FUNCTIONAL MORPHOLOGY AND BIOCHEMISTRY
OF REPTILIAN OVIDUCTS AND EGGS:
IMPLICATIONS FOR THE EVOLUTION OF REPRODUCTIVE MODES
IN TETRAPOD VERTEBRATES

By

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To Sylvia,
for her patience, love, and devotion

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The evolution of shelled amniotic eggs was a major factor facilitating the radiation of vertebrates into terrestrial habitats. This study examined the functional morphology and biochemistry of oviducts and eggs of representative reptilian groups, particularly with respect to albumen and eggshell formation. The results have important implications for our understanding of the evolution of reproductive modes in tetrapod vertebrates.

The oviductal functional morphology and ultrastructure of turtles, a lizard, and an alligator were studied using histochemistry, scanning and transmission electron microscopy. In turtles and lizards, the uterus is dualistic in function, secreting both the fibrous and calcareous layers of the eggshell membrane, as occurs in the monotremes. In alligators, there are two separate oviductal regions for eggshell formation, one specialized for formation of the eggshell membrane (fiber region), and another that secretes the calcareous eggshell layer (uterus). The oviductal functional morphology of

alligators is therefore similar to that of birds. This represents a fundamental divergence in reproductive morphology among higher vertebrates.

The biochemistry of reptilian oviducts and eggs was examined by analyzing the protein composition and secretion of egg albumen. There is much diversity in albumen protein composition among reptiles, although a few proteins are found universally and are immunologically similar to avian albumen proteins. In general, the albumin proteins of alligators and turtles are similar to those of birds, whereas squamate albumen is substantially different in composition. Egg albumin proteins are synthesized and secreted by the endometrial glands of the oviductal tube.

The divergence in reproductive modes between birds and mammals is found within extant reptiles. Evolutionarily, the relationship between crocodilians and birds is evident in oviductal structure and egg formation, whereas turtles and squamates resemble monotremes in formation of the eggshell by the uterus. The egg albumen shows much diversity in protein composition among reptilian groups, which may be related to their nesting ecology. These data refute recent theories concerning the evolution of birds and mammals from a common archosaurian ancestor.

CHAPTER I INTRODUCTION AND LITERATURE REVIEW

An understanding of the functional morphology of oviducts is essential for understanding the evolution of vertebrate reproductive modes. The oviducts influence whether the animal will be oviparous or viviparous, whether the eggs (or young) will be retained, and what kind of investments (coverings) will be applied to the eggs. The oviducts are further involved in fertilization and sperm storage.

Oviductal function is ultimately tied to its morphology and ultrastructure. Gross morphology shows the general layout of the oviducts, such as the number of regions and degree of muscular development. Cytological and ultrastructural data give evidence of the type of secretory material, if any. This can be further investigated with histochemistry in general and immunocytochemistry for specific proteins. The biochemistry of oviductal secretory products is critical to understanding the physiology of the eggs, both while they are retained in the oviducts and following oviposition. This is due to the properties endowed upon the egg by oviductal secretory proteins. These properties may enhance microbial defense, embryonic nutrition, or protection from the physical environment.

Comparisons of oviductal functional morphology and biochemistry are therefore key to understanding the evolution of reproductive modes in vertebrates. The following studies examined oviductal functional morphology and biochemistry of their secretory products in a variety of reptiles. Comparisons among species have revealed remarkable and unexpected similarities, as well as differences. These are ultimately tied to the evolutionary history of the organism and its mode of reproduction, and has direct implications for the evolution of avian and mammalian modes of reproduction.

Evolution of Amniotic Eggs

The amniotic egg not only encloses the embryo but provides an environment suitable for its development. This environment is produced by the mother in the form of egg albumen. Albumen consists primarily of proteins, although carbohydrates and lipids are found in trace quantities. The eggshell consists of a layer of proteinaceous fibers and, usually, a surface layer of calcium carbonate. Although there are variations in the structure of eggs among vertebrates (Fig. 1-1), they all possess albumen and a proteinaceous eggshell membrane, and most exhibit some degree of calcification.

The albumen creates a suitable internal environment for embryonic development, whereas the eggshell reduces the influence of the external conditions but allows gases and water to be exchanged. In later development, the embryo interacts more directly with the external environment through the apposition of extraembryonic membranes (chorion, allantois, yolk sac) to the eggshell and, therefore, plays a greater role in its own homeostasis.

The following review concentrates on oviductal structure, secretory proteins produced by the maternal oviduct and their influence on embryonic development. By far, the most studied proteins are the albumen and eggshell membrane proteins of avian eggs, with those of reptilian eggs only recently coming under investigation.

Reptilian Oviductal Functional Morphology

The oviducts of tetrapods are derived embryonically from the paired Müllerian (paramesonephric) ducts (Hildebrand, 1982). Oviductal morphology varies greatly among sexually mature tetrapod vertebrates. The term oviduct, as defined in comparative anatomical studies and as used throughout this dissertation, refers to the entire female reproductive tract. In most reptiles, both oviducts are functional and separate for their entire length, joining a cloaca posteriorly. Some squamates, primarily snakes, may display a vestigial oviduct, typically the left (Fox, 1977). For important

Figure 1-1. Typical egg structure in birds, crocodilians and chelonians, and lepidosaurians. Redrawn from Packard et al., 1977.

AVES

CROCODILIA & CHELONIA

LEPIDOSAURIA

works and reviews of the female reproductive tracts in reptiles, see Brooks, 1906; Giersberg, 1923; Weekes, 1927, 1935; van den Broek, 1933; Boyd, 1942; Mulaik, 1946; Kehl and Combescot, 1955; Forbes, 1961; Wilkinson, 1965; Cuellar, 1966; Hoffman and Wimsatt, 1972; Botte, 1973; Christiansen, 1973; Veith, 1974; Fox, 1977; Guillette, 1981; Mead et al., 1981; Halpert et al., 1982; Guillette and Jones, 1985; Palmer and Guillette, 1988, 1990a, b; Uribe et al., 1988; Adams and Cooper, 1988; Guillette et al., 1989; and Kumari et al., 1990.

Histologically, the oviduct is organized into three distinct layers (Sacchi, 1888; Hoffmann, 1889). These are, from the coelom to the lumen, the perimetrium, myometrium, and endometrium. The perimetrium consists of a thin layer of loose connective tissue covered by a single layer of squamous epithelium (mesothelium). This layer is continuous with the peritoneal support of the oviduct, which is known as the mesotubarium (Parker, 1884). The serosal layer is highly vascularized, with arteries, veins and lymphatic vessels running parallel to the oviduct along the mesotubarian junction. The serosal layer shows little variation among oviductal regions or vertebrate species.

The myometrium is composed of smooth muscle fibers typically arranged to form an outer longitudinal layer and an inner circular layer. The composition and development of the myometrium is highly variable among specific oviductal regions and among species (Hildebrand, 1982). Usually, the myometrium displays greater development posteriorly, whereas only scattered muscle fibers are present in the most anterior regions (Palmer and Guillette, 1988).

The endometrium is composed of two functionally separate but related layers; the lamina propria and lamina epithelialis (mucosa). The lamina propria in reptiles consists of highly vascularized loose connective tissue that may contain tubulo-alveolar glands (Fox, 1977). The connective tissue of the lamina propria is primarily composed

of collagen (Boyd, 1942). The endometrial gland cells are cuboidal to low columnar with large spherical nuclei (Palmer and Guillette, 1988; Guillette et al., 1989).

The lamina epithelialis is composed of an unstratified layer of columnar epithelial cells which lines the lumen of the oviduct. There are two primary types of cells, ciliated and secretory. The entire mucosa may show extensive folding which may vary in thickness among oviductal regions and with the reproductive status of the animal (Weekes, 1927; Boyd, 1942; Palmer and Guillette, 1988, 1990a; Uribe et al., 1988).

The reptilian oviduct shows considerable variation in morphology between species (Fig. 1-2). In lizards and snakes, three distinct regions have been described; the infundibulum-tube, the uterus, and the vagina (Cuellar, 1966; Fox, 1977; Gist and Jones, 1987), although some authors describe morphologically distinct anterior and posterior uterine regions (Guillette and Jones, 1985). Morphologically, the oviduct of turtles and tortoises form five distinct regions; the infundibulum, the tube (*tuba uterina*), the isthmus or transitional zone, the uterus, and the vagina (Giersberg, 1923; Fox, 1977; Gist and Jones, 1987, 1989), in addition, the infundibulum exhibits distinct anterior and posterior portions (Palmer and Guillette, 1988).

The infundibulum is the most anterior portion of the oviduct. The wide opening of the infundibulum (the ostium) was described in squamates over 100 years ago (Sacchi, 1888). The infundibulum serves to receive eggs from the ovaries, and may actively engulf the ovary prior to ovulation to facilitate reception of eggs (Cuellar, 1970). This region has abundant cilia which were first noted histologically in reptiles in *Lacerta* and *Anguis* by Leydig (1872). The mucosa is slightly folded and composed of ciliated and microvillous secretory cells. In addition, unique bleb secretory cells have been described in the infundibulum of reptiles (Boyd, 1942; Palmer and Guillette, 1988) that may be homologous to similar cells found in the glandular grooves of the avian infundibulum (Giersberg, 1923; Richardson, 1935). All three types of cells may occur on folds, but only bleb secretory cells occur within the grooves between folds (Palmer and Guillette,

Figure 1-2. Morphological characteristics of the oviduct of lizards, snakes, and turtles.
A: Lizard oviduct. B: Snake oviduct. C: Turtle oviduct. Redrawn from Gist and Jones, 1987.

1988). The infundibular epithelial cells increase in height during vitellogenesis (Hansen and Riley, 1941). The infundibulum is very thin walled, with the muscularis being poorly developed and the lamina propria being devoid of glands (Giersberg, 1923; Hansen and Riley, 1941; Palmer and Guillette, 1988; Guillette et al., 1989). The infundibulum serves the important function as the site of fertilization in birds (Aitken, 1971), and presumably also in reptiles.

The major part of the turtle and alligator oviduct consists of the highly pleated tube (Bojanus, 1819-1821; Giersberg, 1923; Palmer and Guillette, 1988, 1990a). The myometrium is reduced to only a few circular muscle fibers scattered among the connective tissue underlying the serosa. The endometrium is slightly pleated forming longitudinal grooves. The lamina propria contains numerous saccular or tubular glands (Giersberg, 1923; Hansen and Riley, 1941; Hattan and Gist, 1975; Palmer and Guillette, 1988; Guillette et al., 1989). Several authors have suggested that these glands produce albumen in reptiles (Gegenbaur, 1878; Christiansen, 1973; Botte et al., 1974; Botte and Granata, 1977; Palmer and Guillette, 1988, 1990a, b). Histological studies have found that the glands of the tube of *Chelonia mydas* (Aitken and Solomon, 1976) and *Gopherus polyphemus* (Palmer and Guillette, 1988) are structurally similar to the albumen secreting glands in the magnum of birds. These gland cells contain basal nuclei and spherical membrane bound granules of varying electron densities. After the passage of eggs, there are fewer granules in most cells. The tube is uniform along its length and lacks the terminal mucous region which produces the thick layer of albumen in birds. Layering of the "albumen" has not been described in reptilian eggs.

The tube is greatly reduced and aglandular in squamates (Hoffman and Wimsatt, 1972; Christiansen, 1973; Mead et al., 1981; Guillette and Jones, 1985; Adams and Cooper, 1988; Uribe et al., 1988; Guillette et al., 1989; Kumari et al., 1990), and is difficult to distinguish from the infundibulum. Squamates also have diminished quantities of albumen in their eggs (Packard et al., 1977; Tracy and Snell, 1985). Early

reports even suggested squamates lacked albumen entirely (Weekes, 1927; Giersberg, 1923).

The epithelium consists of tall columnar cells of which there are proportionately more secretory than ciliated cells (Aitken and Solomon, 1976). Although most of the cilia beat posteriorly, Parker (1928, 1931) observed in the turtle *Chrysemys picta* there is a narrow band of ciliated cells (2mm) that beat anteriorly. These pro-ovarian cilia move sperm toward the infundibulum where fertilization presumably takes place. The position of the pro-ovarian band is marked externally by the vascular edge of the oviduct. The posterior beating abovarian cilia are probably involved in sloughing mucus and the transport of eggs. Crowell (1932) also identified a single band of pro-ovarian cilia in several species of turtles (*C. picta* and *Pseudemys scripta elegans*) and lizards (*Phrynosoma cornutum* and *Sceloporus undulatus*). In the turtle *Mauremys japonica*, Yamada (1952) identified two pro-ovarian bands which lie on opposite edges of the tube.

In turtles, the tube is joined posteriorly to the uterus by a constricted region termed the isthmus or transitional zone. The isthmus is a very short, aglandular segment which probably has no secretory function associated with albumen or eggshell formation (Aitken and Solomon, 1976).

The uterus is the region where eggs are retained until oviposition. It has an extremely well developed myometrium composed of an inner circular and an outer longitudinal layer. The myometrium serves to move the oviductal wall over the egg's surface (Parker, 1928) during gravidity as well as aid in oviposition. The uterine mucosa exhibits tall villous-like folds, particularly posteriorly. The thickness of the oviduct and degree of folding of the mucosa increases from the infundibulum to the vagina (Bojanus, 1819-1821; Giersberg, 1923; Weekes, 1927; Boyd, 1942; Cuellar, 1966; Halpert et al., 1982; Guillette and Jones, 1985; Palmer and Guillette, 1988; Guillette et al., 1989). Within the uterus, the lamina propria is thick and glandular. In the turtles *Chelonia*

mydas (Aitken and Solomon, 1976) and *Gopherus polyphemus* (Palmer and Guillette, 1988), the uterine glands resemble those of the isthmus in birds. These glands contain numerous secretory granules during vitellogenesis, but become depleted in gravid animals (Palmer and Guillette, 1990a). The luminal surface of the gland cells also possesses microvilli and pronounced blebbing during gravidity. These glands have been suggested to produce proteinaceous compounds for the formation of eggshell fibers (Weekes, 1927; Boyd, 1942; Aitken and Solomon, 1976; Palmer and Guillette, 1988, 1990a, b; Guillette et al., 1989).

The glands of the avian shell gland lack distinctive ultrastructural features and secretory granules (Johnson et al., 1963) and are hypothesized to supply the "plumping water", which dilutes the albumen to its final concentration (Breen and de Bruyn, 1969). It has been hypothesized that plumping water is supplied by the reptilian uterus (Tracy and Snell, 1985; Palmer and Guillette, 1988).

The uterine epithelium in reptiles is composed of serous secretory cells and ciliated cells (Giersberg, 1923; Weekes, 1927; Boyd, 1942; Hansen and Riley, 1941; Hoffman and Wimsatt, 1972; Cuellar, 1966; Christiansen, 1973; Aitken and Solomon, 1976; Guillette and Jones, 1985; Palmer and Guillette, 1988; Uribe et al., 1988; Guillette et al., 1989). The epithelium of *G. polyphemus* is mostly ciliated during vitellogenesis, but transforms into mostly secretory cells during gravidity (Palmer and Guillette, 1990a). The epithelium of the avian shell gland is often implicated in calcium transport during eggshelling (Aitken, 1971). In *Crotaphytus collaris*, the mucosal epithelium stains for calcium (Guillette et al., 1989) and may produce the calcareous eggshell as predicted by Gegenbaur (1878).

The vagina in reptiles is a very short segment of the oviduct which connects the uterus with the cloaca. In some lizards (*Eumeces*, *Gerrhonotus* and *Chenidophorus*), the myometrium of the vagina forms a sphincter (Brooks, 1906; Fox, 1977), suggesting that it may function in a manner comparable to the mammalian cervix. It may also

function in retaining eggs within the uterus until the time of oviposition. The mucosa of the vagina is nearly aglandular.

The development of the oviducts has implications for the stimulation of hypertrophy in mature specimens. The oviducts of juvenile *Testudo graeca* and *Mauremys caspica leprosa* are devoid of glands (Argaud, 1920; Kehl, 1944). As these turtles mature, the luminal epithelium invaginates to form crypts, which differentiate into endometrial glands. This indicates that glands form under a stimulus associated with maturity and that the glands of the lamina propria are of epithelial origin. This pattern is similar to that described for squamates, where it was observed that ovarian tissue was capable of stimulating formation of new glands from luminal epithelia in oviductal tissue cultured *in vitro* (Ortiz and Morales, 1974). Hypertrophy of uterine endometrial glands can be induced *in vivo* with estrogen administration (Christiansen, 1973; Fawcett, 1975; Mead et al., 1981).

Oviductal Secretory Proteins

Biological Properties of Albumen Proteins

A functional approach will be used to examine albumen proteins, rather than a discussion of each protein individually. Although avian egg albumen proteins have received intense investigation, specific biological functions have not been found for all, including the most abundant avian egg white protein, ovalbumin (Baker, 1968; Nisbet et al., 1981; Li-Chan and Nakai, 1989). Since little is known concerning reptilian albumen proteins, much of this discussion will be based on what can be interpreted from birds. A summary of characteristics of avian albumen proteins is given in Table 1-1. Most are glycoproteins, with carbohydrate contents ranging between 2 and 22 percent. Functions described for albumen proteins have largely been determined *in vitro*, with virtually nothing known concerning *in vivo* functions. Further, some proteins may have multiple functions.

Table 1-1. Composition and physicochemical characteristics of major albumen proteins of the hen^a.

