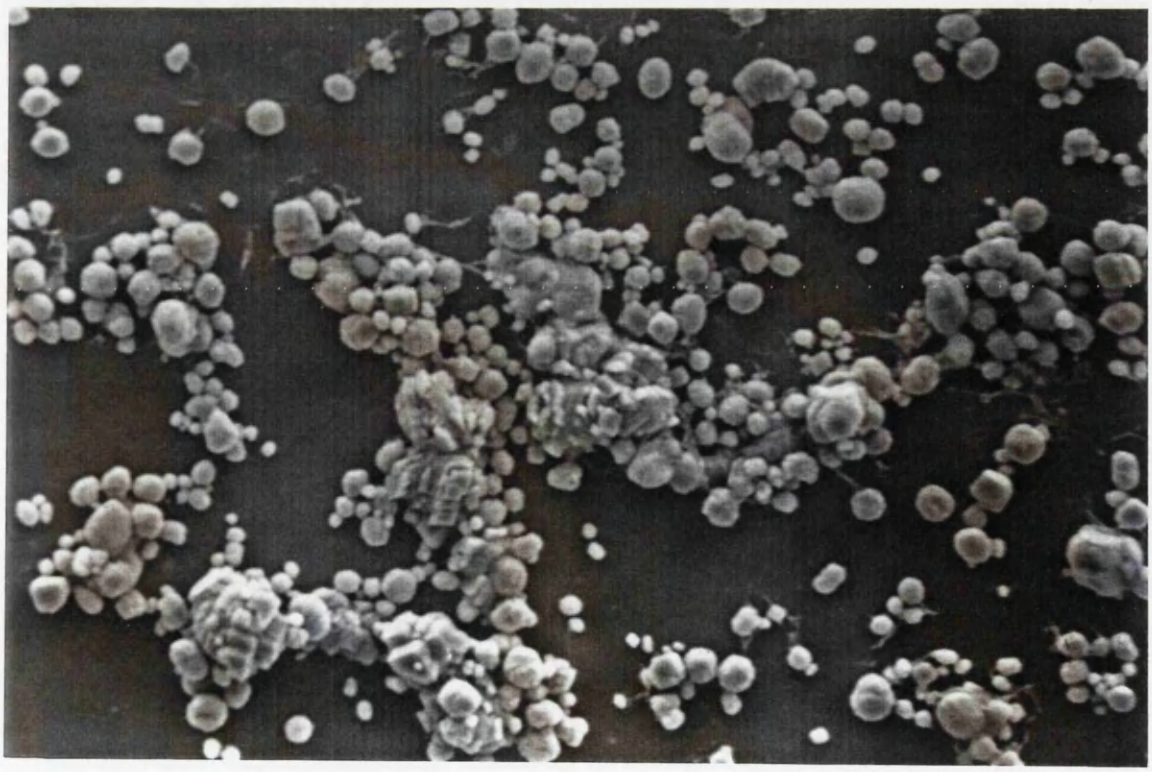


Figure 43: 53µg/ml soluble intramineral protein from poor quality, nonetched shells exerts more of an influence on crystal morphology than its etched counterpart (Figure 42). The crystals are more rounded (x720).

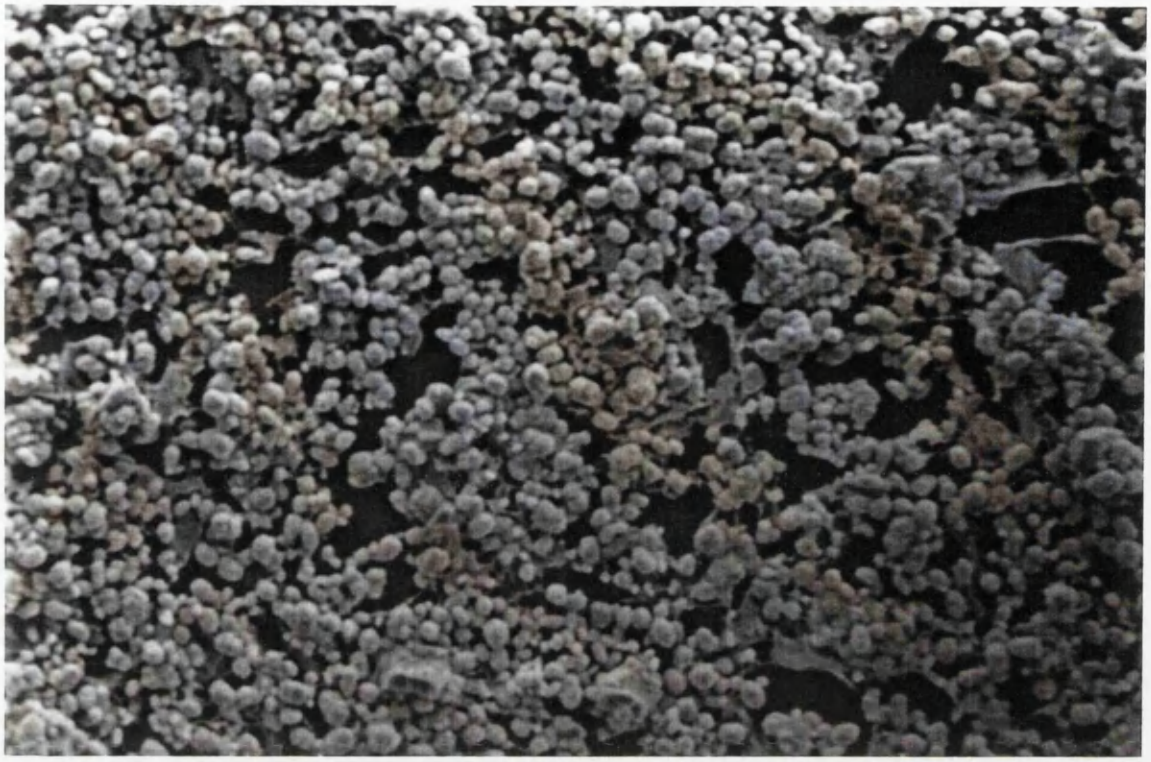


Figure 44: 40μg/ml soluble intramineral, good quality extract from nonetched shells results in a more extreme alteration to crystal morphology than its poor quality (higher concentration) associate (Figure 43) (x720).

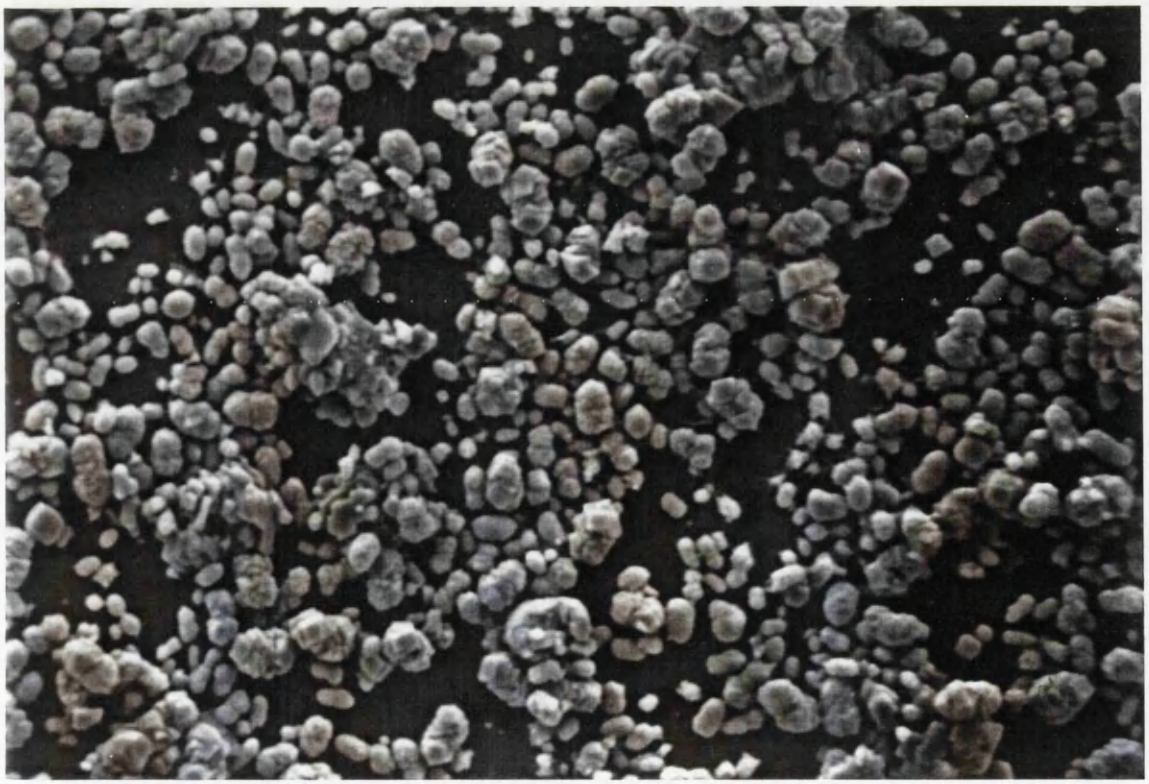


Figure 45: 54μg/ml soluble intramineral, good quality, extract from etched shells exerts less of an effect than its nonetched counterpart (Figure 44) but more than its poor quality equivalent (Figure 42) (x720).