	% of albumen	Molecular Weight (kd)	Isoelectric point	% Carbohydrate
Avidin	0.05	68.3	10.0	8
Cystatin	0.05	12.7	5.1	0
Lysozyme	3.4-3.5	14.3	10.7	0
Ovalbumin	54.0	45	4.5	3
Ovoglobulin G ₂	4.0(?)	49	5.5	~ 6
Ovoglobulin G ₃	4.0(?)	49	5.8	~ 6
Ovoglycoprotein	0.5-1.0	24.4	3.9	16
Ovoinhibitor	0.1-1.5	49	5.1	6
Ovomacroglobulin	0.5	760-900	4.5-4.7	9
Ovomucin	1.5-3.5	230-8,300	4.5-5.0	19
Ovomucoid	11.0	28	4.1	22
Ovotransferrin	12-13	77.7	6.0	2
Riboflavin-Binding Protein	0.8	32	4.0	14

^aCompiled from Gilbert, 1971; Osuga & Feeney, 1977; Powrie & Nakai, 1986; and Li-Chan & Nakai, 1989

Antimicrobial proteins

Albumen provides for microbial defense of yolk and developing embryo in two ways, mechanically and chemically (Board and Tranter, 1986). Mechanically, albumen supports and surrounds the yolk, keeping it from contact with eggshell membranes. Additionally, albumen forms a colloid which acts as a barrier to invading bacteria due to its viscous and fibrous nature. Ovomucin is largely responsible for the high viscosity of albumen (Robinson, 1972, 1987), which is further augmented by lysozyme complexes (Kato et al., 1981; Miller et al., 1982; Hayakawa et al., 1983).

Chemically, albumen may prevent microbial infection by directly killing bacteria or by creating an environment unfavorable for their growth. Most studies on the antimicrobial properties of albumen proteins have been conducted *in vitro*, and it is uncertain if these properties are applicable *in vivo*. Lysozyme, the only albumen protein known to directly affect bacteria, does so by hydrolyzing $\beta(1-4)$ glycosidic bonds (Geoffroy and Bailey, 1975), a component of the cell wall of certain bacteria, thereby disrupting cell wall integrity.

There are several ways in which albumen can create an unfavorable environment for bacterial growth (Tranter and Board, 1982b; Board and Tranter, 1986). Albumen is quite alkaline, having a pH of about 9.5 (Heath, 1977), which is generally unsuitable for growth of many microorganisms (Board and Tranter, 1986). Additionally, some proteins bind required nutrients, such as minerals or vitamins, making them unavailable for bacterial use. Iron is found in very low concentrations in egg albumen. Ovotransferrin strongly (dissociation constant (K_D) $\sim 10^{-29}$ M) binds iron (Chasteen, 1977, 1983; Aisen and Listowsky, 1980; Brock, 1985), especially at the alkaline pH of albumen, and is effective in inhibiting bacterial growth by creating an essentially iron-free environment (Weinberg, 1977; Tranter and Board, 1982b). Additionally, ovotransferrin also binds copper, which may maintain the bactericidal properties of lysozyme, which is inhibited by

this metal (Gilbert, 1971). Ovotransferrin has been identified as the major antibacterial component of fowl egg albumen (Tranter and Board, 1982b).

Other proteins bind vitamins, making them inaccessible to bacteria. Avidin, well known for its ability to strongly bind ($K_D \sim 10^{-15} \text{M}$) the vitamin biotin (Green, 1963, 1975; Elo and Korpela, 1984), is strongly antibacterial, occurring largely in unbound (apoprotein) form (Tranter and Board, 1982a, b; Banks et al., 1986). Avidin also binds to the surface of some bacteria (Korpela, 1984; Korpela et al., 1984). A recently described thiamin-binding protein (Muniyappa and Adiga, 1979; Adiga and Murty, 1983) occurs largely in the apoprotein form in albumen, and may inhibit bacterial growth, although its affinity for thiamin is low ($K_D \sim 3 \times 10^{-7} \text{M}$). Another egg protein, riboflavin-binding protein, binds the vitamin riboflavin. Although it has been suggested that this apoprotein binds riboflavin too weakly ($K_D \sim 10^{-9} \text{M}$) to inhibit bacterial growth (Rhodes et al., 1959), *in vitro* studies have demonstrated inhibition (Li-Chan and Nakai, 1989).

Another group of antimicrobial proteins are protease inhibitors. Albumen has a very low concentration of free nitrogen, which is required by bacteria for protein synthesis. Substantial nitrogen for bacterial growth is found in albumen proteins, and many bacteria have proteases which are used to free nitrogen for incorporation into their own proteins. However, several of the albumen proteins have been demonstrated to be protease inhibitors (Li-Chan and Nakai, 1989), thereby preventing degradation and release of nitrogen for bacterial use. Ovomucoid, the third most common protein of fowl eggs, inhibits trypsin (Lineweaver and Murray, 1947; Feeney and Allison, 1969; Feeney, 1971). Ovoidin, although a minor component of avian eggs, simultaneously binds two molecules of trypsin or chymotrypsin, (Rhodes et al., 1960; Liu et al., 1971; Zahnley, 1980), as well as several types of bacterial and fungal proteases (Matsushima, 1958; Feeney et al., 1963; Tomimatsu et al., 1966; Feeney, 1971). Both ovomucoid and ovoidin belong to a family of serine proteinase inhibitors. Based on its primary

structure, ovalbumin has been placed into a superfamily of serine proteinase inhibitors (Breathnach et al., 1978; Hunt and Dayhoff, 1980; Woo et al., 1981; Carrell and Boswell, 1986; Ye et al., 1989), although no biological function has been found for this protein (Li-Chan and Nakai, 1989). Ovomacroglobulin also has serine protease inhibitory activity (Kitamoto et al., 1982) and is believed to be evolutionarily related to human serum α_2 -macroglobulin, which is inhibitory to trypsin and certain other proteases (Li-Chan and Nakai, 1989). Avian ovomacroglobulin also may be related to the crocodilian counterpart, which inhibits trypsin, subtilisin and papain (Ikai et al., 1983). Thiol proteases, which include ficin, papain, cathepsin C, B₁, H and L, bromelain, chymopapain, papaya proteinase III, and actinidin are inhibited by the minor egg protein cystatin (Fossum and Whitaker, 1968; Sen and Whitaker, 1973; Keilová and Tomášek, 1974, 1975; Barrett, 1981; Anastasi et al., 1983; Barrett et al., 1986).

Although most defenses are directed against bacterial or fungal invasion, two proteins have been suggested to possess antiviral properties. Ovomucin has been shown to be antiviral in that it inhibits viral hemagglutination (Lanni and Beard, 1948; Lanni et al., 1949; Gottschalk and Lind, 1949a, b). Cystatin, a thiol proteinase inhibitor, also may prevent viral infection (Barrett et al., 1986). These properties may be important as egg albumen proteins have been found in embryonic blood (Marshall and Deutsch, 1951; Wise et al., 1964), where they may be involved in prevention of viral hemagglutination. The combined antimicrobial effect of different albumen proteins may be synergistic in preventing microbial attack (Banks et al., 1986).

Nutritive proteins

The albumen proteins may represent a substantial supply of nutrients to the embryo (White, 1990). By binding vitamins and minerals, albumen proteins may not only act in defense of microbial attack, but in supplying nutrients to the developing embryo. Albumen proteins may be ingested by the embryo directly or selectively taken up (phagocytotically) by extraembryonic membranes (Marshall and Deutsch, 1951; Wise

et al., 1964). This uptake may supply the embryo with either the protein itself, its amino acid constituents, or other molecules which are bound to the protein.

Several proteins may be involved in selective transport of vitamins to the embryo. Avidin is well known to bind the vitamin biotin (Green, 1975), and may be used to transport biotin from mother to embryo (Adiga and Murty, 1983; White, 1987; White et al., 1987; Bush and White, 1989), although albumen in the fowl contains only 10% of egg biotin (Romanoff and Romanoff, 1949), with yolk supplying the remaining 90%. Riboflavin-binding protein is present in the albumen in approximately equal proportions of bound and unbound forms (Rhodes et al., 1958, 1959). The albumen of fowl eggs contains about 50-70% of the riboflavin in the egg (Stamberg et al., 1946), indicating that it is a major source of this nutrient (White and Merrill, 1988). Riboflavin-binding protein is synthesized in the oviduct (Mandeles and Ducay, 1962), and riboflavin is transferred to it from the blood. Thiamin-binding protein in albumen may exist largely in the apoprotein form, since the majority of thiamin in fowl eggs is concentrated in yolk (White, 1987). This may limit the value of albumen thiamin-binding protein in embryonic nutrition.

One protein which may be used to transport specific minerals to the embryo is ovotransferrin. Ovotransferrin belongs to a family of iron-binding proteins, the transferrins, and may be involved in the transport of iron to the embryo as occurs with a related mammalian blood protein, transferrin (Faulk and Galbraith, 1979). Ovotransferrin also is identical in amino acid structure to transferrin (Williams, 1962; Williams et al., 1982), differing only in carbohydrate moieties (Williams, 1968) and absence of sialic acid in ovotransferrin (Osuga and Feeney, 1968). Uteroferrin, an iron containing protein from the pig, is secreted by the uterus and is taken up by the fetus as a source of iron (Roberts et al., 1986; Roberts and Bazer, 1988). However, since ovotransferrin in albumen is predominantly in the apoprotein form, it is unlikely to

supply much iron to the embryo, although it can release iron to embryonic red blood cells (Li-Chan and Nakai, 1989).

Support and cushioning proteins

The albumen supports the yolk and embryo within the shell, cushioning them from mechanical injury. In birds, the chalaza (a fibrous ligament), suspends the yolk within the center of the albuminous mass. The chalaza is formed from the twisting of ovomucin fibers as the egg rotates while descending through the oviduct (Conrad and Phillips, 1938; Scott and Huang, 1941). The remaining albumen aids in cushioning the yolk due to its viscosity. The role of albumen in support and cushioning of reptilian eggs is variable. A chalaza has not been reported in reptilian eggs, and the quantity and viscosity of albumen in reptilian eggs is extremely variable at oviposition. In species which have a thick albumen layer, it may help cushion the yolk when the egg drops into the nest cavity.

Water binding proteins

Most avian albumen proteins are water soluble, indicating that they are at least partially hydrophilic. Most also are glycoproteins, which are known to tightly bind water to the hydrophilic side groups of the peptide chain, as well as the carbohydrate residues (Fennema, 1977). Because of the physical structure of the proteinaceous mass, some water is trapped by surrounding protein molecules. There also is free water which is in the albumen due simply to osmotic principles. It is, therefore, the combined qualities of albumen as a whole which enable it to act in water storage, creating an osmotic impediment between the embryo and surrounding environment.

In reptiles, there is no single scheme for storage of water in albumen. Crocodilians, some chelonians and some squamates lay rigid-shelled eggs (Ferguson, 1982; Packard et al., 1982; Packard and Hirsch, 1986; Hirsch, 1983; Schleich and Kästle, 1988; Packard and DeMarco, 1990). In these, albumen is relatively hydrated in the

uterus before oviposition (Tracy and Snell, 1985; Webb et al., 1987a, b; Manolis et al., 1987; Palmer and Guillette, 1988), although additional water may be absorbed from the substrate, such as sand, soil, or organic matter (Guggisberg, 1972; Packard et al., 1977). In those reptiles with parchment-shelled (most squamates) or pliable-shelled (many turtles) eggs, the egg may absorb substantial quantities of water during development (Packard et al., 1977; Packard et al., 1980; Morris et al., 1983; Packard and Packard, 1984; G. C. Packard et al., 1985; M. J. Packard et al., 1985; Packard and Packard, 1987). In fact, eggs of many reptilian species must absorb water for successful embryonic development (Packard et al., 1977; Tracy, 1980; Andrews and Sexton, 1981; Tracy and Snell, 1985), which is at least partially taken up by albumen in some species (Tracy et al., 1978; Tracy, 1980, 1982; Snell and Tracy, 1985).

Albumen proteins of avian eggs are hydrated and bind substantial quantities of water. In the fowl, 88% of the weight of albumen is water (Shenstone, 1968; Osuga and Feeney, 1977), which is three times the amount of water present in yolk and constitutes the major source of water for development of the avian embryo. Comparatively little water is exchanged with the atmosphere as water vapor, although there must be a net loss of water from the avian egg in order for successful embryonic development (Rahn and Ar, 1974; Ar and Rahn, 1978, 1980; Board, 1982).

In birds, albumen is initially secreted as a viscous, concentrated protein mixture in the magnum, which later is saturated with "plumping" water by the shell gland (Breen and de Bruyn, 1969; Wyburn et al., 1973; Solomon, 1983). This has also been observed in turtles (Agassiz, 1857), who noted the swelling of albumen of *in utero* eggs. In squamates, albumen may be relatively unhydrated at oviposition, but obtain water from the substrate (Packard et al., 1977; Tracy, 1980; Tracy and Snell, 1985).

Reptilian Albumen Proteins

Egg albumen proteins exhibit a wide variety of functional properties. Alterations in composition of proteins present in eggs will change the functional properties of the

albumen as a whole. This may have important consequences on the embryo's ability to survive under different nest and incubation conditions (Muth, 1980; Palmer and Guillette, 1990b). Embryos whose eggs have a protein composition with properties suitable to available nest and incubation conditions will have a selective advantage. It is well known that there is variability in composition of albumen proteins among eggs of different avian (Sibley, 1970; Sibley and Ahlquist, 1972) and reptilian species (Palmer and Guillette, 1990b).

When compared to birds, reptiles have more ancient origins, are found in extremely diverse habitats, and exhibit various parity modes (oviparity versus viviparity), suggesting a less conservative pattern of reproductive anatomy and physiology. This is easily seen in the reproductive anatomy of reptiles (Fox, 1977), where studies on oviductal anatomy in lizards (Guillette and Jones, 1985; Adams and Cooper, 1988; Palmer and Guillette, 1988; Uribe et al., 1988; Guillette et al., 1989), snakes (Mead et al., 1981), chelonians (Aitken and Solomon, 1976; Palmer, 1987; Palmer and Guillette, 1988) and crocodilians (Palmer and Guillette, unpublished data) have demonstrated substantial differences in functional morphology among reptilian orders. In addition, there also must be differences in physiological control among these reptilian groups (Trauth and Fagerberg, 1984; Palmer and Guillette, 1988). These phylogenetic, anatomical, and physiological differences may affect the types and ratios of extraembryonic proteins.

The reptilian eggshell is variable in structure and permeability among reptilian groups (Schleich and Kästle, 1988). This is important to the embryo in that the eggshell is the mediator of environmental effects (Packard et al., 1977; Packard et al., 1982; Packard and Packard, 1984; Ackerman et al., 1985a, b; Tracy and Snell, 1985; Packard and Hirsch, 1986; Ratterman and Ackerman, 1989; Packard, 1990; Ackerman, 1990). Some reptilian eggshells have extremely thick calcareous layers, and act as a substantial barrier to water and gas exchange between embryo and environment. Other reptiles lay

eggs with little calcium outside the fibrous membranes (Schleich and Kästle, 1988; Packard and DeMarco, 1990). These differences in eggshell structure may greatly affect the role of extraembryonic proteins due to differences in water conductance rates and need for antimicrobial agents, thus selecting for differences in protein composition of the albumen (Packard and Packard, 1980; Tracy, 1980, 1982; Tracy and Snell, 1985; Palmer and Guillette, 1990b).

The nest site also is extremely variable among reptilian species. Whereas most birds exhibit some degree of parental care, most reptiles do not. In most cases, the eggs are abandoned and allowed to develop without further parental assistance. Eggs may be buried in a substrate or attached to exposed surfaces. Therefore, the egg must be adapted to withstand large variations in moisture content and microbial communities. Again, the need for extraembryonic fluids to buffer these different conditions is implied. It is reasonable that since reptiles have more ancient origins, different reproductive anatomies and physiologies, and diverse eggshell structures and nest conditions, their eggs will exhibit much greater variations in albumen proteins than have been reported in birds.

Reptilian albumen has been recently shown to exhibit substantial differences among orders (Palmer and Guillette, 1990b). An ovotransferrin-like molecule has been detected in turtle (*Pseudemys floridana*) albumen (Palmer, 1988), based on molecular weight data, although more rigorous identification is required. Neither ovalbumin nor ovotransferrin was detected in eggs of *Crocodylus porosus* (Burley et al., 1987), although a major protein of 59,000 molecular weight was identified and has been subsequently detected in the eggs of both alligators and turtles (Palmer, unpublished data). An α_2 -macroglobulin-like protein has been found in the eggs of *Crocodylus rhombifer* (Ikai et al., 1983), *C. porosus* (Burley et al., 1987) and other reptiles (Palmer, unpublished data). Although avidin synthesis was observed in oviducts of the lizard *Lacerta s. sicula* (Botte et al., 1974; Botte and Granata, 1977), neither avidin, riboflavin-binding protein, nor

thiamin-binding protein has been detected in the albumen of *A. mississippiensis* (Abrams et al., 1988, 1989; White, 1990).

Clearly, the study of albumen proteins of reptiles is in its infancy. There appears to be a greater diversity in composition of albumen proteins of reptiles (Burley et al., 1987; Palmer, 1988, 1989; Abrams et al., 1989; Palmer and Guillette, 1990b) than that of birds, and possibly also in the functional role of albumen in embryonic development. Albumen of reptiles has been suggested to act in water storage (Tracy and Snell, 1985), resist lethal rates of water exchange (Tracy and Snell, 1985), and possess antimicrobial properties (Movchan and Gabaeva, 1967; Ewert, 1979). These properties, and the protein bases for them, may have evolved in response to nest and incubation conditions (Packard and Packard, 1980; Tracy, 1980, 1982; Tracy and Snell, 1985).

Albumen Protein Formation

The formation of albumen proteins has been extensively studied in the domestic fowl, on which the following discussion is largely based. In birds, the majority of the albumen is secreted by the magnum (Fig. 1-3) following ovulation, although initial layers are formed in the infundibulum (Dominic, 1960; Wyburn et al., 1970). The egg remains in the infundibulum for approximately 15-30 min and in the magnum for 2-3 hr (Warren and Scott, 1935; Woodward and Mather, 1964). Anatomically, the infundibulum is mostly aglandular, but "glandular grooves" occur posteriorly (Giersberg, 1923). True tubular glands occur near the infundibulum/magnum junction which resemble "glandular grooves" in general cell structure (Dominic, 1960), although characteristic differences have been reported (Aitken and Johnston, 1963). Within the magnum, there are tubular endometrial glands in addition to a secretory luminal epithelium (Richardson, 1935; Wyburn et al., 1970). The endometrial glands are composed of three types of cells; A, filled with electron dense granules, B, filled with low electron density secretory material and C, with prominent Golgi bodies and extensive rough endoplasmic reticulum,

Figure 1-3. Morphological characteristics of a typical avian (*Gallus domesticus*) oviduct.

although types A and C may simply reflect different phases of secretory activity within the same cell type (Wyburn et al., 1970).

Albumen proteins are secreted by specific cells within the magnum (Gilbert, 1979). Most albumen proteins are thought to be secreted by endometrial glands of the magnum. Ovalbumin is secreted by A-cells whereas lysozyme is released by B-cells (Kohler et al., 1968; Oka and Schimke, 1969; Wyburn et al., 1970). The cells of the endometrial glands also are known to secrete ovotransferrin and ovomucoid (Schimke et al., 1977), whereas the luminal epithelium secretes both avidin and ovomucin (Kohler et al., 1968; Wyburn et al., 1970; Tuohimaa, 1975).

The magnum synthesizes the albumen proteins which it secretes, unlike yolk proteins which are manufactured in the liver and transported to the ovary via the circulatory system. This has been shown by *in vitro* incorporation of radiolabeled amino acids into albumen proteins (ovalbumin, ovotransferrin, ovomucoid, lysozyme, and riboflavin-binding protein, Mandeles and Ducay, 1962; avidin, O'Malley, 1967). Additional work has shown *in vivo* incorporation of radioactive lysine and glycine into ovalbumin, ovotransferrin and lysozyme (Mandelés and Ducay, 1962). Some albumen proteins secreted by the oviduct are either identical (avidin) or similar (ovotransferrin) to proteins found in other body tissues or fluids (Green, 1975; Chasteen, 1977, 1983; Aisen and Listowsky, 1980; Elo and Korpela, 1984; Brock, 1985); however, these are known to be synthesized directly by the oviduct and not transported there by blood (Williams, 1962; O'Malley, 1967).

The enormous amount of protein synthesized for egg albumen has given molecular biologists an excellent model to study the regulation of genetic mechanisms involved in protein formation. Under stimulation from the adenohypophysis, ovarian follicles synthesize and release estrogen (Norris, 1985). This is transported to the oviduct by the circulatory system and readily passes through the plasmalemma of cells in the endometrial glands. Once inside the cell, estrogen is bound by a nuclear receptor

protein, which binds to chromatin, stimulating transcription (O'Malley et al., 1979; Chambon et al., 1984; Gorski et al., 1987; Leavitt, 1989). Estrogen stimulates the gland cells of immature chicks to differentiate (Brant and Nalbandov, 1956) and to synthesize ovalbumin, ovotransferrin, ovomucoid, and lysozyme (Palmiter and Gutman, 1972; Palmiter and Schimke, 1973). Further, treatment with progesterone and estrogen causes the luminal epithelium to secrete avidin (Kohler et al., 1968). It has been demonstrated that estrogen treatment stimulates transcription of the ovalbumin gene (Roop et al., 1978).

The cellular mechanism of albumen release has recently been elucidated for ovalbumin and ovotransferrin. Inhibition of glycosylation does not block the release of ovalbumin or ovotransferrin (Kato et al., 1987), indicating that these proteins are free within the endoplasmic reticulum (ER) and Golgi bodies, and not bound to the organelle membranes. Further, inhibition of protein transport from the ER to the Golgi bodies blocks release of these proteins, as may disrupting microtubules which have been implicated in secretory granule transport (Kato et al., 1987). Thus, these data suggest that ovalbumin and ovotransferrin are enclosed within secretory granules which are transported from the golgi complex to the plasmalemma by microtubules.

The signal for secretion of albumen proteins is still poorly understood. The pressure of the descending yolk on the oviductal wall is generally thought to stimulate albumen secretion (Sturkie and Mueller, 1976; Laugier and Brard, 1980). Artificial mechanical stimulation created by introducing objects into the oviduct does induce the formation of albumen and eggshell, although these are grossly abnormal (Wentworth, 1960; Pratt, 1960). However, albumen is also secreted by isolated loops of the oviduct while an egg passes through the intact portion (Burmester and Card, 1939, 1941), indicating that neuronal or endocrine stimulation may play a role in secretion. Although the oviduct is known to be innervated by the autonomic nervous system, administration of acetylcholine (parasympathomimetic) and ephedrine sulfate (sympathomimetic) had

negligible effects on albumen secretion (Sturkie and Weiss, 1950; Sturkie et al., 1954). Diffusible yolk proteins can affect oviductal metabolism *in vitro* (Eiler et al., 1970), but it is unknown if they can alter albumen synthesis *in vivo*. Steroid hormones, which are involved in the ovulatory process, have been shown to induce albumen synthesis (see above), but not release.

It remains to be determined how other hormones involved during ovulation, such as arginine vasotocin and prostaglandins (PG), may be involved in albumen release. Prostaglandins are potent paracrine hormones which influence blood flow and contraction of the female reproductive tract (Poyser, 1981). Stretching the reproductive tract causes PG release which can influence contractile patterns throughout the oviduct. Prostaglandin synthesis immediately after ovulation may be essential for yolk transport down the reproductive tract, release of oviductal proteins and egg rotation for normal shell formation. The reproductive tract of lizards is capable of synthesis and secretion of large amounts of PGF and PGE₂ during the first 24 hrs after ovulation (Guillette, unpublished data). In birds, a similar pattern is observed on a more rapid time scale (Hertelendy et al., 1984). Clearly, mechanical stimulation of the oviduct alone is insufficient to induce release of albumen around the descending yolk, but it may trigger a complex series of paracrine events responsible for albumen and eggshell protein release. Further research into the mechanisms of protein release needs to be conducted.

Albumen of avian eggs is composed of four distinct portions: the chalaziferous layer, which is attached to the yolk and suspends it, the inner liquid layer, the thick layer, and the outer thin layer. These different layers are not obvious during their formation in the magnum. The layers are the result of secretion of different components by consecutive regions of the oviduct (Gilbert, 1971), by the mechanical action of the egg twisting as it descends through the oviduct (Romanoff and Romanoff, 1949) and chemical changes during plumping in the shell gland (Sturkie and Mueller, 1976). The chalaza is formed by the mechanical twisting of ovomucin fibers as the egg

spirals down the magnum (Conrad and Phillips, 1938; Scott and Huang, 1941). The thin albumen layers are produced by addition of plumping water after the egg enters the shell gland (Sturkie and Polin, 1954). The fibrous chalazae and gelatinous nature of the thick albumen are due to their high concentration of ovomucin (Robinson, 1987).

In contrast to birds, the formation of albumen proteins in reptiles is poorly understood. Avidin is the only albumen protein detected in oviductal secretions of a reptile, the lizard *Lacerta s. sicula* (Botte et al., 1974), and is induced by combined effects of estrogen and progesterone, as in the fowl (Botte et al., 1974; Botte and Granata, 1977). Poly(ADPribose)transferase (ADPRT) activity, an indicator of gene expression, increases under estrogen stimulation in the oviduct of the lizard *Podarcis s. sicula*, and is maximal at oviductal morphological maturity (Ciarcia et al., 1986). Enhanced ADPRT activity precedes protein synthesis in immature quail oviducts under estrogen stimulation (Müller and Zahn, 1976). Clearly, much work needs to be done on the process of albumen synthesis and secretion in reptiles.

Eggshell Membranes

The proteinaceous fibers of the eggshell membranes play an important role in the maintenance of a suitable environment for the developing embryo. The eggshell membrane exhibits several functions, which are due to the mechanical structure of the membrane as a whole, rather than from individual chemical properties, as with albumen. In fact, the protein of the eggshell membrane has proven difficult to investigate as it is highly insoluble (DeSalle et al., 1984). The amino acid composition of eggshell membrane protein is known for a variety of species, including fowl (Candlish and Scougall, 1969; Balch and Cooke, 1970; Britton and Hale, 1977; Starcher and King, 1980; Crombie et al., 1981; Leach et al., 1981), green sea turtle, *Chelonia mydas* (Solomon and Baird, 1977), and lizards, *Iguana iguana* (Cox et al., 1982), *Eumeces fasciatus* and *Opheodrys vernalis* (Cox et al., 1984), but the protein has been assigned to various classes over the years, including keratin (Balch and Cooke, 1970; Britton and

Hale, 1977), collagen (Candlish and Scougall, 1969; Wong et al., 1984) and elastin (Starcher and King, 1980; Leach et al., 1981; Crombie et al., 1981). This has led to the conclusion that eggshell membranes are composed of unique proteins (Leach, 1978; Tullett, 1987).

Eggshell membranes have a variety of functions in reptiles, including water balance (Tracy, 1980, 1982; Tracy and Snell, 1985), support (Packard and Packard, 1980), and microbial defense (Packard and Packard, 1980; Tracy and Snell, 1985). As part of the eggshell, the fibrous membrane acts in water balance by forming a barrier to gas exchange and increasing the diffusion distance, thereby slowing down water movement (Ackerman et al., 1985a, b). The membrane, however, has no effect on direction of water movement, which is determined by relative osmotic potential across the eggshell. The fibrous membrane also serves to maintain egg shape, support egg contents, and protect the embryo from mechanical injury (Packard and Packard, 1980; Tracy, 1982). Since fibers of the membrane form a dense crisscrossing mat, they act as a barrier to invading bacteria and fungi (Board and Tranter, 1986). Functions of eggshell membranes may be enhanced by the organic matrix of protein and carbohydrates surrounding the fibers. It is likely that albumen and eggshell coevolved in response to the environmental conditions to which eggs were subjected (Packard and Packard, 1980; Tracy, 1980, 1982; Tracy and Snell, 1985).

Fibers are produced by endometrial glands of the isthmus in birds (Misugi and Katsumata, 1963; Simkiss, 1968; Simkiss and Taylor, 1971; Solomon, 1975; Aitken, 1971; Draper et al., 1972; Solomon, 1983). Secretory granules are released into the lumen of the endometrial glands and coalesce as they are extruded from the glandular duct into the oviductal lumen (Solomon, 1983). It has been proposed that the uterus of reptiles produces the fibrous membranes (Aitken and Solomon, 1976; Palmer and Guillette, 1988, 1990b; Guillette et al., 1989). These fibers, in both birds and reptiles, have been described as possessing a protein core with a mucopolysaccharide mantle (Simons and

Wiertz, 1963; Candlish, 1972; Solomon and Baird, 1977). However, in some reptilian eggs, proteinaceous fibers lack the mantle and exhibit a pitted appearance (Solomon and Baird, 1977; Andrews and Sexton, 1981; Trauth and Fagerberg, 1984; Sexton et al., 1979; DeSalle et al., 1984).

Birds are sequential ovulators, and each layer of the eggshell is applied by subsequent regions of the oviduct. The entire process takes approximately 22-25 hours (Aitken, 1971) and is complete before the egg passes through the vagina. Most reptiles are simultaneous instead of sequential ovulators. This implies that there are also differences between the physiological processes of eggshelling in reptiles and birds (Palmer and Guillette, 1990b). Eggshell formation is disrupted in the lizard *Cnemidophorus uniparens* if ovulation is followed by deluteinization (Cuellar, 1979). Although the thickness of the fibrous layer remains unchanged, its structure is altered. Particularly, only thick fibers are produced, instead of a gradation of thick to thin fibers. Upon oviposition, the eggs leak fluid and dehydrate quickly. Deluteinization decreases plasma progesterone concentration and increases myometrial activity (Jones et al., 1982; Guillette and Fox, 1985; Fox and Guillette, 1987). How the corpus luteum and progesterone concentrations affect eggshell fiber formation and what physiological mechanisms control the sequential formation of eggshell fibers and calcareous layer in the reptilian uterus remain to be studied.

CHAPTER II

ULTRASTRUCTURE AND FUNCTIONAL MORPHOLOGY OF TURTLE OVIDUCTS

Oviductal ultrastructure and functional morphology are poorly understood in reptiles, particularly with respect to albumen deposition and eggshell formation. Studies on the green sea turtle, *Chelonia mydas* (Aitken and Solomon, 1976), gopher tortoise, *Gopherus polyphemus* (Palmer and Guillette, 1988), and the lizards, *Eumeces obsoletus* and *Crotaphytus collaris* (Guillette et al., 1989) suggest that although albumen is formed in a region (tube) structurally similar to that of birds (magnum), eggshell formation is quite different. In these reptiles, there is only one region (uterus) in which all layers of the eggshell are formed, whereas in birds the fibrous membrane and calcareous layers are formed sequentially in separate regions, the isthmus and shell gland, respectively.

Only two studies have examined reptilian oviductal ultrastructure using transmission electron microscopy (TEM), although it is a fundamental tool in the study of functional morphology. One of these was limited to the sperm storage glands of the garter snake *Thamnophis sirtalis* (Hoffman and Wimsatt, 1972), whereas the other examined the tubal and uterine structure of the green turtle *Chelonia mydas* (Aitken and Solomon, 1976), but was based on only two specimens. Previous works on chelonian oviductal functional morphology (Aitken and Solomon, 1976; Palmer and Guillette, 1988) are based on species whose populations are endangered or reduced in size. Therefore, they are inappropriate for detailed ultrastructural and physiological examination of albumen and eggshell formation. This study examined oviductal ultrastructure at the histological and TEM level using species that are readily available (stinkpot turtles, pond sliders, and painted turtles) throughout the United States, either by field collection or through dealers. This will enable future physiological studies on

these species to be interpreted in relation to known functional morphological characteristics.

Methods and Materials

Specimens

Three species of common water turtles were used for this study. Female pond sliders (*Pseudemys scripta*; n=5) and stinkpot turtles (*Sternotherus odoratus*; n=5) were caught using baited funnel traps in Alachua county, Florida. Additionally, female painted turtles (*Chrysemys picta*; n=10) were obtained from dealers. The specimens were anesthetized using 10mg/kg pentobarbital i.p., and the oviducts and ovaries surgically excised. Oviductal regions were identified as infundibulum, tube (*tuba uterina*), transition zone, uterus, and vagina (Palmer and Guillette, 1988). Representative samples of those regions involved in albumen and eggshell formation (tube and uterus) were prepared for histochemistry and electron microscopy.

Histochemistry

Tissue biopsies were fixed in 10% neutral buffered formalin or Bouin's fixative, washed, transferred through graded alcohols, cleared in xylene, and embedded in paraffin (Humason, 1979). Specimens were serial sectioned at 7 μ m for a total of 10 slides (approximately 200 sections) and stained as in Table 2-1.

Electron microscopy

Specimens for electron microscopy were fixed in 2% glutaraldehyde in 0.1M cacodylate HCl buffer for 3 hrs. For scanning electron microscopy, the tissues were washed in 0.1M cacodylate buffer (3 X 20 min.), dehydrated in graded alcohols (1 hr ea), critical point dried (Anderson, 1951), and sputter coated with gold. Examination was performed on a Hitachi S-450, operated at 15kV.

Table 2-1. Staining techniques employed and their general interpretation¹.

Hematoxylin & eosin	Nuclei and general cytology
Alcian blue (pH 2.5)	Glycosaminoglycans
Fast green	Connective tissue
Orange G.	Red blood cells
Beibrich scarlet	Muscle, and proteinaceous secretory material

¹Humason, 1979

For transmission electron microscopy (TEM), oviductal tissues were minced (1mm^3), and fixed as above. Specimens were then washed in cacodylate buffer (3 X 15 min), fixed in 1% OsO_4 for 30 min, washed in cacodylate buffer (3 X 15 min), dehydrated in graded alcohols (15 min ea), treated with 100% acetone (2 X 1 hr), and embedded in Spurr's resin. Thick sections ($1\mu\text{m}$) were prepared and stained with toluidine blue. Thin sections were cut on a LKB ultramicrotome at $600\text{\AA}$, and poststained with uranyl acetate and lead citrate. Examination of tissues was performed on a Hitachi HU-11E or a Philips EM-300.