CHAPTER 7 - THE USE OF THE AVIDIN BIOTIN (ABC) COMPLEX METHOD TO IDENTIFY OC-17 LOCALISATION IN THE EGGSHELL MATRIX.

7. THE USE OF THE AVIDIN BIOTIN COMPLEX (ABC) METHOD TO IDENTIFY OC-17 LOCALISATION IN THE EGGSHELL MATRIX.

7.1. INTRODUCTION.

It is known that the egg sequentially acquires each of its layers as it passes through the oviduct (Solomon, 1991). The mammillary knobs are formed on the outer surface of the outer shell membrane in the tubular shell gland and it is likely that their organic content is secreted here by the granular epithelial cells (Wyburn *et al.,* 1973). The mineral fraction and matrix proteins of the palisade layer are then deposited during the 20 hours that the egg remains in the shell gland pouch. The organic matrix is known to be intimately involved in the process of eggshell mineralization (see **1.2.4., 6.1.**), although its exact mode of action remains to be established.

The organic matrix of the eggshell contains a complex array of distinct proteins and macromolecules. Purification and identification of these individual components would allow for the demonstration of specific effects in terms of organic-inorganic interaction within the shell, thus enabling a more precise functional role for the various proteins to be determined. To date, only two particular organic matrix proteins have been identified, namely ovocleidin-17 (OC-17) and ovalbumin, Hincke *et al .,* (1993, 1995) and Hincke (1995). Of these, only OC-17 has been purified to homogeneity thereby enabling the production of antibodies, raised in the rabbit, for immunohistochemical investigation (see **1.2.4.**).

The use of immunohistochemistry in the identification and localisation of eggshell matrix components has been pioneered by Arias *et al.,* (1992, 1993), Arias and Fernandez (1993, 1995), Hincke *et al .,* (1993, 1995) and Hincke (1995). Such technology enables the detection and identification of distinct molecules (antigens) in tissue sections by means of specific antibody-antigen interactions. This can be achieved by means of either direct or indirect labelling of the antibody (Marriot and Carlton, 1990; Robinson *et al.,* 1990).

The direct method is the fastest (and least sensitive) means of antigen detection. The primary antiserum is conjugated directly with a tracer molecule such as horseradish peroxidase. However, yields of conjugated antibody can be low using this method and there may be problems associated with background staining.

In the indirect method of antibody labelling, the primary unconjugated antibody is allowed to bind to the antigen in the tissue section. A second trace conjugated antibody is raised in another animal host, this being specific for the animal and immunoglobulin class of the primary antibody. This is then applied to the section and allowed to bind with the primary antibody. The resulting complex can then be visualised by incubating the section with an appropriate fluorochrome (Robinson *et al.*, 1990). The indirect method is more sensitive than the direct, yet is still rapid and inexpensive.

Using specific monoclonal antibodies Arias *et al,* (1992, 1993) and Arias and Fernandez (1993, 1995) employed indirect labelling to demonstrate the presence of collagen X in the shell membranes and the co-localised occurrence of collagen X with the proteoglycan keratan sulphate in the outer portion of the membranes (region of thick fibres). These authors also identified the proteoglycans keratan and dermatan sulphate in the organic matrix (see **1.2.4.**). Hincke *et al.*, (1993, 1995) used a similar methodology to demonstrate the presence of OC-17 in the oviduct and eggshell of the domestic fowl.

The Avidin Biotin Complex (ABC) method is an alternative indirect means of antibody labelling, which relies on the marked affinity of the albumin glycoprotein avidin (molecular weight 67kDA) for the vitamin biotin. As this affinity is over one million times higher than that of antibody for most antigens, the binding of avidin to biotin (unlike antibody-antigen interactions) is essentially irreversible. In addition to this high affinity, the ABC method can be effectively exploited as avidin has 4 binding sites for biotin and most proteins (including antibodies and enzymes) can be conjugated with several molecules of biotin. This affinity enables mounted sections to be stored indefinitely, while the sensitivity allows for high dilutions of the primary antiserum and enables visualisation without the aid of a fluorescence microscope. Because of these advantages, it was decided to use the ABC technique as opposed to the methodology utilised by Arias *et al.,* (1992,

1993), Arias and Fernandez (1993, 1995) and Hincke *et al*., (1993, 1995).

Using rabbit-anti OC-17 provided by Dr. M. Hincke, University of Ottawa, Canada, the current research set out to establish the distribution and localisation of OC-17 in etched and nonetched shells classed as good and poor quality in ultrastructural terms. The aim of this work was to complement and add to data obtained in chapters **4**, **5** and **6**, thereby enabling further development of existing hypotheses regarding the structure and functional role of organic matrix proteins within the eggshell of the domestic fowl.

7.2. MATERIALS AND METHODS.

The eggshells used in this study were etched and nonetched samples classed as good and poor quality in ultrastructural terms, from the commercial battery system as described in chapters **2** and **4**.

7.2.1. DECALCIFICATION PROCEDURE.

The eggshells were decalcified using a 1:1 solution of 150mM NaEDTA (pH.7) and Lana's Fixative (4% paraformaldehyde, 0.4% picric acid in 0.16M phosphate buffer). The process took 3-4 days to complete with a daily change of solution. Once decalcified the samples were stored in PBS.

7.2.2. PARAFFIN WAX EMBEDDING.

The decalcified shells were dehydrated through a graded series of alcohols, cleared and mounted in paraffin wax blocks. Transverse sections were then cut at 2μm using a Leitz rotary microtome and mounted on glass slides.

7.2.3. THE ABC METHOD.

For the following procedure a VECTASTAIN ABC Kit (Vector Laboratories Inc. 1993) was used in conjunction with rabbit-anti OC-17 (Dr. M Hincke, University of Ottawa, Canada).

Sections were deparaffinised, rehydrated through a graded series of alcohols and then rinsed for 5 minutes in distilled water. Endogenous peroxidase activity was quenched by incubating the sections in 0.3% H_2O_2 in methanol then rinsing in buffer for 20 minutes. Following this, the sections were incubated for 20 minutes with normal rabbit serum.

After blotting, the sections were incubated for 30 minutes with antiserum (rabbit anti OC-17) diluted in PBS (1:1000 to 1:16000). In the controls, normal rabbit serum was used in order to ensure that no positive reaction occurred due to the presence of naturally occurring antibodies in the nonsensitised rabbit. The sections were then washed in phosphate buffer for 10 minutes, prior to incubation for 30 minutes with diluted biotinilated antibody solution.

They were subsequently washed as before and incubated for one hour with the ABC reagent. The sections were then washed for 10 minutes in buffer, then incubated for a further 5 minutes in peroxidase substrate solution. Following a 5 minute wash in tap water the sections were counterstained with toluidine blue, cleared and mounted prior to viewing with a Leitz Laborlux S microscope with a camera attached.

The presence of a brown staining reaction indicates a positive result, with a negative result showing only the blue colouration of the counterstain.

7.3. RESULTS AND DISCUSSION.

Figures 46 (control/blue) and 47 (positive/brown) show transverse sections of eggshell matrix from a good quality nonetched shell. Figures 48 and 49 illustrate corresponding staining reactions of matrix, extracted from a good quality etched shell. Figure 50 represents a positively stained transverse section of a poor quality unetched shell.

The high staining density associated with the mammillary bodies reported by Hincke *et al.,* (1993, 1995) is apparent in the positively stained sections (Figures 47, 49, 50). Contrary to the findings of Hincke *et al.,* (1993, 1995), the shell membranes (ME) have also stained strongly positive for OC-17 (see Figures 47 and 50). In Figure 47, a strong positively staining band can clearly be identified at the inner level of the palisade layer (PA), indicating a concentration of OC-17 in this area. This may correspond to the region, one third way into the shell, where the matrix is particularly concentrated as described by Arias *et al.*, (1993). Above this layer OC-17 appears to be relatively uniformly distributed. In figures 49 and 50, this band is less apparent and OC-17 is more regular in its distribution throughout the palisade, these observations being more consistent with the findings of Hincke *et al.*, (1993, 1995). These results serve to underline an apparent variability as regards matrix protein distribution in shells of different origin, a feature which has also been observed by Bain *(pers comm)*.