Results

Luminal Epithelium

The general morphological characteristics of the female turtle reproductive tract are presented in Fig. 2-1. The luminal epithelium of the tube and uterus consists of microvillous secretory and ciliated cells (Fig. 2-2A, B). The ciliated cells have central nuclei, are eosinophilic, and are typically compressed laterally into a funnel-like shape, with the apical end expanded (Fig. 2-3A). Most ciliated cells are nonsecretory, although some have electron light secretory granules (Fig. 2-3B). The cilia exhibit a typical 9+0 triplet microtubular arrangement below the cell surface and a 9+2 doublet arrangement once they penetrate the plasmalemma (Fig. 2-4A). The apical membrane of the ciliated cells have some microvilli, particularly if an apical cone is present, which projects outward from the center of the cell (Fig. 2-4B). The lateral plasmalemma of the luminal epithelial cells shows extensive interdigitations, tight junctions, and desmosomes (Fig. 2-4A).

The secretory epithelial cells stain positively with Alcian blue (pH 2.5) for glycosaminoglycans (GAGs). Ultrastructural examination reveals that there are two distinct types of microvillous secretory cells. The first is characterized by a basal nucleus with prominent nucleolus and abundant membrane-bound electron-light secretory

Figure 2-1. Gross morphology of the turtle oviduct. Modified from Palmer and Guillette, 1988.

Figure 2-2. Histology and scanning electron microscopy of the luminal epithelium of the turtle oviduct, showing ciliated cells and secretory cells. **A:** Paraffin histology of the luminal epithelium (2,000X). **B:** SEM of the apical membrane of the luminal epithelium (8,000X). E, epithelium; G, endometrial glands.

Figure 2-3. Ultrastructure of the ciliated cells of the luminal epithelium. A:
Transmission electron micrograph of nonsecretory ciliated cells (13,750X). **B:**
TEM of ciliated secretory cells, which have electron light secretory granules near
the cell apex (8,900X). N, nuclei; arrow, secretory vesicles.

Figure 2-4. Transmission electron micrographs of the apical portion of ciliated cells. A: TEM showing extensive interdigitations, desmosomes and tight junctions of the lateral plasmalemma (23,750X). B: TEM of microvillous cone present on the apical membrane of ciliated cells (35,250X).

Figure 2-5. Microvillous secretory cells of the luminal epithelium characterized by electron light secretory granules. **A:** Basal aspect of cell showing nucleus, paranuclear vacuole and electron light secretory granules (13,750X). **B:** Apical membrane showing release of secretory granules by exocytosis (35,250X). N, nucleus; SG, secretory granules; V, paranuclear vacuole.

Figure 2-6. Microvillous secretory cells of the luminal epithelium characterized by electron dense secretory granules. **A:** Transmission electron micrograph showing nucleus and characteristic electron dense secretory material (13,750). **B:** Apical membrane showing release of secretory material (23,750X). N, nucleus; SG, secretory granule; arrow, release of secretory material.

Figure 2-7. The lamina propria surrounding the endometrial glands of both the uterus and tube is highly vascularized. **A:** Transmission electron micrograph of an arteriole adjacent to the tubal endometrial glands (4,250X). **B:** Scanning electron micrograph of a small uterine endometrial blood vessel (1,200X). E, erythrocyte; G, endometrial gland; arrow, endothelial cell.

Figure 2-8. Histology and ultrastructure of the tubal endometrial glands. **A:** The endometrial glands (G) are branched acinar or branched tubular, and occupy the entire lamina propria between the luminal epithelium and the myometrium (1,800X). **B:** Most tubal endometrial glands are characterized irregularly shaped, electron dense secretory granules (10,000X). N, nuclei; SG, secretory granules.

Figure 2-9. Transmission electron micrographs of the tubal endometrial glands. **A:** Some tubal endometrial glands have secretory material of various electron densities (11,375X). **B:** The apical membrane of the endometrial gland cells exhibits short microvilli (11,375X). L, glandular lumen; N, nucleus; SG, secretory granules.

Figure 2-10. Histology and ultrastructure of the uterine endometrial glands. **A:** The uterine endometrial glands are branched tubular, connected to the lumen by short ducts (500X). **B:** The glands are characterized by spherical, electron dense secretory granules and numerous mitochondria (11,375X). N, nucleus; SG, secretory granules.

granules throughout the cytoplasm (Fig. 2-5A), which are released through exocytosis (Fig. 2-5B). Frequently, a paranuclear vacuole is present immediately apical to the nucleus (Fig. 2-5A), as are lipid droplets in the narrow zone between the nucleus and basal plasmalemma. The second type of microvillous secretory cell is characterized by basal nuclei, but these cells have electron-dense secretory granules near the apical membrane (Fig. 2-6A), which are secreted by exocytosis (Fig. 2-6B), but no paranuclear vacuoles have been identified.

Endometrial Glands

The lamina propria below the glands is highly vascularized, with the vessels in proximity to the glands (Fig. 2-7A, B). The endometrial glands of the tube are branched acinar or branched tubular (Fig. 2-8A). The endometrial gland cells are cuboidal and have extensive secretory granules, and a basal, irregularly shaped nucleus. The secretory vesicles, which fill most of the cytoplasm apical to the nuclei, are irregular in size and shape, but usually electron dense (Fig. 2-8B). These cells are further characterized by having abundant rough endoplasmic reticulum (RER) and ribosomes surrounding the nucleus. The lateral plasmalemma is extensively interdigitated. However, some cells possess secretory granules with various electron densities (Fig. 2-9A). The apical membrane of both types of endometrial gland cells bear numerous short microvilli (Fig. 2-9B).

The endometrial glands of the uterus are distinct from those of the tube. These are branched tubular glands with a duct that is histologically similar to the luminal epithelial cells (Fig. 2-10A). The glandular cells are cuboidal, with numerous eosinophilic secretory granules. The nuclei are basal and spherical, instead of irregular in shape as in the tube. The secretory granules are dispersed throughout the cytoplasm, and are uniformly spherical and electron dense (Fig. 2-10B). The cytoplasm is characterized by extensive RER, Golgi bodies and mitochondria. Further, the lateral

membranes show fewer interdigitations than observed for the tubal glands. The apical membranes of the endometrial glandular cells exhibit microvilli.

Discussion

The formation of albumen and eggshell has been extensively studied in birds, in which the albumen, shell membranes and calcareous shell are formed sequentially as the egg passes through separate regions of the oviduct; magnum, isthmus, and shell gland, respectively (Solomon, 1983). Although the same basic components are present in reptilian eggs, regions similar to those observed in birds have not been identified in the chelonian oviduct. Additionally, all eggs of a clutch are ovulated simultaneously in turtles, instead of sequentially as in birds. These differences have suggested the hypothesis that albumen and eggshell formation is substantially different in birds and turtles (Aitken and Solomon, 1976; Palmer and Guillette, 1988).

Luminal Epithelium

Several distinct types of luminal epithelial cells (bleb secretory cells, secretory and nonsecretory ciliated cells, two types of microvillous secretory cells) have been identified in the oviducts of turtles. Within the infundibulum and tube, different components of the albumen may be produced by these cell types. Bleb secretory cells, found within the grooves of the posterior infundibulum, have previously been identified in infundibular grooves of the lizard *Hoplodactylus maculatus* (Boyd, 1942) and the tortoise, *Gopherus polyphemus* (Palmer and Guillette, 1988). These cells may be homologous to the cells of the glandular grooves in the infundibulum of birds (Aitken and Johnston, 1963, Aitken, 1971). Although the ultrastructure of bleb secretory cells in reptiles remains unknown, they may function in the formation of initial albumen layers in the infundibulum (Palmer and Guillette, 1988, 1990b) as occurs in birds (Aitken and Johnston, 1963; Aitken, 1971).

The ciliated cells have distinctive secretory granules, which also have been noted in avian oviducts (Gilbert, 1979; Solomon, 1983), but were not described in the green turtle *Chelonia mydas* (Aitken and Solomon, 1976). The ultrastructure of the cilia is similar to that found in the sea turtle (Aitken and Solomon, 1976).

The microvillous cells with electron light secretory material resemble ultrastructurally that of mucus (glycosaminoglycan) secreting cells of the avian magnum (Wyburn et al., 1970; Gilbert, 1979). Although these cells are involved in mucus secretion, they do not have the compressed basal nuclei and cytology of typical goblet cells. The general histological appearance of the tubal epithelial cells and their positive staining for GAGs further resembles the avian magnum cells (Solomon, 1971; Wyburn et al., 1970). These mucus secreting cells in the avian oviduct have been identified as secreting avidin and ovomucin (Kohler et al., 1968; Wyburn et al., 1970; Tuohimaa, 1975). Avidin has also been identified in the secretions of the tube in lizards (Botte et al., 1974; Botte and Granata, 1977).

The other microvillous secretory cells, which have electron dense secretory material, are typical of glycoprotein secreting cells of the avian oviduct (Wyburn et al., 1973; Gilbert, 1979). These cells are found in the tube of the reptilian reproductive tract, although in birds they are not found in the magnum. Structurally similar cells were identified in the oviduct of the garter snake (Hoffman and Wimsatt, 1972). Unfortunately, the luminal secretory epithelial cells were not described in the sea turtle (Aitken and Solomon, 1976). The function of these cells is not clear, although in the tube they may secrete some albumen proteins, which are thought to be mostly glycoproteins.

Endometrial Glands

The endometrial glands of the turtle tube are ultrastructurally and histochemically like those of the avian magnum, supporting earlier hypotheses that the tube is involved in albumen formation in reptiles (Aitken and Solomon, 1976; Palmer

and Guillette, 1988; Guillette et al., 1989). In birds, the endometrial glands of the magnum are composed of three types of cells; A-cells, filled with electron dense granules, B-cells, filled with low electron density secretory material and C-cells, with prominent Golgi bodies and extensive RER. Types A and C may merely reflect different activity phases of the same cell type (Wyburn et al., 1970). The different cell types of the avian magnum are identified as secreting specific albumen proteins; A-cells secrete ovalbumin whereas lysozyme is secreted by B-cells (Kohler et al., 1968; Oka and Schimke, 1969; Wyburn et al., 1970). No secretory protein for C-cells has been identified. Most of the tubal endometrial gland cells in turtles have secretory granules that are similar to the avian type A-cells, which are electron dense. However, some tubal gland cells have lighter material intermixed with the dense secretory granules. This electron light material may (1) represent a different stage in processing of the secretory product, (2) be similar to the avian type B-cell secretory material, which is also electron light (Wyburn et al., 1970; Gilbert, 1979), or (3) be a unique secretory product. The latter two hypotheses suggest that the cells of the turtle magnum are less specialized than those of birds, with some cells producing a mixture of albumen proteins. Clearly, detailed biochemical studies are needed to determine the composition of reptilian albumen. Moreover, immunochemical studies are needed to resolve the secretory nature of the tubal epithelia and endometrial glands.

The homogeneity of endometrial gland cell structure along the reptilian tube suggests that the albumen secreted from different portions of the tube is also relatively uniform. In contrast, along the avian magnum separate structurally distinct subregions can be identified, which correspond to the characteristic layering of the albumen into thick and liquid albumen layers and fibrous chalaza (Aitken, 1971; Gilbert, 1979; Solomon, 1983). Reptilian eggs lack a chalaza, and the albumen appears relatively uniform in consistency, although it is laid down in concentric layers around the yolk (Webb et al., 1987; Palmer, unpublished data).

The formation of proteinaceous fibers of the eggshell membrane is not understood in reptiles. The uterine endometrial glands of turtles possess spherical, electron dense secretory granules and general cytological features that are comparable to those of the avian isthmus (Draper et al., 1972; Gilbert, 1979; Solomon, 1983), which secrete the eggshell fibers. This agrees with the findings of Aitken and Solomon (1976) in the sea turtle, and implies that the uterine endometrial glands of turtles, and possibly other reptiles as well, secrete the fibers of the proteinaceous eggshell membrane, as occurs in the isthmus of birds.

However, the eggshell fibers of birds and many reptiles are composed of two parts, an inner proteinaceous core and an outer carbohydrate sheath. This implies involvement of both RER (for syntheses of the proteinaceous components) and Golgi bodies (which are involved in glycosylation proteins and packaging carbohydrates for secretion) in fiber formation (Aitken and Solomon, 1976). The uterine endometrial glands of turtles show both well developed RER and Golgi bodies, suggesting that they are responsible for formation of both layers of the fibers. In some reptiles, the fibers appear pitted under TEM, which may represent a crude mixture of proteinaceous and carbohydrate fiber components (Solomon and Baird, 1977; Sexton et al., 1979; Andrews and Sexton, 1981; DeSalle et al., 1984; Trauth and Fagerberg, 1984). The fibers of the green turtle are pitted (mixture of protein and carbohydrate) while retained in the oviduct, but show the typical protein core and carbohydrate sheath morphology following oviposition, suggesting that all components are secreted simultaneously, but segregate over time (Solomon and Baird, 1977). This is similar to the fiber formation in birds, in which both components are present in the lumen of the glands (Solomon, 1983).

It remains to be determined how the eggshell membrane proteins are formed into a fibrous membrane surrounding the yolk in reptilian eggs. Agassiz (1857) concluded that the eggshell fibers polymerize directly around the yolk in turtles. This was later reported for various other reptiles (Giersberg, 1923; Weekes, 1927). However,

in birds, the eggshell proteins polymerize within the lumen of the endometrial glands and are extruded from the glands' duct as intact fibers (Solomon, 1983).

Although it is known that the reptilian uterus secretes the calcareous layer of the eggshell, the mechanism of calcium deposition remains to be determined. The uterine glands have little resemblance to those of the avian shell gland, which have been proposed to secrete the calcareous shell (Breen and de Bruyn, 1969). However, others contend that the luminal epithelium of the avian shell gland is responsible for calcium ion secretion (Gay and Schraer, 1971; Solomon et al., 1975), and that the endometrial glands are involved in "plumping water" transport (Solomon, 1983), the dilution of the albumen proteins by the addition of water and possibly enzymes (Solomon, 1979). Recent evidence in lizards suggests that the luminal epithelium of the reptilian uterus is the source of calcium ions for eggshell formation (Guillette et al., 1989). These data support the hypothesis that the uterus in turtles and lizards is dualistic in function, producing both the fibrous membrane and calcareous shell while eggs are held *in utero* (Aitken and Solomon, 1976; Palmer and Guillette, 1988, 1990b; Guillette et al., 1989), the endometrial glands forming the fibrous membranes and the luminal epithelium secreting the calcareous layer.

How the uterus is able to accomplish eggshell formation on an entire clutch simultaneously is unknown. There is frequently a spatial separation of eggs undergoing shelling along the length of the uterus, each within its own "egg chamber". This spatial separation may allow each egg to be shelled in isolation from the others in the uterus. The physiological controls that are involved in the secretion of both fibrous membranes and calcareous eggshell by the uterus remain unknown.

CHAPTER III

OVIDUCTAL MORPHOLOGY AND EGGSHELL FORMATION IN THE LIZARD, *SCELOPORUS WOODI*

Recent studies have suggested that eggshell formation in reptiles occurs strictly in the uterus (Aitken and Solomon, 1976; Fox, 1984; Palmer and Guillette, 1988; Guillette et al., 1989). This is in contrast to the pattern of eggshell formation in birds, in which the proteinaceous fibers are produced by the isthmus and the calcareous layer is subsequently secreted within the shell gland (Solomon, 1983). The endometrial glands of the chelonian uterus, and possibly of other reptiles as well, are ultrastructurally similar to those of the avian isthmus, possessing spherical, electron dense secretory granules (Chapter II). The uterine luminal epithelium has been shown to stain histochemically for calcium during gravidity in the lizard *Crotaphytus collaris*, suggesting that the luminal epithelium secretes the calcareous eggshell (Guillette et al., 1989). It has therefore been hypothesized that the reptilian uterus is dualistic in function, first producing the fibers of the eggshell membrane from the endometrial glands, and subsequently secreting calcium ions from the luminal epithelium for deposition of the calcareous eggshell layer while the eggs are retained in the uterus (Fox, 1984; Guillette and Jones, 1985; Palmer and Guillette, 1988; Guillette et al., 1989).

The mechanism for fiber formation in reptiles has received very little attention in recent years. Agassiz (1857) conducted extensive examinations on the formation of albumen and eggshell in a variety of turtles. He concluded that the proteins of the eggshell membranes gradually polymerized directly on the egg surface, first appearing as small particles that continue to increase in size and coalesce with one another until a long fiber is formed. This theory was later supported in several species of reptiles (Giersberg, 1923; Weekes, 1927). Recent authors have not speculated on how the fibers

were formed, but describe the fibers as branched and interwoven (Packard et al., 1988), which presumably could only occur by polymerization of fibers directly surrounding *in utero* eggs.

This hypothesis explaining fiber formation in reptiles is quite different from the pattern observed in birds, which has been extensively studied. It has been conclusively shown that the fibers of the eggshell membrane are produced by the endometrial glands of the avian isthmus, and that the proteinaceous material coalesces within the lumen of the gland (Solomon, 1983). The fused material is extruded from the glandular duct as an unbranched, completely formed fiber, which is wrapped around the egg by the twisting action of the myometrium. Definitive evidence for the mechanism of formation of the fibrous eggshell membrane remains to be determined in reptiles.

This study used the lizard *Sceloporus woodi* to examine oviductal functional morphology and the process of eggshell formation throughout gravidity, in order to assess the role of the uterus in eggshell formation. These results are correlated with a concurrent study on the timing and sequence of eggshell formation in the same specimens (DeMarco and Palmer, unpublished data).