Arias *et al.,* (1992, 1993) and Arias and Fernandez (1993, 1995) have demonstrated the presence of keratan sulphate proteoglycan in the outer membrane fibres. As the mammillary cores are also known to contain keratan sulphate (Arias *et al.,* 1992, 1993; Arias and Fernandez, 1993, 1995) and give a strong reaction for the presence of the OC-17 antibody (Hincke *et al.,* 1995), this would suggest that the membrane fibres should also give a positive reaction to the presence of OC-17. This was observed in the current study (see Figures 47 and 50). Furthermore, if as discussed in section **6.3**, OC-17 is a matrix subunit protein formed upon complete dissociation of organic matrix molecules such as keratan and dermatan sulphate, it is not surprising that these molecules should give a positive reaction to OC-17 antigen. The positive reaction to OC-17 in the dermatan sulphate rich palisade layer clearly adds to this subunit hypothesis.

Hincke *et al.,* (1995) have indicated that OC-17 may be a glycoprotein, possibly a sulphated glycoprotein similar to those described by Krampitz and Grasser (1988) and Arias *et al.,* (1992). Indeed, dermatan sulphate proteoglycan, following digestion and fractionation, has been shown to contain glycopeptide fragments (Yanagishita and Haskall, 1983a; cited by Beeley, 1985). These observations support the hypothesis (see **6.3**) that OC-17 (and the other proteins identified by SDS-PAGE in chapter **5**) may be subunits of keratan and dermatan sulphate proteoglycans.

The positively staining membranes illustrated in Figures 47 and 50 also give credence to the results described in chapter **5**, which indicate the presence of OC-17 in the extramineral fraction of the organic matrix. This fraction is known to contain membrane derived components (Gautron, 1994). The presence of OC-17 in the shell membranes also ties in with the observation that the extramineral fraction influences the crystallization of calcium carbonate *in vitro*, as outlined in chapter **6**.

Figure 49 shows a strong positive reaction for OC-17 at the region of the vertical crystal layer, indicating the presence of organic molecules in this domain. This reinforces the findings outlined in chapter **4**, which indicate that this region contains matrix material.

Figures 47 and 50 clearly show a negatively staining cuticular layer, suggesting that the matrix and vesicles contained within the cuticle (see chapter **4**) do not contain the protein OC-17. This provides further evidence to support the hypothesis that the matrix material laid down in the final stages of mineralisation is different to that found in the rest of the shell.

There appears to be little difference in the distribution of OC-17 within shells classified as good and poor quality in terms of the organisation of the inorganic component at the level of the mammillary layer (see Figures 47 and 50). That such differences exist, however, is clearly evident from the micrographs presented in section **1.2.4.**. These changes in the ultrastructural quality of the eggshell may be linked to more subtle changes in matrix molecular structure as outlined in chapter **6** and not to differences in specific protein distribution within the shell.

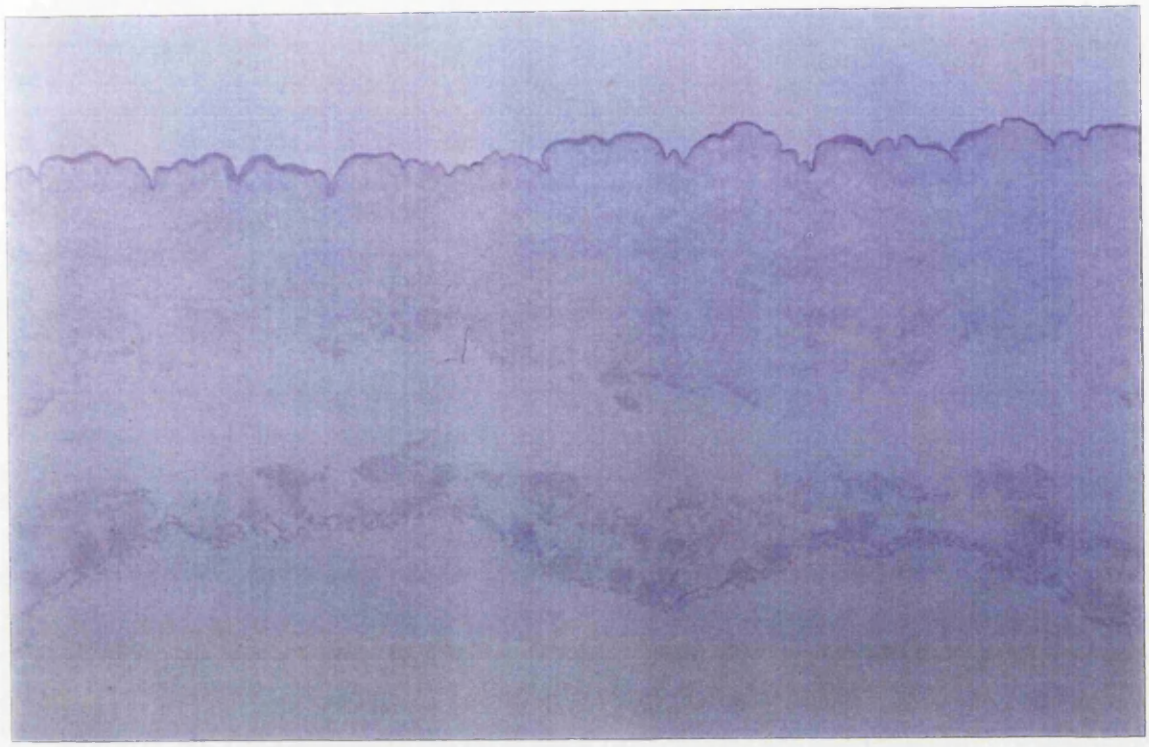


Figure 46: Negative control of good quality nonetched shell showing no reaction for the presence of OC-17 (x90).

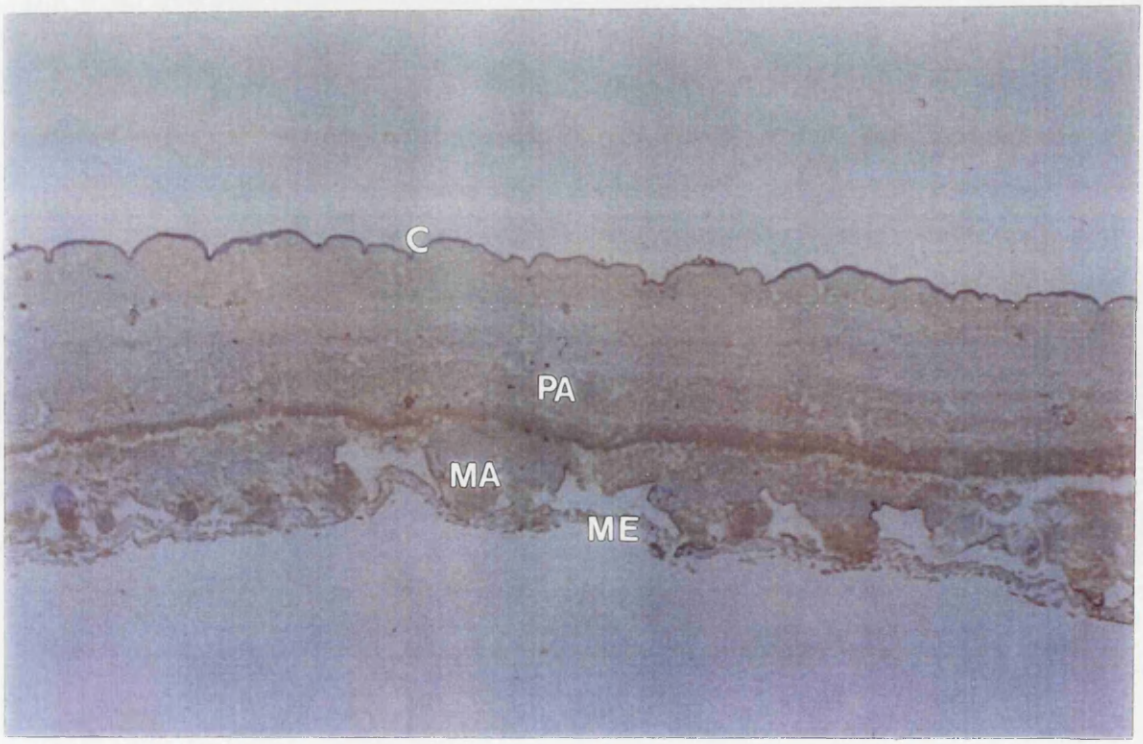


Figure 47: Transverse section of good quality, nonetched shell showing positively staining (brown) membranes (ME), mammillae (MA) and a strongly positive inner palisade layer (PA). The cuticle (C) has stained negatively (blue) (x90).

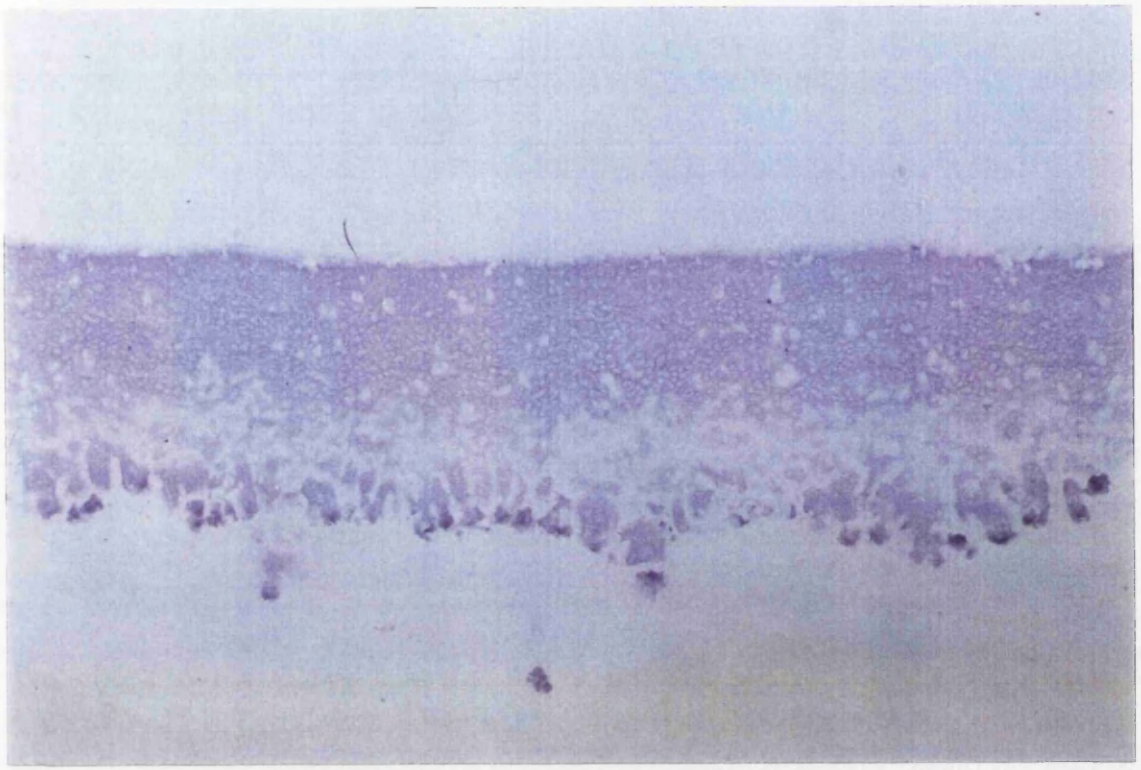


Figure 48: Negative control of good quality etched shell, which like its unetched counterpart (Figure 46) shows no reaction for OC-17 (x90).

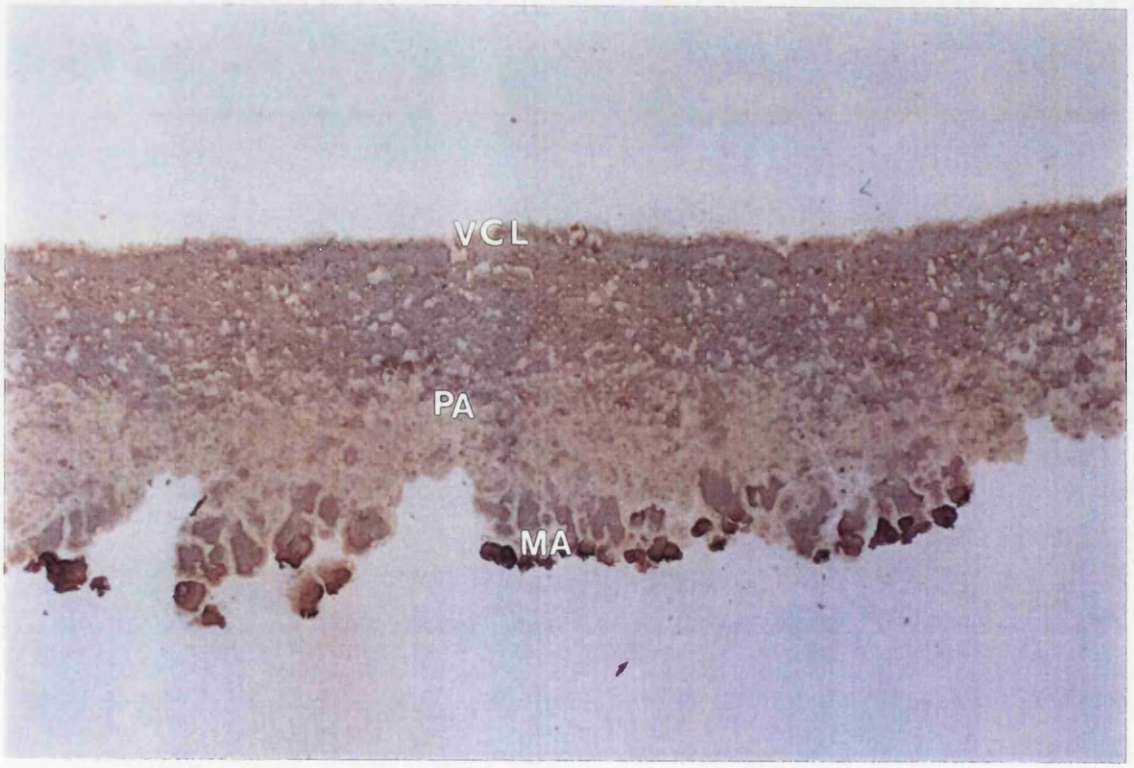


Figure 49: Transverse section of good quality etched shell showing positively staining mammillae (MA), palisade (PA) and vertical crystal layer (VCL). In this case the cuticle is absent.

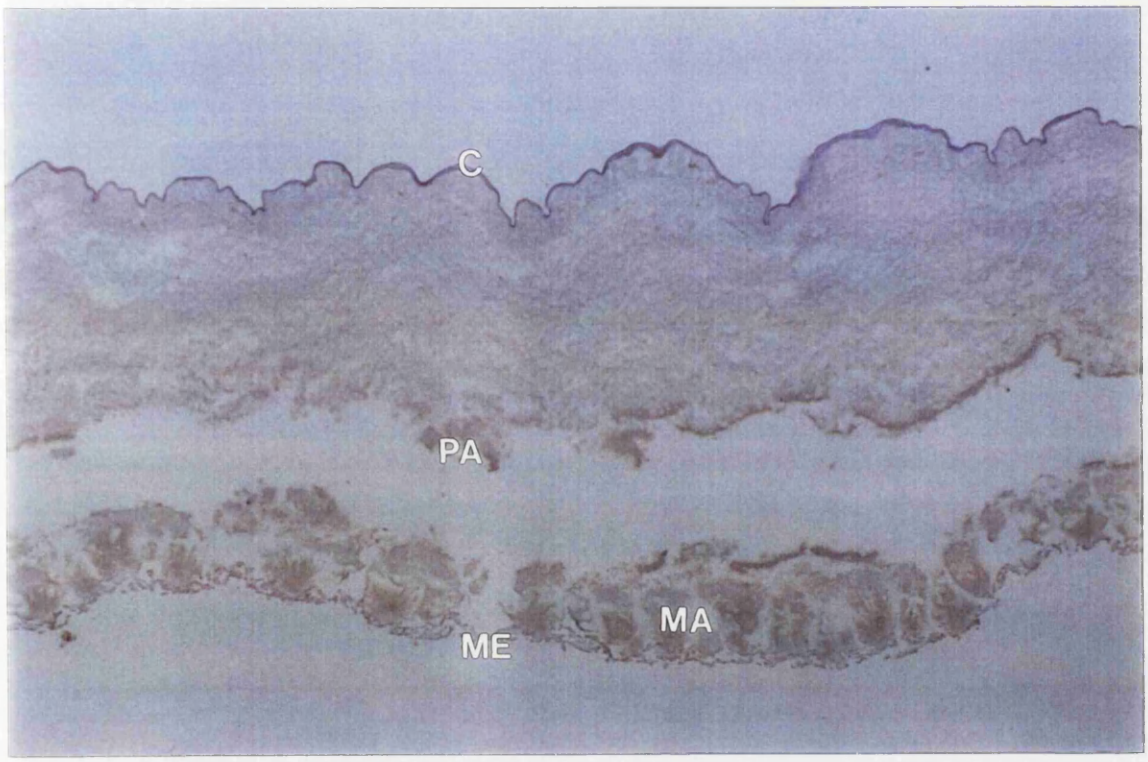


Figure 50: Transverse section of a poor quality shell with positive staining membranes (ME), mammillae (MA), pallisade (PA) and a negative staining cuticle (C) similar to that observed in a good quality shell (Figure 47) (x90).