Methods and Materials

Specimens

Florida scrub lizards (*Sceloporus woodi*) were collected within the Ocala National Forest, FL, and palpated to determine relative reproductive condition. Eighteen mid- to late vitellogenic (3-5mm diameter follicles) females were returned to the laboratory, where they were housed with mature males, maintained under natural light conditions, and fed *ad libitum*. As the females neared the end of vitellogenesis, they were palpated approximately every 4 hours to determine the time of ovulation. Following ovulation, females were killed by decapitation at selected time intervals (Table 3-1), and their oviducts (with eggs *in situ*) removed and fixed in 10% neutral buffered formalin.

Table 3-1. Identification of the females used in study of oviductal functional morphology and eggshell formation.

Specimen #	Duration from ovulation (days or hours)
3-18	vitellogenic
3-19	vitellogenic
5-12	9 (hrs)
5-9	12 "
4-17	1 day
4-20	2 "
5-8	2 "
3-9	3 "
7-17	3 "
6-15	4 "
7-16	5 "
4-6	6 "
5-11	7 "
6-13	9 "
2-14	11 "
2-17	14 "
2-13	15 "
4-18	post oviposition

Histochemistry

Following fixation, the oviducts were dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin (Humason, 1979). The tissues were serial sectioned at $7\mu\text{m}$ for a total of 10 slides (approximately 200 sections), and stained using various techniques (Table 2-1).

Scanning Electron Microscopy

The oviducts were fixed and dehydrated as for histology and CO_2 critical point dried (Anderson, 1951). The specimens were sputter coated with a gold-palladium alloy in a Denton Vacuum Desk-1. Examination and analyses were performed on a Hitachi S-415A SEM operated at 15kV.

Results

The infundibulum-tube is thin and flaccid, lacking endometrial glands, although posteriorly the endometrium forms longitudinal grooves. Throughout the infundibulum-tube and the uterus, two types of cells are apparent, ciliated and microvillous secretory. In the infundibulum proper, the epithelium is composed of cuboidal, eosinophilic cells, whereas in the tube, the secretory cells become slightly hypertrophied and distended, compressing the shorter ciliated cells (Fig. 3-1A, B). The uterine luminal epithelium also consists of ciliated and microvillous secretory cells (Fig. 3-2A). Extensive branched saccular or branched tubular glands are present in the uterine endometrium (Fig. 3-2B), with spherical, eosinophilic secretory granules. Posteriorly, the vagina is aglandular, with extremely tall, thin, longitudinal folds (Fig. 3-3A). The vaginal epithelium is almost entirely ciliated (Fig. 3-3B).

The oviductal changes occurring during gravidity are correlated with events following ovulation. The egg is coated with oviductal secretions as soon as it enters the ostium, as indicated by an egg already coated with secretory material when it had only partially entered the oviductal ostium from the coelomic cavity. Once the eggs are in

the uterus, deposition of the fibrous membranes of the eggshell are evident. By 9 hours post-ovulation, long, unbranched proteinaceous fibers are extruded from the ducts of most of the endometrial glands (Fig. 4A, B). At any one location, the fibers are being pulled in the same direction across the luminal epithelium, as is evident by the indentations formed in the apical membranes of adjacent epithelial cells. Most glands have stopped secreting long, continuous fibers by 24 hours post-ovulation, with only short pieces of fibrous material obvious in the mouths of glands and littering the surface of the luminal epithelium. Also by this time, the fibrous eggshell membrane is largely complete, with stratified layers of inner thick fibers, middle thin fibers, and an outer layer of proteinaceous particles (DeMarco and Palmer, unpublished data; Fig. 3-5A). For up until 6 days post-ovulation, small fragments of fibrous material can still be observed extruding from the ducts of occasional endometrial glands (Fig. 3-6B).

The luminal epithelium shows little hypertrophy during the first 24 hours post-ovulation (Fig. 3-6A). The apical membranes are relatively flat, with short microvillous projections. Later during gravidity, however, the apical membranes of the secretory cells become greatly distended as the cells hypertrophy, and the microvilli become less pronounced (Fig. 3-6B). By the end of gravidity, the epithelial cells have returned to a less distended appearance.

Discussion

By examining the functional morphology of the oviduct throughout the course of gravidity, several hypotheses concerning the formation of the shell are supported. Although it has been previously suggested that the eggshell fibers polymerize around the egg (Agassiz, 1857; Giersberg, 1923; Weekes, 1927) by an unknown mechanism that is distinct from that of birds, our data strongly support the hypothesis that the formation of the eggshell fibrous membrane in the lizard *Sceloporus woodi*, and perhaps other reptiles as well, occurs by a mechanism similar to that in birds. Indeed, the extrusion of

Figure 3-1. The luminal epithelium of the infundibulum and tube consists of ciliated and secretory cells. **A:** Histology of the infundibulum (2,000X). **B:** Scanning electron micrograph of the tubal epithelium, showing the distention of the secretory cells (2,400X). CT, connective tissue; E, luminal epithelium.

Figure 3-2. Histology and scanning electron microscopy of the uterus. **A:** The uterine luminal epithelium consisting of ciliated and microvillous secretory cells (2,400X). **B:** The uterus is characterized by branched acinar or branched tubular glands (2,000X).

Figure 3-3. Histology and scanning electron microscopy of the vagina. **A:** The vagina is aglandular and the endometrium forms tall, longitudinal folds (1,250X). **B:** The luminal epithelium of the vagina is largely composed of ciliated cells (4,800X). E, luminal epithelium.

Figure 3-4. Formation of the fibrous eggshell membrane. **A:** Extrusion of the eggshell fibers from the ducts of the uterine endometrial glands. The indentations are evident (arrows) where the fibers rested during eggshell formation (1,600X). **B:** Scanning electron micrograph of the extrusion of the eggshell fibers (6,000X).

Figure 3-5. Structure of the eggshell of *Sceloporus woodi*. **A:** Cross-section of the eggshell, showing the inner boundary membrane, inner thick fibers, middle thin fibers, and the outer particulate matter (4,000X). **B:** Surface of the eggshell with deposition of blocks of calcium carbonate apparent (1,000X). Ca, calcium deposits.

Figure 3-6. Changes in the gross morphology of the uterine epithelium during eggshell formation. **A:** The secretory cells of the luminal epithelium at 9 hrs post-ovulation, during formation of the fibrous eggshell membrane (2,400X). **B:** At 6 days post-ovulation, when calcium deposition is at its greatest, the luminal secretory cells bulge outward. A short fragment of eggshell fiber (arrow) is still apparent in the mouth of a glandular duct (1,200X).

intact fibers from the endometrial glands, which are similar in both morphological appearance and dimension to those present on the surface of the egg undergoing shell formation, is obvious. In a process that is similar to that of birds, the proteinaceous material secreted by the endometrial glands coalesces into a fiber as it is forced out of the neck of the gland (Solomon, 1983). The intact fiber must then be wrapped around the egg by an undetermined mechanism. Since fibers are pulled in the same direction away from the glandular duct, it is likely that the activity of the myometrium is rotating the egg within the oviduct, or at least siding the oviductal wall across the egg's surface. It is known that the reptilian myometrium is highly active following ovulation (Guillette, Matter and Palmer, unpublished data). Since the egg is within the uterus within 9 hours post-ovulation (when a substantial layer of fibers has already been deposited), and the fibrous membrane of the eggshell is essentially complete by 24 hours post-ovulation, the deposition of fibers appears correlated with the maximum activity of the myometrium (Guillette, Matter and Palmer, unpublished data).

The mechanism for deposition of albumen and formation of eggshells on an entire clutch simultaneously by the reptilian oviduct remains unclear. Albumen deposition begins immediately as the eggs enter the oviduct, as indicated by an egg that was only half inside the ostium that already had secretory material (albumen) deposited on the portion inside the duct. This indicates that the infundibulum-tube forms the egg albumen. However, the stimulus for albumen release is unknown, even in birds. The pressure of the descending yolk on the oviductal wall is generally thought to initiate albumen secretion in birds (Sturkie and Mueller, 1976; Laugier and Brard, 1980). Mechanical stimulation created by the introduction of foreign objects into the oviduct does induce albumen and eggshell formation in birds, although these are grossly abnormal (Wentworth, 1960; Pratt, 1960). However, isolated loops of avian oviduct also secrete albumen while an egg passes through the intact portion (Burmester and Card, 1939, 1941), indicating that neuronal or endocrine stimulation may affect albumen

secretion. Steroid hormones have been shown to induce albumen synthesis, but not release (Palmiter and Gutman, 1972; Palmiter and Schimke, 1973). Although the avian oviduct is innervated by the autonomic nervous system, administration of acetylcholine (parasympathomimetic) and ephedrine sulfate (sympathomimetic) had negligible effects on albumen secretion (Sturkie and Weiss, 1950; Sturkie et al., 1954). Prostaglandins (PGs) may be involved in initiation of protein release, as stretching of the tissues (such as caused by descending eggs) triggers PG secretion in reptilian tissues (Guillette and Palmer, unpublished data), which may signal neighboring cells to release albumen proteins. Additionally, explant tissue cultures of avian magnum (Mandeles and Ducay, 1962; O'Malley, 1967) and the tube of turtles (Chapter 7) and lizards (Palmer and Guillette, unpublished data) can secrete albumen proteins *in vitro*, without any apparent stimulation. As ovulation in most reptiles is thought to be simultaneous, all the eggs of the clutch must be coated with albumen within a short period. In *Sceloporus woodi*, all eggs are within the uterus 9 hours after ovulation. This indicates that either all the proteinaceous material for albumen formation must be stored in the cells prior to ovulation, or that there must be an additional, rapid synthesis of albumen protein by the tube immediately following ovulation. In the lizards *Crotaphytus collaris* and *Eumeces obsoletus*, and the tortoise, *Gopherus polyphemus*, there is not a significant decrease in the size of the tubal endometrial glands or the height of the luminal epithelium following ovulation (Guillette et al., 1989; Palmer and Guillette, 1990a). In birds, there is a rapid buildup of albumen within the magnum following each sequential ovulation (Gilbert, 1979).

The proteinaceous fibers of the eggshell membrane are deposited on all the eggs of a clutch within 24 hours in *Sceloporus woodi* (DeMarco and Palmer, unpublished data). It is unclear if eggshell formation begins on each egg as they sequentially enter the uterus, or if the process is switched on once all eggs are in place, as there was no distinguishable difference between the eggshell membranes of those eggs in different

regions of the uterus (DeMarco and Palmer, unpublished data). The stimulus for release of the eggshell fibers in birds is thought to be the pressure of the egg against the oviductal wall (Solomon, 1983). Release of fibers can be achieved in alligator and turtle reproductive tracts by mechanically stimulating excised portions of the fiber forming portion of the oviduct (Palmer, unpublished data). This suggests a role for prostaglandins in stimulating the secretion of eggshell proteins, as mechanical stimulation of reptilian oviducts is known to induce PG production and release (Guillette and Palmer, unpublished data). In *Gopherus polyphemus*, the endometrial gland cells (but not the diameter of the glands themselves) decrease in size and show fewer secretory granules following formation of the eggshell membrane (Palmer and Guillette, 1990a). This may indicate that most or all of the protein required for eggshell formation is stored in the glands. Further support for this hypothesis comes from the decrease in thickness of the fibers, and subsequent production of only particulate material, suggesting diminished stores of secretory material or polymerizing enzymes as eggshell formation proceeds. However, it has been shown that in the lizard *Cnemidophorus uniparens*, deluteinization immediately following ovulation disrupts the formation of the fibrous eggshell layer (Cuellar, 1979). It is unlikely that disruption of eggshell formation was due to shortened gestation length (6 days; Cuellar, 1979), as the eggshell membranes are formed in *Sceloporus woodi* within 24 hours. Further, the total thickness of the fibrous membrane was unchanged, but only thick fibers were laid down, instead of a progression from thick fibers basally to a top layer of fine fibers and particles, forming a dense mat. This suggests that control of fiber formation was disrupted, and that luteal hormones, such as progesterone, may be involved in the control of eggshell formation. This hypothesis is supported by the occurrence of a progesterone spike at ovulation (near the initiation of eggshell formation) in *Sceloporus woodi* (Guillette and DeMarco, personal communication).

The source of the calcareous eggshell layer in reptiles remains unknown. Even in birds, there has been considerable disagreement about the source of calcium for eggshell formation. The endometrial gland cells of the avian uterus are believed to secrete large quantities of "plumping water" (Breen and de Bruyn, 1969), and have many mitochondria, which are known to sequester calcium ions (Hohman and Schraer, 1966), suggesting that the endometrial gland cells are involved in the secretion of calcium ions. However, others have proposed that the surface epithelial cells transfer calcium to the oviductal lumen (Gay and Schraer, 1971; Solomon et al., 1975).

Several authors have hypothesized that in reptiles the secretory cells of the luminal epithelium may be involved in calcium transport (Guillette and Jones, 1985; Palmer and Guillette, 1988; Guillette et al., 1989). This is supported circumstantially by our data that during the periods of maximal calcification (days 3-9), as indicated by the buildup of calcium crystals on *in utero* eggs (DeMarco and Palmer, unpublished data), the secretory cells of the luminal epithelium become greatly distended and hypertrophied. Further evidence for the role of secretory cells is their proportional increase in number from vitellogenesis to gravidity. In the turtle *Gopherus polyphemus*, the uterine luminal epithelium is tallest and possesses the greatest proportion of secretory cells during gravidity. This is also known in several species of lizards (Christiansen, 1973; Fawcett, 1975; Guillette et al., 1989). Finally, the uterine secretory cells of the lizard *Crotaphytus collaris* stain positively for calcium during gravidity. Additional studies are clearly warranted to localize the source of calcium for eggshell deposition.

These data indicate that the uterus of lizards, and probably of turtles as well (based on ultrastructure; Chapter II), is dualistic in function, producing both the fibrous and calcareous layers of the eggshell sequentially. This is substantially different from the situation in birds, where each layer of the eggshell is produced by a separate, highly specialized region. It remains to be determined if this is a universal condition in all

reptiles, or if different reproductive anatomies occur in other groups. Particularly, as crocodilians are the closest living relatives of birds (Benton, 1985), the functional morphology and ultrastructure of their reproductive tract would be worthwhile investigating.

CHAPTER IV

OVIDUCTAL ULTRASTRUCTURE AND EGG FORMATION IN ALLIGATORS (*ALLIGATOR MISSISSIPPIENSIS*)

The process of eggshell formation has been extensively studied in birds and mammals, but little has been known concerning the formation of eggshell components in reptiles (Weekes, 1927; Boyd, 1942; Aitken and Solomon, 1976; Palmer and Guillette, 1988; Guillette et al., 1989). In birds, three oviductal regions exist, and each produces a separate component of the egg investments (albumen, fibrous eggshell membrane, and calcareous eggshell layer; Solomon, 1983). In egg-laying mammals, the monotremes, which produce reptile-like eggs (C. J. Hill, 1933; Hughes, 1977), the tube produces egg albumen and the uterus produces all layers of the eggshell (C. J. Hill, 1933, 1941; Hughes and Carrick, 1978). Likewise, eggshell formation in turtles, lizards, and the tuatara (*Sphenodon punctatus*), occurs strictly in the uterus (Palmer and Guillette, 1988, 1990b; Packard et al., 1988; Guillette et al., 1989; Chapter II and III). The uterine endometrial glands secrete intact fibers that are wrapped around the egg forming the eggshell membranes (Chapter III). Structural and histochemical evidence suggests that the secretory cells of the luminal epithelium secrete calcium ions for crystallization of the eggshell (Palmer and Guillette, 1988; Guillette et al., 1989; Chapter II and III). All the eggs of a clutch are shelled simultaneously, with each egg resting within its own uterine "chamber" (Chapter III). This supports the conclusion that the uterus of turtles and squamates is dualistic in function, forming both the fibrous and calcareous layers of the eggshell on an entire clutch simultaneously. This is substantially unlike eggshell formation in birds, in which separate regions secrete each eggshell component, and only one egg of a clutch is shelled at a time. The physiological control of eggshelling in

reptiles is largely unknown, although a luteal product, such as progesterone, is strongly implicated (Cuellar, 1979; Fox, 1984; Guillette and Jones, 1985; Guillette et al., 1989).

These major differences in oviductal gross morphology, ultrastructure, and presumed physiological control between reptiles and birds raises important questions about the evolution of avian eggshell formation from that of reptilian ancestors. The examination of chelonians and lepidosaurians has given little indication of the evolution of avian reproductive anatomy and physiology. In this study, oviductal functional morphology and ultrastructure were examined during the reproductive cycle in a crocodilian, the American alligator (*Alligator mississippiensis*). Crocodilians are archosaurs, as are the birds, and represent the closest living reptilian relatives of birds (Benton, 1985; Gauthier et al., 1988).

Methods and Materials

Specimens

Thirteen alligators (*Alligator mississippiensis*) exhibiting various reproductive conditions (vitellogenic, early gravid, late gravid, immediately post-oviposition and reproductively quiescent) were collected from several lakes (Griffin, Orange, and Okeechobee) in central Florida (Permit #W88063). Within 24 hours of capture, the specimens were anesthetized with 20mg/kg sodium pentobarbital, and the oviducts surgically removed under sterile conditions. Representative tissues from each oviductal region were dissected free and immediately fixed for histochemistry, scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

Histochemistry

Representative tissues from each region were fixed in 10% neutral buffered formalin or Bouin's fixative. Tissues were then washed, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin (Humason, 1979). Specimens were serial

sectioned at $7\mu\text{m}$ on a rotary microtome, for a total of 10 slides per tissue specimen, and stained as in Table 2-1.