CHAPTER 8 - GENERAL DISCUSSION AND CONCLUSIONS.

CHAPTER 8 - GENERAL DISCUSSION AND CONCLUSIONS.

8.1. GENERAL DISCUSSION AND CONCLUSIONS.

The enigma as to what came first, the chicken or the egg? has puzzled both scientists and laymen for centuries. As yet, this matter remains unresolved. However, on commencing this final chapter a similar question is posed by the author, "what came first, the jungle or the jungle fowl?" In an attempt to answer this, the following points are deemed worthy of consideration.

It is known that the wild jungle fowl (*Gallus gallus*) is a predominantly ground dwelling bird, living in the dense tropical rain forests of Southeast Asia. It makes extensive use of cover, especially for roosting and nesting, emerging mainly to feed (Appleby *et al.,* 1992). Thus, it would appear that the wild jungle fowl evolved as a successful species within the jungle environment through a process of natural selection. It can, however, show considerable variation within habitat and possesses a wide variety of behavioural mechanisms as a species, thereby enabling adaptation to a wide range of environments. This has led to the successful domestication of the group worldwide for provision of both eggs and meat, using a diversity of production systems. Although the initial question as to what came first regarding the jungle fowl may appear rather simplistic to the classical Darwinian evolutionist, it is nevertheless obvious that interaction between environmental factors and biological processes are extremely important in terms of biological success in the natural habitat. It is not, therefore, unreasonable to assume that such interactions would be important to the well being of the commercial laying hen in her production environment. Indeed, although todays modern hybrids are far removed from their wild ancestors in terms of performance, many of their behavioural requirements remain the same (see **1.4.2.**).

It was hoped that, during the course of this study, a definitive ranking order could be obtained for four systems of egg production (battery, perchery, modified and traditional free range) in terms of traditional, material and ultrastructural product quality. Bird welfare within each system was also an important consideration. Such a definitive order was not, however, apparent.

In the attempt to devise such a classification for the four housing systems under investigation, the traditional free range would come first with respect to its total ultrastructural score, contrary to the findings of previous authors (Mohumed, 1986; Bain, 1990; Solomon, *pers comm*). Nevertheless, using traditional and material measures of quality, this was not always the case. Stiffness measurements revealed that elastic properties of the eggshells from birds housed in the alternative systems showed a high degree of variability compared to eggs from the battery system. These observations were reflected in terms of elastic modulus values (see Tables 1-2, Graphs 5 and 7), thereby highlighting the need to examine the relationship between the inorganic and organic fractions of the shell (see **2.4.**).

In commercial terms, predictability of quality of end product combined with a promise of quality standardisation throughout the laying period are highly desirable features. Although rarely displaying the highest (or lowest) scores, the eggs from the battery system best met these criteria, probably as a result of the high degree of environmental control available within such a management system.

In contrast, the alternative systems generally appeared less predictable in terms of overall shell quality. They also offered less environmental control and social order stability than the battery system. This was most evident for the perchery system during the end of lay period, when shells contained a preponderance of detrimental characteristics (see **1.2.5.**). The author has personal experience of working in each of the systems under study and has noted that birds housed in percheries often exhibit a degree of aggression and flightiness not commonly observed in other systems, especially under commercial stocking densities and colony sizes. He has also observed severe problems relating to social order dysfunction (vent and feather pecking/cannibalism) and lack of environmental control (wet litter/disease/dirty and floor eggs) in free range and modified free range systems. Indeed, Harrison (1989) has expressed concern that, although many alternative systems show promising results under experimental use, the systems deteriorate under commercial conditions, often as a consequence of enterprises attempting to adapt the systems to the same economies of scale as the battery system. This has resulted in gross overcrowding of birds in very large flocks, the complete elimination of litter and a reduction in light intensity in order to reduce cannibalism. For example, the perchery system used in the

current study gave excellent production figures combined with low mortality levels when operated as a prototype (Michie, *pers comm*). These have not always been obtained by commercial producers (Scott, 1995; Darrah and Johnston, *pers comm),* thus reinforcing the concerns outlined by Harrison (1989). The results of the current thesis appear to highlight such problems within the perchery system and suggest that management of this particular system be reviewed. In addition, it should be pointed out that many alternative systems remain untried and untested to any significant level within the commercial framework and there is a real need for further research and development work on the management of semi-intensive and modern free range systems. It must be remembered that these systems are very far removed from the simple farmyard scenario of yesteryear.

At present, the commercial egg industry normally assesses egg and eggshell quality in physical terms either directly, as in the case of shell breaking strength or indirectly, as in the case of egg weight, shell thickness, nondestructive deformation and specific gravity measurements. The latter two are the most widely used in industry as they are noninvasive, inexpensive, rapid and allow for multiple measurements to be made (Bain, 1990). According to the latter author eggshell quality can most accurately be assessed using a range of these indicators, though in practice rarely more than one is used. The study of shell ultrastructure gives an additional direct method which is more sensitive than traditional measures, as shown by the results of the current research. As such it must be considered an innovative tool for forward thinking breeders and producers who wish to benefit from a better understanding of the influence of genetic, environmental and nutritional manipulation upon shell quality. Undoubtedly, refined egg quality assessment such as outlined in this thesis will play a valuable role in future research.

In the domestic situation, as in the jungle, different aspects of the environment interact. Social considerations are important to the way in which birds relate to the structures around them. It is known that if more space is given to birds then a greater extent and variety of behaviours can be expressed. However, care must be taken that negative behaviours are discouraged and that enrichment of the environment can stimulate positive behavioural expression (Baxter, 1994). The provision of a perch provides for a behavioural expression found in both modern strains and the wild jungle fowl. It is easy to install, inexpensive and unlike other cage modifications,

does not interfere with the automated processes associated with intensive production. It has also been used as a successful marketing tool in the commercial situation, albeit for a small, family run concern (Slaughter, *pers comm*).

The results herein have shown that the provision of a perch for birds stocked 5 to a cage (450 cm^2/bird) resulted in eggs of poorer ultrastructural quality. Existing knowledge on stress related ultrastructural features (see Watt, 1989) and observations of the birds in question fighting over a position on the perch (Slaughter, *pers comm*), again reinforce the importance of a stable social order. The introduction of a perch into these cages altered environmental conditions in such a way as to disrupt the previously held social order stability, as the birds did not fight when the perch was absent. The provision of a perch for birds stocked 4 birds to a cage (600 cm^2/bird) did not affect social harmony and shell quality improved in ultrastructural terms.

An additional comparison of 4 birds in cages (600 cm^2/bird), with and without perches, confirmed that it was the presence of a perch which had improved total ultrastructural scores, indicating that the results of the initial trial were not simply a reflection of the lowered stocking density. The current data reveal that the ultrastructural makeup of the shell and the incidence of certain stress related forms such as aragonite, changed membrane and type B bodies provide an insight into the birds physiological harmony with its environment, as hypothesised by Bain and Fraser (1993). As such, the ultrastructural integrity of the eggshell may well provide another useful factor in the complex equation that constitutes "welfare".

In practical terms, these results emphasise the fact that provision of a perch should not be considered in isolation from other environmental features, in particular stocking density. The current EC legislation regarding stocking density sets a minimum of 450cm^2/bird. The data provided indicate that a stocking density of 600cm^2/bird would be more appropriate in both production and welfare terms, especially if the provision of perches also become a requirement as has been suggested (Baxter, 1994).

The domestic laying fowl is a complex and highly evolved animal. It has been the subject of much scientific research and a great deal is now known about its environmental, physiological and behavioural requirements. The current work indicates that birds housed under more extensive alternative systems tend to be affected by diffuse stressors, such as those those associated with social interaction. Battery caged birds, however, are subject to the more specific and intense stressors associated with behavioural and environmental deprivation. In summation, no one system offers the bird all that it requires from a welfare point of view, with economic constraints exerting a major influence on the compromises that are reached. It is the opinion of the author that enhancing the environment of battery cages will ultimately provide for a more satisfactory egg production system in terms of improved bird welfare. These improvements should enable the bird to exhibit natural behaviour to an acceptable level which, when combined with the advantages currently exhibited by the battery system in terms of ease of management, would allow good financial returns. Extensive and alternative systems will always occupy a small but significant niche in the egg production market because of their perceived welfare advantages. This thesis points out, however, that such systems are not without problems regarding product performance and welfare, highlighting the need for further research in this area.