Electron Microscopy

For transmission electron microscopy (TEM), tissue samples were minced (1 mm^3) and fixed in 2% glutaraldehyde with 0.1M cacodylate HCl buffer for 3 hours, washed in buffer (3 X 15 min), treated with OsO_4 for 30 min, and washed in buffer (3 X 15 min). The specimens were dehydrated in graded alcohols, treated with 100% acetone (2 X 1 hr), and embedded in Spurr's resin. Thick sections ($1\mu\text{m}$) were prepared and stained with toluidine blue. Thin sections were cut at $600\text{\AA}$, and poststained with uranyl acetate and lead citrate. Examination of tissues was performed on a Hitachi HU-11E or a Philips EM-300.

Specimens for scanning electron microscopy (SEM) were cut into 1 cm^3 , and fixed for 24 hours in 2% glutaraldehyde in 0.1M cacodylate HCl buffer. Tissues were dehydrated in graded alcohols, CO_2 critical point dried (Anderson, 1951), and sputter coated with gold. Examination was performed on a Hitachi S-450.

Results

General Oviductal Morphology

The general morphological characteristics of the alligator oviduct are presented in Fig. 4-1. There are seven clearly distinguishable regions along the length of the oviduct: anterior infundibulum, posterior infundibulum, tube (*tuba uterina*), transitional region, fiber region, uterus, and vagina. The anterior infundibulum is funnel shaped with thin, transparent walls and opens to the coelom across the distal margin. The posterior infundibulum is tubular, with thicker, more muscular walls and greater endometrial folding. There are no true glands in the endometrium, although it contains secretory cells. The demarcation between the infundibulum and the tube (*tuba uterina*) is indicated by the occurrence of endometrial glands in the tube, which are discernible at

the gross morphological level by a milky coloration. The tube is long and convoluted, although not to the extent of that in chelonians. The endometrium of this region is folded longitudinally, creating grooves which run along the length of the tube. At the posterior end of the tube is a short, narrow region, the transition zone, which is visually distinguished by being translucent due to the lack of endometrial glands. The "fiber region" is narrower than the tube and its walls are more muscular. Whereas the tube is flat in cross-section with a wide lumen, the fiber region is rounded with tall endometrial folds. The transition between the fiber region and the uterus is gradual. The outer diameter increases to that of the uterus, and the color (of fresh tissue) changes from pale cream or gray in the fiber region to a darker shade of reddish-gray in the uterus. The uterine lumen is greater in diameter than the fiber region, and the endometrium is formed into tall random folds. The vaginal lumen is extremely narrow and spirals through muscular walls. Each vaginal canal opens separately into the cloaca.

Tube

The luminal epithelium of the tube stains intensely with Alcian blue for glycosaminoglycans (GAGs), and consists of two types of tall simple columnar cells, ciliated and microvillous secretory (Fig. 4-2a, b). The ciliated cells have apical nuclei whereas the secretory cells have central, or occasionally basal, nuclei. The glands of the endometrium are branched acinar, often with extensive ducts connecting them to the surface (Fig. 4-3a). The duct cells are cuboidal and slightly eosinophilic. The endometrial gland cells are cuboidal or low columnar with basal nuclei. Ultrastructurally, the endometrial glands exhibit roughly spherical-shaped secretory granules of a wide range of electron densities (Fig. 4-3b). The apical membrane of the endometrial gland cells bears numerous microvilli.

Fiber Region

In the fiber region, the luminal epithelium consists of a simple columnar layer of ciliated and secretory cells, as in the tube, although they are lower and stain less intensely with Alcian blue. The endometrial glands are branched tubular, with short ducts connecting them to the lumen (Fig. 4-4a). The endometrial gland cells of the fiber region are cuboidal, with basal nuclei and numerous eosinophilic granules. TEM demonstrates that these granules are spherical and electron dense (Fig. 4-4b). During early gravidity, small (1cm^3) runt eggs, consisting of albumen surrounded by proteinaceous fibers, were observed within this region. In these early gravid specimens proteinaceous fibers were observed being extruded from ducts of the endometrial glands (Fig. 4-5a, b). These fibers are identical in structure and diameter to those of the eggshell membrane.

Uterus

The luminal epithelium of the uterus is composed of low columnar epithelial cells similar to those described for other regions. However, very few cells stained positively for GAGs using Alcian blue. The alligator's uterine endometrial glands are branched acinar with cuboidal cells that are not eosinophilic (Fig. 4-6a). The glandular cells have basal nuclei and contain numerous small, electron light secretory vesicles (Fig. 4-6b). These cells have numerous tall microvilli on the apical membrane and extensive interdigitations occur on their lateral cell junctions. Eggs are retained in the uterus for most of gravidity, where eggshell calcification was apparent. The thickness of the calcareous layer increases from early to late gravidity, as indicated by diameter and condition of the corpora lutea. Most eggs are calcified simultaneously, as all eggs from a single female had approximately equal shell thicknesses. However, in the earliest gravid female, those eggs from the ends of the uterus had thicker shells than those from the middle.

Figure 4-1. Gross morphology of the oviduct of the alligator (*Alligator mississippiensis*).

Figure 4-2. Histology and scanning electron microscopy of the oviductal luminal epithelium. **A:** The epithelium consists of two cell types, ciliated and secretory (2,000X). **B:** Scanning electron microscopy of ciliated and secretory cell types (4,000X). **E,** luminal epithelium; **G,** endometrial glands.

Figure 4-3. Histology and transmission electron microscopy of the oviductal tube. **A:** The tubal endometrial glands are branched acinar and completely fill the lamina propria (625X). **B:** The gland cells are characterized by secretory granules of various electron densities (18,750X). E, luminal epithelium; G, endometrial glands; N, nucleus; SG, secretory granules.

Figure 4-4. Histology and transmission electron microscopy of the fiber region of the oviduct. **A:** The endometrial glands of the fiber region are branched tubular and contain numerous eosinophilic secretory granules (500X). **B:** The glandular cells have numerous spherical, electron dense secretory granules (42,500X). E, luminal epithelium; G, endometrial glands; SG, secretory granules.

Figure 4-5. Formation of the fibrous membrane of the eggshell. **A:** The proteinaceous fibers of the eggshell membrane are extruded from the ducts of endometrial glands (2,400X). **B:** The fibers are complete and unbranched as they are extruded from the ducts (1,200X).

Figure 4-6. Histology and ultrastructure of the uterus. **A:** The uterine endometrial glands are branched acinar with cuboidal cells (850X). **B:** The glandular cells have numerous electron light secretory granules, apical microvilli and extensive interdigitations of the lateral and basal membranes (4,250X). **E,** luminal epithelium; **L,** glandular lumen.

Discussion

In all other reptiles, a single oviductal region has been identified in the production of eggshell components (uterus), whereas albumen is secreted by the tube. In alligators, the existence of a distinct secretory region between the uterus and tube, as occurs in birds, suggests that the ultrastructure and function of the alligator oviduct is more like that of avian species than of other reptiles. The gross morphology and ultrastructure of the alligator tube resembles the magnum of birds (Wyburn et al., 1970; Solomon, 1971, 1983; Gilbert, 1979), which is known to secrete albumen proteins, and the tube of turtles and squamates, which has been theorized to perform this function (Giersberg, 1923; Weekes, 1927; Botte et al., 1974; Aitken and Solomon, 1976; Botte and Granata, 1977; Palmer and Guillette, 1988, 1990a; Guillette et al., 1989). However, cells of the endometrial glands exhibit secretory granules that are similar to both avian type-A and type-B cells. This suggests that the cells of the alligator tube are more generalized, and secrete a more complex mixture of proteins. Recent immunocytochemical data support the conclusion that the tube functions in albumen formation in both turtles and the alligator (Palmer, 1988, 1989; Palmer and Guillette, 1990b; Chapter 7). The presence of the translucent transition zone between the albumen and eggshell membrane forming regions of the oviduct also occurs in birds and turtles (Aitken, 1971; Gilbert, 1979; Solomon, 1983; Palmer and Guillette, 1988).

The fiber region of alligators histochemically and ultrastructurally resembles the avian isthmus (Draper et al., 1972; Wyburn et al., 1973; Solomon, 1975, 1983) and the uterus of other reptiles, which secretes the eggshell fibers (Palmer and Guillette 1988, Guillette et al., 1989; Chapters 2, 3). The extrusion of eggshell fibers from the endometrial glands conclusively demonstrates that the fiber region of the alligator oviduct produces the proteinaceous fibers of the eggshell membranes.

The mechanism for formation of the complete eggshell membrane remains unclear. In birds, the isthmus produces the entire fibrous membrane on a single egg at

a time (Solomon, 1983). In the lizard *Sceloporus woodi*, the uterus secretes the eggshell fibers around an entire clutch simultaneously (Chapter 2). There are two possible hypotheses concerning the formation of the eggshell membrane in alligators. First, the fibers could be wrapped around the egg as it descends along the length of the fiber region. The anterior end would therefore produce the thicker underlying fibers and the posterior end would produce the thinner, more tightly packed fibers. The extrusion of intact fibers and presence of runt eggs were predominantly found in the anterior fiber region, where eggs first enter. This supports the first hypothesis, although it may also be due to other physiological mechanisms. Second, the eggs could enter the fiber region where each is coated with fibers as it is held within its own egg chamber, as occurs in the uterus of other reptiles (Chapter 3). The fiber forming region of the oviduct in alligators is about as long as the uterus, and presumably can contain all the eggs for that side. No specimens were collected with any eggs in the fiber region, so no data supporting either hypothesis are available.

The alligator uterus is ultrastructurally similar to the shell gland of birds (Breen and de Bruyn, 1969; Wyburn et al., 1973; Solomon, 1975, 1983), which secretes the calcareous eggshell, but is distinct from the uterus of other reptiles (Aitken and Solomon, 1976; Chapter 2). Although the alligator uterus resembles that of birds, an entire clutch is shelled simultaneously, as occurs in other reptiles (Ewert, 1979; Fox, 1984; Guillette and Jones, 1985; Guillette and Fox, 1987; Palmer and Guillette, 1988; Guillette et al., 1989; Chapter 3). The luminal epithelium of turtles and squamates is thought to secrete ions for calcification of the shell (Fox, 1984; Palmer and Guillette, 1988; Guillette et al., 1989). It is still unclear in birds if the endometrial glands (Breen and de Bruyn, 1969) or luminal epithelium (Gay and Schraer, 1971; Solomon et al., 1975) produce the calcareous shell. It is known that the endometrial glands of the avian shell gland secrete the plumping water in birds (Breen and de Bruyn, 1969), which hydrates the albumen proteins. The uterus of many reptiles also hydrates the albumen

before oviposition (Tracy and Snell, 1985; Palmer and Guillette, 1988, 1990b). The structure of the alligator's oviduct is similar to that of birds in general characteristics, such as separate regions for the production of each egg component, and in particular the uterine ultrastructure greatly resembles that of birds in both luminal epithelial and endometrial glandular characteristics. It is, therefore, possible that both plumping water and calcium ion secretion is similar to that of the avian shell gland. This similarity in structure, and possibly in function, between the oviducts of alligators and birds may be due to their common archosaurian ancestry.

In birds, the reproductive tract is essentially an assembly line, with each region performing only one specialized task, such as fibrous membrane formation or calcium secretion (Solomon, 1983). This condition also exists in crocodilians, which also exhibits an assembly line morphology, with separate regions for formation of albumen, fibrous eggshell membranes, and the calcareous shell. The other reptiles, including turtles, squamates, and the tuatara (*Sphenodon punctatus*), have oviducts that are different in functional morphology and eggshell formation than birds and crocodilians (Giersberg, 1923; Weekes, 1927; Boyd, 1942; Cuellar, 1966; Aitken and Solomon, 1976; Fox, 1977; Cuellar, 1979; Fox, 1984; Packard et al., 1988; Palmer and Guillette, 1988, 1990b; Guillette et al., 1989). These other reptile groups more closely resemble the reproductive anatomy of the egg-laying mammals, the monotremes (C. J. Hill, 1933, 1941; Hughes and Carrick, 1978; Hughes and Shorey, 1972; Hughes et al., 1975).

Although avian and crocodilian oviducts show great similarities in structure, and may be homologous, their reproductive modes differ in ovarian function. Birds ovulate only one egg of a clutch at a time (Gilbert, 1979), whereas crocodiles and most other reptiles ovulate an entire clutch simultaneously (Jones et al., 1979; Lance, 1989). The oviducts of alligators must still coat all the eggs of a clutch with albumen and eggshell simultaneously. The evolution of sequential ovulation in birds, and the associated changes in functional morphology and physiology involved, remains obscure.

These data represent a fundamental divergence in oviductal functional morphology in higher vertebrates. Clearly, more work is required on how the eggshell is formed in crocodilians and other reptiles. Particularly, it is still unknown how the eggshell fibers are wrapped around the egg, where calcium ions are secreted for eggshell formation, and how these processes are controlled. Further, little is understood about reptilian egg albumen biochemistry and its secretion (Palmer and Guillette, 1990b). This also may have important phylogenetic and ecological implications.

CHAPTER V REPTILIAN EGG ALBUMEN BIOCHEMISTRY

Egg albumen in birds exhibits a wide variety of functional properties (Palmer and Guillet, 1990b). Albumen is a complex mixture of proteins, each having unique characteristics, which contribute to the diverse properties of complete albumen. Alterations in protein composition in eggs change the functional properties of albumen as a whole. This may have important consequences on the embryo's ability to survive under different nest and incubation conditions (Palmer and Guillet, 1990b). There is considerable variability in protein composition, both in kinds of proteins present and their concentrations, in eggs of different avian species (Sibley, 1970; Sibley and Ahlquist, 1972). Embryos whose eggs have a protein composition with properties suitable to available nest and incubation conditions will have a selective advantage (Palmer and Guillet, 1990b). Selective pressures and/or phylogenetic inertia may influence albumen composition.

In birds, several albumen properties have been identified as vital to embryonic survival, including antimicrobial, nutritive, water balance, support and cushioning. Antimicrobial proteins may have antibacterial, antifungal, and/or antiviral characteristics (Lanni and Beard, 1948; Lanni et al., 1949; Gottschalk and Lind, 1949a, b; Matsushima, 1958; Feeney et al., 1963; Tomimatsu et al., 1966; Feeney, 1971; Geoffroy and Bailey, 1975; Tranter and Board, 1982a, b; Banks et al., 1986; Board and Tranter, 1986; Li-Chan and Nakai, 1989). Other albumen proteins supply nutrients through either direct consumption by the embryo or selective uptake. These nutritive proteins may be required for their amino acid and carbohydrate content, or other molecules bound to them, such as vitamins or minerals, or for the proteins themselves, which are transported

intact to the embryonic blood stream (Marshall and Deutsch, 1951; Wise et al., 1964; Adiga and Murty, 1983; White, 1987, 1990; White et al., 1987; Bush and White, 1989). Proteins that maintain water balance or support and cushion the developing embryo act by insulating the embryo from the environment, both mechanically and chemically (Romanoff and Romanoff, 1949; Palmer and Guillette, 1990b).

The albumen of reptilian eggs has been suggested to function in several capacities, although it remains largely unstudied. These functions include water storage and reduction in the rate of water exchange (Packard et al., 1977; Tracy, 1980, 1982; Andrews and Sexton, 1981; Snell and Tracy, 1985; Tracy and Snell, 1985; Webb et al., 1987a, b; Manolis et al., 1987; Ackerman, 1990; Packard, 1990; Palmer and Guillette, 1990b), antimicrobial properties (Movchan and Gabaeva, 1967; Ewert, 1979; Mandal et al., 1989) and support and cushioning (Palmer and Guillette, 1990b). These properties may have evolved in response to environmental pressures (Palmer and Guillette, 1990b).

Although the albumen proteins of avian eggs are among the most intensively studied proteins in biochemistry (Li-Chan and Nakai, 1989), the proteins of reptilian egg albumen have received little attention in the past. Ovalbumin, the major component of avian eggs, has not been identified in reptilian eggs. The occurrence of ovotransferrin, another major avian albumen protein, was recently suggested in the turtle *Pseudemys floridana* (Palmer, 1988). Neither ovalbumin nor ovotransferrin was detected in the albumen of *Crocodylus porosus* (Burley et al., 1987), although a major protein of 59,000 molecular weight was identified. In two species of crocodilians, *Crocodylus rhombifer* (Ikai et al., 1983) and *C. porosus* (Burley et al., 1987), an α_2 -macroglobulin-like molecule was detected. Avidin, a biotin-binding protein common in vertebrate eggs and other tissues, is synthesized by oviducts of the lizard *Lacerta s. sicula* (Botte et al., 1974; Botte and Granata, 1977), although neither avidin nor other vitamin-binding proteins (riboflavin- or thiamin-binding protein) have been detected in the albumen of *Alligator mississippiensis* (Abrams et al., 1988, 1989; White, 1990).

Biochemistry of reptilian albumen may be crucial to understanding not only the physiological ecology and developmental biology of eggs and embryos, but may also shed light on their evolutionary history. This study used modern biochemical techniques to separate and identify the proteins of egg albumen from representative reptiles into individual components. This is a first step in an intensive ongoing investigation into the biochemistry and molecular biology of reptilian oviducts and their secretions, such as egg albumen (Palmer and Guillette, 1990b).

Methods and Materials

Specimens

Eggs of various reptilian species were obtained for analysis, as indicated in Table 5-1. Sufficient clutches (5) and numbers (25) of alligator eggs were available to allow comparison of variations in albumen components both within and between clutches. The eggs of alligators and turtles were collected immediately post-oviposition, removed from gravid females by surgery or administration of arginine vasotocin (AVT), or determined to be infertile from natural nests. Eggs from squamate species were allowed to develop to embryonic stages 35-40 (Dufaure and Hubert, 1961; Hubert and Dufaure 1968; Hubert, 1985) in order to facilitate collection of the minute quantities of albumen, which swells with water during incubation. Tuatara albumen was collected immediately from artificially oviposited (induced with AVT) eggs of late-gravid females. Unlike the eggs of other reptiles, which are retained for only several weeks, the tuatara retains eggs *in utero* for 7-8 months (Thompson, personal communication). The entire albumen was separated from shell and yolk, frozen in liquid N₂, lyophilized, ground with a mortar and pestle, and stored desiccated at 0-5°C.

Table 5-1. Reptilian eggs analyzed for albumen protein composition.

Specimens	# of Eggs (Clutches)
<u>CROCODYLIA</u>	
American alligator (<i>Alligator mississippiensis</i>)	25 (5)
Morelet's crocodile (<i>Crocodylus moreletii</i> *)	2 (1)
<u>TESTUDINES</u>	
Florida peninsula cooter (<i>Pseudemys floridana peninsularis</i>)	12 (3)
Chicken turtle (<i>Deirochelys reticularia</i>)	5 (1)
Galapagos tortoise (<i>Geochelone elephantopus</i>)	3 (2)
<u>SQUAMATA</u>	
Ringneck snake (<i>Diadophis punctatus</i>)	5 (1)
Florida scrub lizard (<i>Sceloporus woodi</i>)	5 (1)
Striped plateau lizard (<i>Sceloporus virgatus</i>)	5 (1)
Bunch grass lizard (<i>Sceloporus scalaris</i>)	5 (1)
Brown anole (<i>Anolis sagrei</i>)	5 (1)
<u>SPHENODONTIA</u>	
Tuatara (<i>Sphenodon punctatus</i>)	2 (1)

*Positive identification of species was not possible, but *C. moreletii* is highly probable.

Electrophoresis

The albumen samples were solubilized with 2% sodium dodecyl sulfate (SDS) and 10% 2-mercaptoethanol in 10mM Tris buffer (pH 7.4) and separated by molecular weight using one-dimensional polyacrylamide gel electrophoresis (1D-SDS-PAGE). Separation gels were made from 7, 8, 9, and 10% T (total acrylamide) to facilitate analyses of both low and high molecular weight proteins. Protein samples (40 μ g dry weight) were loaded onto a discontinuous PAGE apparatus (Smith, 1984), stacked at 10 mV/slab for approximately 1-2 hours and then run at 20mV/slab for 3-5 hours, or until complete. The gels were fixed, and stained with Coomassie blue (Smith, 1984). Molecular weights were determined using protein standards (Table 5-2) and determination of R_f values (Shapiro et al., 1967).

Electroblotting

In order to identify proteins with specific antibody cross-reactivity, proteins were transferred to nitrocellulose immediately following PAGE. The gels were equilibrated in transfer buffer (25mM Tris, 192 mM glycine, and 20% v/v methanol) immediately following electrophoresis and the proteins transferred to nitrocellulose under a 300mA electrical field. The nitrocellulose was equilibrated in Tris buffered saline (TBS; 25mM Tris, 0.3M NaCl, pH 7.4) for 15 min, blocked with 2.5% powdered milk in buffer (1 hour), and incubated for 6-8 hours with antibodies (diluted 1:500 in 2.5% buffered powdered milk) specific for ovalbumin (see purification methods below), whole hen albumen (Sigma C6534), human α_2 -macroglobulin (Sigma M1893), and human transferrin (Sigma T6265). The nitrocellulose was washed in buffer (3 X 5 min), and incubated overnight with either goat anti-rabbit or rabbit anti-goat antibodies (diluted 1:1000 in 2.5% buffered powdered milk) conjugated with alkaline phosphatase. The nitrocellulose was washed again in buffer (3 X 5 min), and incubated at 37°C in 0.1 g/l nitroblue tetrazolium (NBT), 0.05 g/l 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and

Table 5-2. Proteins used as molecular weight standards for one-dimensional polyacrylamide gel electrophoresis.

Protein	Molecular Weight X 10 ³
Thyroglobulin	335
β-Galactosidase	116
Phosphorylase B	92.5
Transferrin	76
Bovine serum albumin	66
Ovalbumin	45
Carbonic anhydrase	30
Soybean trypsin inhibitor	20
Cytochrome c	12.5

2mM MgCl_2 in 0.1M Tris buffer (pH 8.8 for 15 min) for localization of cross-reactive proteins.

Purification of α -Ovalbumin Antibodies

A 1 ml column was prepared by swelling 0.3g Sepharose-4B-CNBr in 1mM HCl for 15 min., rinsed with 70 ml of 1mM HCl, and washed three times with 1 ml coupling buffer (0.1M NaHCO_3 and 0.5M NaCl). Ovalbumin (Sigma C6534) was dissolved in coupling buffer (12 mg/2 ml), added to the Sepharose, agitated for 2 hours, and rinsed with coupling buffer. The column was flushed with 50 ml (1 ml/min) of blocking buffer (1M 2-aminoethanol, 0.1M NaHCO_3 and 0.5M NaCl), rinsed with Tris buffered saline (TBS), and flushed three times with 1 ml of glycine buffer (0.05M glycine, 0.5M NaCl, pH 2.6). The column was equilibrated with 25 ml TBS, and rinsed with glycine buffer and TBS sequentially 4 times.

Following column preparation, antibodies specific to whole hen egg albumen (Sigma C6534) was drawn through the column at 1 ml/min, and washed with TBS (25 ml). The column was eluted progressively with 7 ml of 100, 200, 300, and 400 mM sodium chloride, followed by 7ml of glycine buffer. Antibodies were collected in 1 ml aliquots, and 30 μl 0.1M Tris-HCl (pH 7.4) was added to each aliquot.

Specificity of purified α -ovalbumin antibodies was tested against ovalbumin (Sigma A-5503), ovotransferrin (Sigma C-1005), and ovomucoid (Sigma T-2011), the most abundant proteins of avian egg albumen. Each protein (50 μl at a concentration of 1 mg/ml in TBS) was individually added to wells of a culture plate for 15 min, and rinsed three times with TBS. Powdered milk (2.5%) in TBS was added to the wells as a blocking agent for 15 min and rinsed three times with TBS. Both salt and glycine wash fractions of the α -ovalbumin antibodies purified above were added to separate wells and agitated for 30 min before rinsing the wells (3X) with TBS. Goat anti-rabbit antibodies conjugated with alkaline phosphatase were diluted 1:1000 in 2.5% buffered milk and added to each well for 30 min, and rinsed (3X) with TBS. The culture plates were

incubated at 37°C in 0.1 g/l nitroblue tetrazolium (NBT), 0.05 g/l 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and 2mM MgCl₂ in 0.1M Tris buffer (pH 8.8 for 15 min) for localization of cross-reactive proteins. Controls included wells without protein antigen (blocked with milk), and omission of primary antibodies. The purified antibody fraction with the highest affinity for ovalbumin (glycine wash) was used in immunodetection. This fraction showed minimum cross-reactivity to other antigens, and no activity with either control.

Results

The albumen of reptilian eggs demonstrates extensive variation among reptilian orders (Fig. 5-1, 5-2), with greater similarities found within orders. However, differences are apparent even within genera. The specifics of each are discussed below.

Crocodilians

The separation of alligator egg albumen by relative molecular weight is shown in Fig. 5-1. There is a low molecular weight protein of 26kd, a complex of three proteins ranging from 40-50kd, major protein bands at 57kd and at 75kd, and three high relative molecular weight (Mr) proteins at 116, 166, and 338kd. Little variation in protein composition either within or between clutches was detected (Fig. 5-3).

The albumen of *Crocodylus moreletii* showed many similarities with that of alligators (Fig. 5-4). The three highest molecular weight proteins observed in *C. moreletii* were similar to those in alligators, with molecular weights of 116, 166, and 330kd, respectively. Several other proteins which are similar to those found in alligator eggs were exhibited, with molecular weights of 74, 52, and 45kd.

Testudines

Many more proteins were identified as part of the albumen of turtle eggs than were detected in the albumen of alligators (Fig. 5-1, 5-2, 5-3). In *Pseudemys floridana*,

two proteins were detected at a Mr of 41 and 47kd, with a minor band at 56kd. Two proteins were found at a Mr of 72 and 79kd, whereas another pair occurred at 98 and 104kd. There was also a high molecular weight protein at 174kd.

Another emydid species of water turtle, *Deirochelys reticularia*, had many similar proteins to the above species, including those with a Mr of 174, 79, and 41kd, with other proteins varying slightly, having relative molecular weights of 166 and 63kd. In the Galapagos tortoise, *Geochelone elephantopus*, the egg proteins were similar to those of *Pseudemys* showing Mr of 174, 107, 98, and 79kd, but differing slightly with the remaining proteins at Mr 69 and 43kd.

Squamates

Squamates exhibited the greatest amount of variation in egg albumen proteins among species (Fig. 5-1). The snake, *D. punctatus*, exhibited the greatest number of proteins in its albumen. There was a major band at a Mr of 28kd, and several minor bands ranging from a Mr of 30 to 47kd. A pair of protein bands appeared at Mr 55 and 63kd, and a single protein band at a Mr of 78kd. Three proteins over 100kd were apparent, with relative molecular weights of 116, 174 and 214kd.

The albumen of lizard eggs was dominated by proteins of low molecular weight (Fig. 5-1), particularly with protein bands in the 27 to 31kd range. *Sceloporus woodi* and *S. scalaris* had two very similar proteins at 28 and 30kd, whereas another member of the genus, *S. virgatus*, exhibited 3 proteins in the range from 27 to 30kd. A lizard from an unrelated genus, *Anolis sagrei*, had 4 proteins within the range of 29 to 31kd. Albumen from all lizards examined were consistent in higher relative molecular weight proteins, occurring at 45kd, 56kd, 76kd, and one at approximately 175-200kd.

Sphenodon

The albumen of the tuatara, *Sphenodon punctatus*, had only 5 proteins detectable by 1D-SDS-PAGE (Fig. 5-2). Two of these were over 100kd, occurring at Mr

of 129 and 112kd. There were two intermediate Mr proteins at 54 and 39kd, and low Mr protein at 26kd.

Immunodetection

Immunodetection using antibodies specific to ovalbumin indicated cross-reactivity with the protein band at the approximately Mr of 45kd in albumen of all reptile groups examined (except for the tuatara, which was not tested). In turtles and alligators, this represented a relatively abundant albumen protein. However, in squamates, this protein was barely detectable using 1D-SDS-PAGE, indicating that it forms a very low percentage of the albumen in these species.

The other antibodies used for immunodetection had greater variability among species. An α_2 -macroglobulin-like protein was detected using western blots only in eggs of *Crocodylus moreletii*. Polyclonal antibodies to human transferrin, a protein similar to avian ovotransferrin, bound to proteins of approximately 76kd in both crocodilians tested (*Alligator mississippiensis* and *Crocodylus moreletii*), and in turtles (*Pseudemys floridana* and *Deirochelys reticularia*), but not in the Galapagos tortoise (*Geochelone elephantopus*), snake (*Diadophis punctatus*; other squamates were not tested) or the tuatara (*Sphenodon punctatus*).

Discussion

Egg albumen proteins exhibit substantial differences among reptilian orders. However, based on analyses of alligator eggs, little variation in protein composition occurs either within or between clutches. Although there are clearly proteins that are apparent across reptilian taxa, there are, however, two distinct subgroups among reptiles, those whose albumen composition is dominated by higher molecular weight components (turtles and crocodilians), and those that have predominantly lower molecular weight proteins (squamates, particularly lizards). The squamate albumen was collected following some embryonic development, which may have altered its composition,

Figure 5-1. Separation of egg albumen proteins from representative reptilian groups by molecular weight using 10%T one-dimensional SDS polyacrylamide gel electrophoresis to demonstrate low molecular weight components. Lanes: 1, *Alligator mississippiensis*; 2, *Pseudemys floridana*; 3, *Diadophis punctatus*; 4, *Sceloporus woodi*; 5, *Sceloporus virgatus*; 6, *Sceloporus scalaris*; 7, *Anolis sagrei*.

Figure 5-2. Separation of egg albumen proteins from representative reptilian groups by molecular weight using 7%T SDS-PAGE to demonstrate high molecular weight components. Lanes: 1, standards; 2, *Alligator mississippiensis*; 3, *Crocodylus moreletii*; 4, *Geochelone elephantopus*; 5, *Pseudemys floridana*; 6, *Deirochelys reticularia*; 7, *Sphenodon punctatus*; 8, *Anolis sagrei*.

Figure 5-3. Comparisons of *Alligator mississippiensis* albumen proteins of eggs from clutches of 5 different females, separated by molecular weight by 1D-SDS-PAGE. Lanes: 1, standards; 2-6, eggs removed from uterus of female #2; 7-11, infertile eggs collected from natural nest, clutch 100; 12-16, infertile eggs collected from natural nest, clutch OKS-09-88; 17-21, eggs removed from uterus of female #4; 22-26, infertile eggs collected from natural nest, clutch JE-61; 27, standards.

Figure 5-4. Comparisons of albumen proteins of eggs from a crocodilian and three species of turtles separated by 1D-SDS-PAGE. Lanes: 1, standards; 2-3, *Pseudemys floridana* eggs from clutch 1; 4-9, *Pseudemys floridana* eggs from clutch 2; 10-14, *Deirochelys reticularia* eggs from a single clutch; 15-18, *Geochelone elephantopus* eggs from 2 clutches; 19-20, *Crocodylus moreletii* eggs from a single clutch.

although it is unclear what changes may have taken place. The tuatara (*Sphenodon punctatus*) has proteins in common with each of the other reptilian groups. The tuatara, anatomically the most ancient of all reptiles, may therefore possess egg albumen whose composition is ancestral to the other, more derived groups.

These differences among reptilian taxa may be due to more than phylogenetic inertia. Albumen proteins possess different properties that influence embryonic development, such as microbial defense, water balance, and nutrition (Palmer and Guillette, 1990b). There is much diversity in egg structure and nest microhabitat among reptilian groups. The differences in albumen composition may be related to these other factors, and represent adaptations to their nesting ecology. Turtles and crocodilians usually have thicker eggshells and proportionally more albumen than most squamates (Packard et al., 1977). The thicker eggshell may reduce both microbial invasion and water loss, while the abundant albumen can serve as a temporary water reserve under desiccating conditions. Further research into both the ecological and physiological significance of reptilian albumen proteins is clearly warranted.

A protein similar in molecular weight to the major avian albumen protein, ovalbumin, and exhibiting cross-reactivity to α -ovalbumin antibodies is present in crocodilians, chelonians and squamates. This represents the first demonstration of an ovalbumin-like molecule in non-avian eggs. Although it was not quantified, it is clearly not as abundant in alligator and turtle eggs as it is in avian eggs (it comprises 54% of hen albumen), and is found in minute quantities in squamate eggs. No protein similar in molecular weight to ovalbumin was detected in the tuatara, although more rigorous tests are required before the presence of an ovalbumin protein in the albumen of tuatara eggs can be conclusively ruled out. Ovalbumin was not detected in eggs of *Crocodylus porosus* (Burley et al., 1987) by gel electrophoresis.

Ovalbumin belongs to a superfamily of serine protease inhibitors (serpins), which function in the inhibition of proteolytic activity (Breathnach et al., 1978; Hunt and

Dayhoff, 1980; Woo et al., 1981; Carrell and Boswell, 1986; Ye et al., 1989). This activity could inhibit bacterial growth by preventing the breakdown of other albumen proteins for bacterial nutrition (Tranter and Board, 1982b; Board and Tranter, 1986). However, even though ovalbumin has been extensively studied, no biologically significant properties have been described for it (Li-Chan and Nakai, 1989).

Ovotransferrin is the second most abundant protein in avian eggs, comprising 11-13% of hen albumen (Li-Chan and Nakai, 1989). Ovotransferrin, a member of the family of iron-binding proteins, is identical in amino acid sequence to the mammalian plasma protein transferrin, differing only in the carbohydrate side chains (Williams, 1968) and the absence of sialic acid (Osuga and Feeney, 1968). The conserved structure and universal appearance of transferrins in many fluids and tissues of vertebrates supports their occurrence in reptilian as well as avian eggs.

An ovotransferrin-like molecule has been detected in turtle (*Pseudemys floridana*) albumen (Palmer, 1988), based on molecular weight data. This is supported by the presence of a protein of similar molecular weight (76-81kd) in all species examined. Further, these albumen proteins demonstrate cross-reactivity to human plasma transferrin in both crocodilians (*Alligator mississippiensis* and *Crocodylus moreletii*) and fresh water turtles (*Pseudemys floridana* and *Deirochelys reticularia*). No cross-reactivity to human transferrin was found in the Galapagos tortoise (*Geochelone elephantopus*), squamates, or the tuatara. No molecules of appropriate molecular weight to ovotransferrin were detected in eggs of *Crocodylus porosus* (Burley et al., 1987), suggesting that vigorous investigation is required into the occurrence of ovotransferrin-like proteins in reptilian species.