In view of these findings, it is suggested that future planning and design of poultry housing systems must therefore incorporate all available knowledge regarding husbandry, behaviour and welfare. This emphasises the need for efficient communication between producers, ethologists and poultry scientists if progress in this area is to be made. The problem involves economic, ethical, aesthetic and practical concepts, which all interact to make up the welfare issue. What is required is an acceptable balance between economic return, bird welfare and consumer acceptability, whatever the production system. There are no simple solutions to the problem and it is unlikely that a production system(s) will be found to please everyone, particularly people with extreme points of view. The aim must be to accommodate the majority and provide them with a quality product in a manner which they find acceptable.

It should not be forgotten that most farmyards of only 30 years ago contained small flocks of hens which ranged freely on fenced off, rotated pasture. They were housed in buildings which contained straw floors, nest boxes and perches at various heights. This environment was, to all intents and purposes, very similar to that of the wild jungle fowl. Indeed, much of what is currently being published in todays scientific papers on welfare has been known to small farmers worldwide for generations. Of course, such small enterprises would no longer be able to compete in economic terms. However, all things considered, those who will be responsible for designing future housing systems for laying hens would be wise not to loose sight of the wild ancestor of todays modern layer, or even its less distant forebears.

The effect of force molting, albeit an imposed protocol, was to exert a beneficial effect on the traditional and ultrastructural properties of the shells under study, whilst also influencing their material properties as described. This is in agreement with the findings of Roberts and Brackpool (1994), who also report improvements in shell ultrastructure post molt. These authors propose that when the process of shell formation is compromised, as occurs during molting, the hen has some capacity for altering the way in which the shell is laid down, optimising the use of available calcium carbonate. Although the exact function of the organic matrix of the shell remains to be elucidated, it is probable that under such conditions its role would be of paramount importance.

The process of evolution does not waste energy manipulating materials and structures that have no function and quickly eliminates those that do not function adequately and economically. Nature designed the avian eggshell as an embryonic chamber and it has been developed and refined over millions of years as part of a very successful cycle of reproduction. As such, it constitutes a naturally formed bioceramic containing several unique substructures which form complex, yet elegant solutions to the variety of problems facing the developing embryo (Fink, 1993). It is therefore well suited to the breeding sector of poultry production, where its role in housing the embryo remains of prime importance. As packaging for a food product, however, the table egg serves a somewhat different purpose. It must be able to routinely withstand insult and injury during the production and distribution processes. This dual role for the eggshell has been discussed at length by Bain (1990), who correlated many of the ultrastructural variations of the shell

with performance under load and showed that not all of these variations at mammillary level are detrimental. In addition, Nascimento (1992) highlighted the role played by specific structural traits in inhibiting bacterial transfer.

As far as production is concerned, genetic selection over many centuries has resulted in modern hybrid strains producing upwards of 320 eggs *per annum*, a quite phenomenal increase from the 22-26 eggs laid by the wild jungle fowl. It is likely that the metabolic process within the bird will have been altered considerably from those of the wild ancestor. However, the nutrient requirements of such modern strains are very well known and the role of dietary calcium and carbonate as regards shell formation have been extensively investigated (Tullett, *pers comm*). Nonetheless, shell quality remains a problem within the industry, especially at the end of lay (Solomon, 1991). It may be that within the present situation we have an organism which is being pushed to its biogenetic limits. Also, the fact that supplementary calcium does not always give a beneficial response in egg quality terms suggests that another factor/s may be involved.

It is known that in addition to the calcium and carbonate fractions of the shell, a carefully controlled sequence of organic matrix molecules are required to regulate the eggshell assembly process. Understanding the relationship of these molecules with the mineral phase, will form a fundamental aspect for establishing a more complete biological basis of understanding eggshell formation.

The results obtained for both light and transmission electron microscopy (TEM) offer descriptions of the organic matrix which agree with the findings of previous researchers (see **4.3.1.** and **4.3.2.**). TEM showed that the morphology of the matrix, particularly at the level of the inner palisade at the end of lay, was distinctly different from that observed in eggs at the beginning of lay. These changes appear to be linked to a decrease in fracture toughness (see battery flock, chapter **2**), thereby adding merit to the hypothesis described by Silyn-Roberts and Sharp (1986) that the organic matrix influences the material and mechanical properties of the shell. This is further supported by the suggestion that the matrix is involved in the spatial localisation of the final structure.

Towards the outer edge of the palisade layer a change in matrix morphology was observed (see Figure 20 and **4.3.3.**), with the perpendicular fibres changing to a vertical orientation within the vertical crystal layer (VCL). This is contrary to the findings of Arias *et al.*, (1993), who speculate that it is the absence of organic matrix material within the VCL that is responsible for the vertical orientation of the crystals. In light of the evidence presented within this thesis, it is suggested that the vertically aligned matrix within this layer is somehow responsible for the change in orientation of the crystals *in situ.*

TEM also revealed the cuticle to consist of two components, vesicular cuticle (VC) and nonvesicular cuticle (NVC) (see **4.3.4.**). It appears that these vesicles may contain hydroxyapatite ($Ca_{10}(PO_4)(OH_2)$), formed by its content of protein lipid complexes in a manner similar to that found in the cellular processes of certain bacteria.

Proposed functional roles for matrix and cuticular vesicles have been outlined, based on their physical and physiological modes of action in lower plants and animals (see **4.3.2., 4.3.4.**). A great deal of research has been conducted into the process of biomineralization in such organisms (Mann, 1986, 1988, 1990; Simkiss, 1986; Simkiss and Wilbur, 1989; Williams, 1989), thereby providing a reservoir of information which may prove applicable to the study of such processes in more advanced species, such as the domestic fowl. Indeed, the current thesis indicates that, in order to more fully understand the nature of the organic matrix within the eggshell, it would be wise to consider its role at the simplest level and build upon this information.

The results of the SDS-PAGE investigation underline the fact that there is a lack of uniformity in terms of technique and nomenclature regarding the eggshell in this field. For example, both Hincke *et al.*, (1992) and Gautron (1994) have used methods of membrane removal which are known to be incomplete or ineffective. Moreover, Gautron (1994) has described the soluble extramineral fraction of the eggshell matrix as consisting of membrane and cuticular components only. The results obtained herein question such a definition and indicate that the soluble extramineral fraction may contain proteins associated with the organic cores, as well as matrix subunit proteins such as OC-17.

A difference between the soluble extramineral fractions of good and poor quality nonetched shells was apparent, although such a difference was not obvious in the case of their etched counterparts (see **5.4.**). This suggests that the difference in the nonetched shells is membrane associated. It may be that the complete removal of the shell membranes by plasma etching (Reid, 1983) gives a more accurate picture of what constitutes the extramineral fraction of the matrix.

OC-17 appears to be present in both the membranes and the mammillary cores, a feature borne out by effect of the extramineral fraction on calcium carbonate crystal morphology *in vitro* and the positive identification of the OC-17 protein in these domains (see chapters **6** and **7** respectively). There is also evidence to suggest that the membranes and intramineral fraction of the organic matrix share a similar compositional origin. This relates well to the hypothesis that patterns of mineral biodeposition have some underlying mechanism in common, Wilbur (1984) and Addadi and Weiner (1989).

The data presented in chapter **5** refer to a period of study conducted in the laboratory of Dr. Yvs Nys at INRA, Tours, France. The time scale available meant that it was only possible to use Coomassie blue staining to identify the main proteins present in each migration. Silver staining of the gels, as described by Gautron (1994) may have added to the profiles by detecting weaker protein bands not revealed by Coomasie blue. It is therefore suggested that future work incorporates this methodology. It is also possible to identify specific calcium binding proteins using Stainsall, a cationic carbocyanine (Cusack, *pers comm*). This might help narrow the field of investigation regarding which proteins play an active role as matrix components and help identify which bands are most relevant for future isolation and purification.

It is also perhaps pertinent to point out at this juncture that, despite using the most advanced and refined technology currently available (Gautron, *pers comm*), the amount of solubilised protein obtained was lower than 0.15% of the total shell weight and corresponded to less than 5% of the total organic fraction. Thus, to date, we have a somewhat limited view of the protein components of the eggshell matrix. It is hoped that future technology may overcome such limitations. In the mean time, it is suggested that studies on

the organic matrix from the calcified shell incorporate investigation into precursor matrix molecules in their soluble phase, such as may be found in the uterine fluid during the various stages of shell formation (see Nys, 1995). Such an investigation might also point out any transformations occurring in matrix structure during the calcification process.

In vitro calcification studies using soluble eggshell matrix proteins revealed that crystals from control wells were perfect single rhombohedra, as described by Wheeler and Sikes (1984, 1989), Addadi and Weiner (1985), Arias *et al.,* (1993) and Gautron *et al.,* (1993, 1995 in press). In the current research controls contained only double deionised water. However, crystallization of calcium carbonate from metastable solutions might be influenced by proteins from sources other than the eggshell, Nys (*pers comm*). Recently, Belcher *et al.,* (1996) used Bovine Serum Albumin as a control whilst investigating the influence of soluble mollusc shell proteins on *in vitro* calcium carbonate crystallization. The latter authors found, that in this case, perfect rhombohedra were obtained identical to those formed in the absence of soluble mollusc shell protein. It is acknowledged that such a mechanism constitutes a more satisfactory experimental control and it is suggested that future work on eggshell crystallization *in vitro* incorporates such a procedure.

Both the etched and nonetched extramineral matrix proteins influenced the precipitation and morphology of calcium carbonate crystals formed *in vitro*, more so in nonetched shells. In both cases the effect was dose dependent (see figures 35-38). Gautron (1994) has stated that extramineral proteins consist of membrane and cuticular components only. More recently, Gautron and Nys (*pers comm*) have suggested that molecules associated with the palisade may also be involved. This is borne out by the results obtained in chapter **5**, which suggest that the extramineral fraction may contain proteins associated with the organic cores (see **5.4.**). One such protein may be OC-17. Although these results are not in agreement with previous researchers (Gautron *et al.*, 1993, 1995 in press), the collagen and proteoglycan components associated with the shell membranes (Arias *et al.*, 1992, 1993; Arias and Fernandez, 1993, 1995; Wu *et al.,* 1992, 1994) suggest an influential role for these extramineral components in the biomineralization of the eggshell, thereby accounting for the reported changes in crystal morphology (see **6.3.**).

SDS-PAGE (**5.3.1.**) revealed that nonetched shells contained extramineral bands at 43 and 80kDa, a feature not observed in their etched counterparts. This suggests that higher molecular weight proteins exert a more significant effect on crystallization than those of lower molecular weight (see section **5.3.**).

Soluble intramineral matrix proteins influenced both crystal size and morphology, this being in agreement with the findings of Gautron *et al.,* (1993, 1995 in press). Soluble intramineral matrix from good quality shells, however, had a greater effect on crystal morphology than that from poor shells, whilst plasma etching appeared to reduce the level of influence. These results imply a difference in intramineral matrix composition between good and poor quality shells, a difference which was not identified by SDS-PAGE.

Again consideration of the proteoglycan nature of the matrix associated with the intramineral fraction, as outlined in section **6.3.**, may account for these observations.

In order to confirm these hypotheses and determine a precise functional role for the organic matrix, it will be necessary to obtain a more complete biochemical characterisation of the molecules and macromolecules present. Nevertheless, the results of the current research undoubtedly indicate that the organic matrix of the eggshell interacts with the process of calcium carbonate mineralization and also influences its mechanical properties. The isolation, identification, purification and localisation of the various constituents involved in organic-inorganic interaction within the eggshell is unquestionably essential to future studies in this field.

Using histochemical techniques, Hincke *et al.,* (1993, 1995) demonstrated a uniform distribution of the OC-17 antigen throughout the palisade layer, with a higher staining intensity at the level of the mammillary bodies. No OC-17 was demonstrated in the shell membranes.

The results presented in chapter **6** clearly show the presence of OC-17 in the membranes, mammillae, the palisade layer and the vertical crystal layer. This confirms that the VCL does contain matrix material, contrary to the findings of Arias *et al.,* (1993, 1995). OC-17, however, was not demonstrated in the cuticular matrix suggesting that it is of a different molecular composition

to that found in other areas of the shell.

Little difference was found in terms of OC-17 distribution between good and poor shells, supporting the hypothesis that observed changes in the ultrastructural quality of the eggshell may be linked to subtle changes in matrix molecular structure (see chapter **7**). Positively staining membranes support the results described in chapter **5**, which indicate the presence of OC-17 in the extramineral fraction of the organic matrix and also the observation that the extramineral fraction influences the crystallization of calcium carbonate *in vitro,* as outlined in chapter **6**. In addition, the results obtained in chapter **6** reinforce the hypothesis that OC-17 and the proteins identified by SDS-PAGE in chapter **5** are subunits of keratan and dermatan sulphate proteoglycans and add merit to the idea that OC-17 is a sulphated glycoprotein, as postulated by Hincke *et al.*, (1995).

It must be remembered that OC-17 is only one of a complex group of molecules making up the shell matrix and a more definitive immunocytochemical appraisal of variations in the nature of the proteins present still needs to be carried out. The methodology used in the present study does not allow for quantification of results and it is suggested that future work use the more refined technique of immunogold labelling (which is semi quantifiable) in a complementary manner, in order to overcome these restrictions. Indeed, Bain (*pers comm*) is currently now applying this technique to demonstrate the distribution of OC-17 in the eggshell.

Although Hincke *et al.,* (1993, 1995) failed to demonstrate the presence of OC-17 in the liver, matrix precursor proteins are known to be synthesised in this organ (Krampitz and Grasser, 1988). The livers of older birds are often affected by reduced efficiency, due to fat accumulation and fatty liver syndrome (Dun, *pers comm*) and it is hypothesised that this may result in metabolic changes which could influence matrix protein composition and ultimately its distribution within the shell. If this is the case then perhaps much of the effort in past research directed at improving the quality of shells at the end of lay has been misguided, as it has only addressed the calcified portion of the shell. The current thesis reveals a change in matrix morphology at the end of lay, with an increase in both fibrillar material and vesicle numbers at this time. If the vesicles of the eggshell play a similar role to those found in lower plants and animals in regulating and maintaining localised ionic

environments (as hypothesised in **4.3.2.**), it may be that as the bird ages more vesicles are required to maintain these conditions. It is known that this change in the matrix was accompanied by a decrease in the fracture toughness of the shells in question, although their elastic properties remained the same.

These results imply that the quality of the organic matrix is altered detrimentally at the end of lay period and that this may in turn influence the shells material properties, subsequently affecting its performance as a food packaging material. It would therefore be appropriate to investigate the synthesis of matrix proteins within the liver and their method of transport to the oviduct and beyond, in an attempt to maintain and perhaps even positively manipulate their composition. Obviously any good structure requires a sound foundation and the organic matrix undoubtedly plays such a role in the eggshell. The decline in egg quality associated with the end of lay would appear to owe something to the change in matrix material as reported. Certainly, this area has been underestimated in egg quality terms for too long and at this stage of the investigation there remain more questions than answers. Current information as regards the distribution of the molecular components of the organic matrix of the eggshell is summarised in Figure 51.

It is widely recognised that changes in oviducal environment such as pH and temperature, can influence the calcitic crystal modification of the eggshell (Watt, 1989). It is also apparent, from the results of this thesis, that the organic matrix ultimately plays a role in modifying the crystal growth pattern of the eggshell (in addition to its function as a reinforcing network). The organic matrix, even in these early days of its investigation, is known to be an admixture of proteoglycan species, Arias *et al.,* (1992, 1993) and Arias and Fernandez (1993, 1995). In principal, proteoglycans have the potential for almost limitless heterogeneity under a range of local environmental conditions. They can differ markedly in protein content, molecular size and the number and types of glycosaminoglycan molecules present, thereby differing in charge, polarization and orientation (see **1.2.4.**). Obviously, the interaction between localised chemical processes and matrix organisation results in the formation of specific mineral states. The main chemical factors involved are solubility, supersaturation, nucleation and subsequent crystal growth (Mann, 1988, 1989, 1993). In addition, biochemical factors such as selective ion transport, complexation, localised pH and redox states, enzymic

action and matrix synthesis can influence crystal size, structure, morphology, composition and orientation (Mann, 1988, 1989, 1993; Birchall, 1993; Williams 1989). Thus, it appears that the underlying heterogeneity of matrix molecules in the changing oviducal environment may ultimately be responsible for the patterns of mineralization which take place in the avian eggshell. This would include the presence or absence of aberrant crystal forms in poor quality shells, thereby modulating the shape, strength, physical and material properties of the resulting structure. It should, however, be pointed out, that by and large, the process of eggshell formation is remarkably constant. Each day millions of hens world wide produce eggs which pass as grade one for human consumption. This would appear to suggest that the process of eggshell formation occurs under carefully controlled conditions. At the centre of this control process lies the relationship between organic molecules interacting with the surfaces of forming inorganic calcium carbonate crystals (see Figure 52). It is suggested that the term organic matrix should include not only the acidic proteoglycan and glycoprotein molecules of the matrix *per se* but also those, as yet unidentified species, associated with the mantles which constitute the boundaries of the matrix vesicles. The vesicles themselves have very intricate morphologies and offer an, as yet, unexplored area in terms of eggshell formation and structure. Indeed, only when it becomes possible to fully isolate and identify the composition of the organic matrix and establish its role *en mass in vivo*, will it be possible to resolve completely the environmental and physiological factors which contribute to the formation of the egg of the domestic fowl *in situ.*

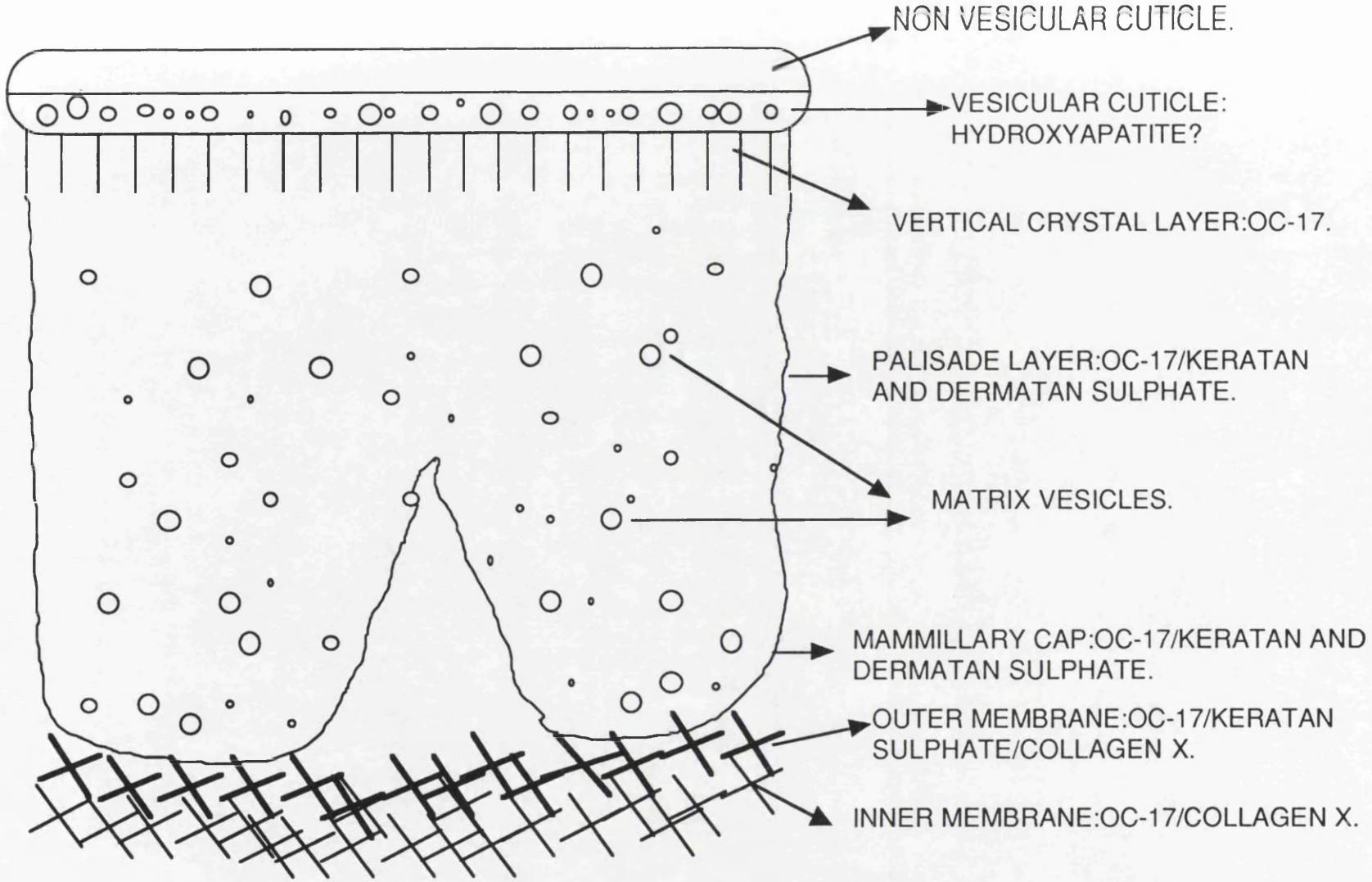


Figure 51. Schematic diagram summarising the distribution of the molecular components of the organic matrix.

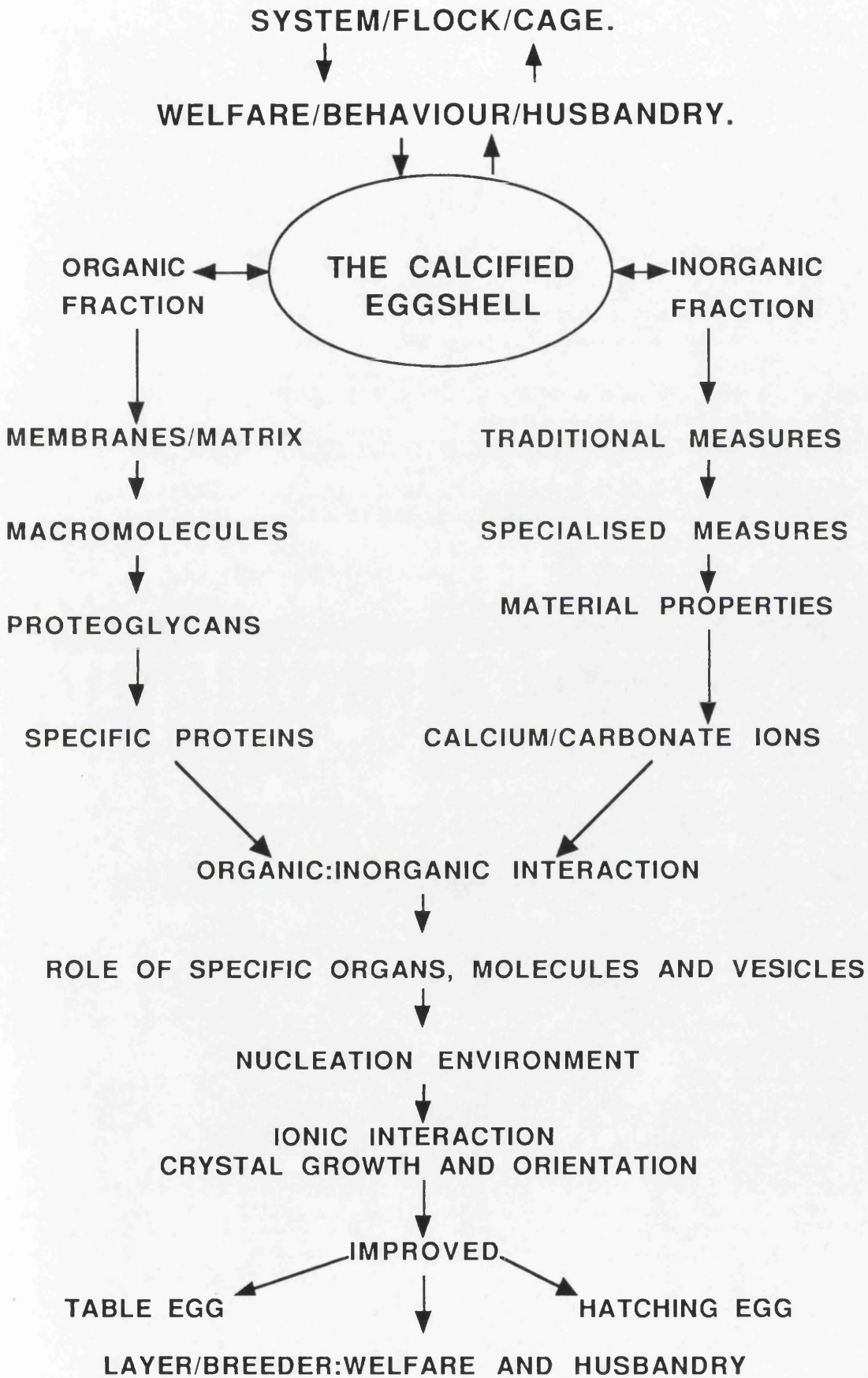


Figure 52. Flow diagram illustrating enviromnental and physiological factors known to influence eggshell formation.

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