The antibacterial nature of ovotransferrin is well known (Weinberg, 1977; Tranter and Board 1982a, b; Board and Tranter, 1986). Due to its high affinity for iron (Chasteen 1977, 1983; Aisen and Listowsky, 1980; Brock, 1985), this protein creates an essentially iron-free environment in which bacterial growth is inhibited. The slight

variations in molecular weight found among and within species may be due to molecules being partially saturated with iron. Clearly, further investigation into the presence, structure, and biological function of ovotransferrin-like molecules in reptilian eggs is warranted.

A major protein band of high molecular weight (approximately 166kd in crocodilians and 174kd in chelonians and squamates) occurred in all specimens examined in the presence of SDS and following reduction with 2-mercaptoethanol. Additionally, in *Crocodylus moreletii*, this protein showed cross-reactivity with antibodies to α_2 -macroglobulin, which has a molecular weight of 725kd. An α_2 -macroglobulin-like protein has been found in the eggs of *Crocodylus rhombifer* (Ikai et al., 1983), *C. porosus* (Burley et al., 1987), and the marine turtle *Caretta caretta* (Mandal et al., 1989), having Mr of 730kd, 150kd (following reduction with 2-mercaptoethanol), and 724kd, respectively.

A protein similar to α_2 -macroglobulin, ovomacroglobulin, is found in avian eggs (Li-Chan and Nakai, 1989), with a molecular weight of 760-900kd. Ovomacroglobulin, like ovalbumin, belongs to the serpin family of proteins, functioning as a protease inhibitor, and is known to play a role in bacterial defense (Tranter and Board, 1982b; Board and Tranter, 1986). The related protein in crocodilians has been shown to inhibit trypsin, subtilisin and papain (Ikai et al., 1983), and may be an important antibacterial agent in reptilian eggs (Burley et al., 1987).

A major protein of relative molecular weight 52-59,000 was detected in the eggs of alligators and crocodiles, the turtle *Pseudemys floridana*, the snake *Diadophis punctatus*, and in the tuatara. A similar protein was previously identified in *Crocodylus porosus* (Burley et al., 1987). It is absent from the eggs of lizards, however, and its functional role remains to be determined.

Avidin, a biotin-binding protein found in many different tissues in vertebrates (Green, 1975; Elo and Korpela, 1984), forms a small fraction (0.05%) of the avian egg

albumen protein (Li-Chan and Nakai, 1989). Although avidin synthesis was observed in oviducts of the lizard *Lacerta s. sicula* (Botte et al., 1974; Botte and Granata, 1977), neither avidin, riboflavin-binding protein, nor thiamin-binding protein has been detected in reptilian egg albumen (Abrams et al., 1988, 1989; White, 1990).

The lizards of the genus *Sceloporus* presented an opportunity to examine the egg albumen of closely related species. Not surprisingly, the protein composition of their albumen shows great similarities. However, some variations occur within related protein bands, such as those from Mr 27-30kd. The protein composition of the snake *Diadophis punctatus* showed similarities with both turtles and crocodilians, and the other squamates examined, the lizards. However, the tuatara also possessed proteins which were found across reptilian groups. The significance of these observations requires additional detailed studies on a broader variety of species.

Clearly, the study of albumen proteins of reptiles is in its infancy. There appears to be a greater diversity in composition of albumen proteins of reptiles (Burley et al., 1987; Palmer, 1988, 1989; Abrams et al., 1989) than that of birds, and possibly also in the functional role of albumen in embryonic development. Albumen of reptilian eggs has been suggested to act in water storage (Tracy and Snell, 1985; Webb et al., 1987; Manolis et al., 1987), to resist lethal rates of water exchange (Tracy and Snell, 1985), and to possess antimicrobial properties (Movchan and Gabaeva, 1967; Ewert, 1979). These properties, and the protein composition from which they are derived, may have evolved in response to nest and incubation conditions (Packard and Packard, 1980; Tracy, 1980, 1982; Tracy and Snell, 1985; Palmer and Guillette, 1990b).

CHAPTER VI REPTILIAN EGG ALBUMEN SECRETION

The site of egg albumen formation has never been definitively demonstrated in reptiles. Conventionally, it has been assumed that the albumen of reptilian eggs is formed in the tubal region (*tuba uterina*) of the oviduct. Indeed, this region bears many histological similarities to that of the avian magnum, with which it may be homologous (Aitken and Solomon, 1976; Palmer and Guillette, 1988; Guillette et al., 1989; Chapter II, IV).

Experiments to delimit the site of albumen formation in reptiles, however, have been inconclusive. Avidin, a biotin-binding protein that is found in avian eggs, and various tissues of many vertebrates, was found to be synthesized and secreted by the tubal region of the oviduct in the lizard *Lacerta sicula* (Botte et al., 1974; Botte and Granata, 1977). Careful analysis of reptilian eggs has failed to demonstrate the presence of avidin in their albumen (Abrams et al., 1988, 1989; White, 1990).

The site of albumen formation in birds has been extensively studied. Although some avian albumen proteins are either identical (avidin; Green, 1975; Elo and Korpela, 1984) or similar (ovotransferrin; Chasteen 1977, 1983; Aisen and Listowsky, 1980; Brock, 1985) to proteins found in other body tissues or fluids, the proteins of egg albumen are synthesized directly by the oviduct. This has been demonstrated by the *in vitro* incorporation of radiolabeled amino acids into albumen proteins (Mandeles and Ducay, 1962; O'Malley, 1967).

The following experiments are part of an ongoing investigation into the functional morphology and biochemistry of reptilian oviducts and their secretions. The

purpose of these experiments was to determine the site of albumen protein synthesis and secretion within the reptilian oviduct.

Methods and Materials

Specimens

Five female Florida peninsula cooters (*Pseudemys floridana peninsularis*) and 5 stinkpot turtles (*Sternotherus odoratus*) were collected with baited funnel traps in Alachua county, FL. The turtles were anesthetized with 20 mg/kg sodium pentobarbital, and the oviducts surgically removed under sterile conditions. Portions of the tube (*tuba uterina*) and uterus were dissected free, minced into 2mm³ pieces, and 0.5 g of tissue was placed into sterile culture using 15 ml modified (leucine deficient) Minimum Essential Media (MEM) to which 100μCi ³H-leucine was added (Nuzzolo and Vellucci, 1983). The tissues were incubated at 30°C on a rocking platform for 24-48 hours.

Sample Preparation

From each culture dish, 0.5 ml samples were collected at 4 hour intervals for 24 hours, then at 6 hour intervals for the next 24 hours. The remaining media was collected at the end of culture and the tissue preserved in 10% neutral buffered formalin. Media samples were dialyzed using Mr 6-8 kd cutoff tubing (Spectra/Por 1) against 50mM Tris/0.6M NaCl buffer (pH 7.5) for 2 X 24 hours at 4°C, and dH₂O for 24 hours at 22°C. The total activity of the sample was determined by scintillation counting. The samples were stored at -85°C.

Electrophoresis

Both one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE) and two-dimensional (2D) SDS-PAGE were used to separate proteins. 1D-PAGE separates proteins, in general, by molecular weight, and is useful for direct comparison of multiple samples, whereas 2D-SDS-PAGE provides more information

about a single protein mixture, separating proteins by isoelectric point and molecular weight. Protein concentration of each dialyzed culture medium was determined (Lowry et al., 1951), and 40 μ g samples were lyophilized and solubilized in 10mM Tris buffer with 2% SDS and 10% 2-mercaptoethanol (pH 6.8). For 1D-SDS-PAGE, solubilized samples were loaded directly on top of a 10% discontinuous slab gel (Smith, 1984) and run at 15mV per slab until stacked (1-2 hours), and for approximately at 20mV per slab or until complete (4-5 hours). For 2D-SDS-PAGE, solubilized samples were loaded into isoelectric tube gels for the first dimension and separated by isoelectric point overnight using ampholytes (pH 3.5-10, 5-7, 9-11). The tube gel was equilibrated, affixed to the top of a 10% discontinuous slab gel and run as described by Roberts et al. (1984). Gels were fixed and stained with Coomassie blue. Molecular weights were determined by use of protein standards and determination of R_f values (Shapiro et al., 1967).

Electroblotting

In order to identify specific proteins that were secreted by the regions, the proteins within the explant culture medium were transferred to nitrocellulose to allow detection of proteins with specific antibodies. The gels were equilibrated in transfer buffer (25mM Tris, 192 mM glycine, and 20% v/v methanol) immediately following electrophoresis and the proteins transferred to nitrocellulose under a 300mA electrical field. The nitrocellulose was equilibrated (15 min) in Tris buffer saline (TBS; 25mM Tris with 0.3M NaCl, pH 7.4), blocked with 2.5% powdered milk in buffer (1 hr), washed in buffer, and incubated with rabbit antibodies (1:500 dilution) specific for ovalbumin (Chapter V) in 2.5% milk for 6 hours. The nitrocellulose was washed in buffer (3 X 5 min), and incubated overnight with goat anti-rabbit antibodies (diluted 1:1000 in 2.5% buffered powdered milk) conjugated with alkaline phosphatase. The nitrocellulose was washed again in buffer (3 X 5 min), and incubated at 37°C in 0.1 g/l nitroblue tetrazolium (NBT), 0.05 g/l 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and

2mM MgCl₂ in 0.1M Tris buffer (pH 8.8 for 15 min) for localization of cross-reactive proteins.

Fluorography

To identify which proteins were synthesized by the oviductal regions while in culture, the 2D-SDS-gels (with 20,000 dpm of activity per sample) were subjected to fluorography to identify the labeled proteins. Following 2D-SDS-PAGE, the gels were equilibrated in an intensifier solution (sodium salicylate), and dried. The dried gel and a plate of radiographic film (Kodak XAR) were clamped securely together between glass plates. This sandwich was stored at -85°C for 6 weeks before development of the film (Roberts et al., 1984).

Statistics

To analyze the synthesis of proteins by the tissues cultured *in vitro*, the rate of ³H-leucine incorporation/gram of tissue was plotted against time in culture for both gravid and vitellogenic turtles. Standard errors were calculated for each mean, and linear regressions plus correlation coefficients (r) were calculated (Montgomery, 1984).

Results

Tritiated leucine was taken up from culture medium by tissue explants in culture, incorporated into proteins, and the synthesized proteins secreted into the media. The incorporation curve for the synthesis and secretion of radiolabeled proteins into the culture medium is presented in Figure 6-1. There is a linear release of nondialyzable radiolabeled material into the media. The best fit line for each tissue culture, and the corresponding correlation coefficient (r), is presented in Table 6-1.

One-dimensional-SDS-PAGE revealed that the major proteins within explant tissue culture media from both the tube and uterus were similar in relative molecular weight (Fig. 6-2). However, this represents not only proteins synthesized by the cells,

but all proteins which were released from mincing the tissues, including cellular components and plasma proteins.

To further analyze the complex mixture of proteins present in the culture medium, 2D-SDS-PAGE was used to separate proteins by both isoelectric point and relative molecular weight. Fluorography demonstrated a variety of proteins that were synthesized and secreted by the explant tissue cultures of both tube and uterus (Fig. 6-3, 6-4). The major protein secreted from both regions has a relative molecular weight of 77kd and an isoelectric point of approximately 7.0. A small quantity of a single heavy protein (over 100kd) exists at pI 6.1. Several medium weight protein groups were also observed; at Mr 50-60kd over a wide range of pI, at Mr 43-47kd with a pI of 4.2-5.7, and at Mr 29-37kd with pI ranging from 5.9 to over 8.5. Two proteins occur at the lowest range Mr detectable, 12-13kd. Similar results were obtained with both species examined.

Subtraction of protein spots, using 2D-SDS-PAGE fluorographs, was used to determine which proteins were synthesized and secreted by one region but not by the other. The major protein secreted largely by the tube, with little or none produced by the uterus, has a relative molecular weight of 47kd and isoelectric point of 4.5 (Fig. 6-3, 6-4).

Discussion

The incorporation of ^3H -leucine into proteins that are subsequently secreted from explant tissue cultures demonstrates that the tubal and uterine regions of the oviduct are actively involved in protein syntheses and secretion. Similar results of *in vitro* incorporation of radiolabeled amino acids into secretory proteins have been reported by tissues of the reproductive tract of birds (Mandeles and Ducay, 1962; O'Malley, 1967; Kohler et al., 1968) and mammals (Bartol et al., 1985; Moffatt et al.,

Table 6-1. Equations for the best fit linear regression and correlation coefficients (r) for the incorporation of ^3H -leucine into secretory proteins by tubal and uterine explant tissue cultures from the turtle *Pseudemys scripta*.

Tissue	Equation	r
<u>Vitellogenic</u>		
Tube	$y = 9.4x + 5.5$	0.92
Uterus	$y = 20.4x - 34.7$	0.89
<u>Gravid</u>		
Tube	$y = 5.2x + 25.1$	0.91
Uterus	$y = 4.5x + 5.0$	0.97

Figure 6-1. Incorporation of ^3H -leucine into proteins by explant tissue cultures from *Pseudemys scripta*.

Figure 6-2. One-dimensional SDS-PAGE separation of proteins present in *Pseudemys scripta* tissue culture medium by molecular weight. Lanes: 1-3, tubal tissue cultures; 4-7, uterine tissue cultures; 8, *Pseudemys floridana* egg albumen; 9, standards.

Figure 6-3. Representative fluorograph of *Pseudemys scripta* tubal explant tissue culture proteins separated by 2D-SDS-PAGE. Arrow, secretory protein indicative of the tubal explant cultures.

Figure 6-4. Representative fluorograph of *Pseudemys scripta* uterine explant tissue culture proteins separated by 2D-SDS-PAGE. Arrow, location of secretory protein indicative of tubal explant cultures.

1987a, b; Roberts et al., 1987; Roberts and Bazer, 1988; Ing et al., 1989; Murray et al., 1989). By using electrophoresis followed by fluorographic detection, it is possible to determine those proteins that were synthesized *in vitro* and secreted by the explant tissue while in culture.

The dominant protein secreted largely by the tube but not the uterus has a relative molecular weight (47kd) and isoelectric point (4.5), similar to ovalbumin in avian eggs (Li-Chan and Nakai, 1989), and in Mr to the major egg protein in both these species of turtles (Chapter V; Palmer, unpublished data), other reptiles (Chapter V). These data support previous ultrastructural and histochemical studies that the tube is the site of egg albumen secretion. It was found that the ultrastructure of the reptilian tube resembles that of the avian magnum, which secretes albumen proteins (Aitken and Solomon, 1976; Palmer and Guillette, 1988; Guillette et al., 1989). In turtles and alligators, it was determined that cells of the tubal endometrial glands resemble type A-cells of the avian magnum (Chapter II, IV), which secrete ovalbumin, and that turtle and alligator albumen contains an ovalbumin-like molecule (Chapter V). The endometrial glands of birds also secrete ovotransferrin (Schimke et al., 1977), another possible component of reptilian eggs (Chapter V), although no specific cell type in birds has been identified.

These data demonstrate that proteins are actually synthesized in the tube, and not transported there by the circulatory system from other organs, as occurs with yolk proteins. In the lizard *Lacerta sicula*, it was demonstrated that avidin, a minor component of avian albumen, is synthesized and secreted in the tubal portion of the oviduct (Botte et al., 1974; Botte and Granata, 1977). However, avidin, or other biotin-binding molecules, have not been identified within the albumen of alligators (Abrams et al. 1988, 1989; White, 1990).

The stimulation of albumen synthesis within the oviduct has been intensively studied in birds, in which ovarian estrogen initiates the synthesis of albumen proteins

(O'Malley et al., 1979). Estrogen is bound by a nuclear receptor protein to create an estrogen-receptor complex (Gorski et al., 1987; Leavitt, 1989), which binds to the chromatin of cells in the tubal endometrium, stimulating transcription (O'Malley et al., 1979; Chambon et al., 1984). Estrogen stimulates differentiation in gland cells of immature chicks, causing the syntheses of ovalbumin, ovotransferrin, ovomucoid, and lysozyme (Palmiter and Gutman, 1972; Palmiter and Schimke, 1973). In addition, treatment with progesterone and estrogen causes the luminal epithelium to synthesize and secrete avidin (Kohler et al., 1968). It has also been demonstrated that estrogen treatment stimulates transcription of the ovalbumin gene (Roop et al., 1978).

In the lizard *Lacerta sicula*, secretion of tubal proteins is induced by the administration of estrogen and progesterone (Botte et al., 1974; Botte and Granata, 1977). Further, in the lizard *Podarcis* (= *Lacerta*) *sicula*, the occurrence of a nuclear protein, poly(ADPribose)transferase (ADPRT), is associated with increased estrogen levels (Ciarcia et al., 1986). ADPRT is associated with DNA replication and repair (Hayaishi and Ueda, 1977; Ueda and Hayaishi, 1985), cell differentiation, and gene expression (Caplan and Rosenberg, 1975; Farzaneh et al., 1982; Mandel et al., 1982). ADPRT activity precedes protein synthesis in immature quail oviducts under estrogen stimulation (Müller and Zahn, 1976). These data strongly suggest that estrogen is vital in the stimulation of albumen synthesis and secretion in reptiles, as has been shown in birds.

The results of this study indicate that the tube is the site of secretion of the major albumen protein in turtles, and probably in other reptiles. Further, these data suggest proteins are synthesized directly by tubal cells. Additional work is required to further characterize the secretory proteins of the tube. Also, localization of specific cells involved in formation of albumen proteins is still unknown. In lizards, there is only a secretory luminal epithelium, and the quantity of albumen in the eggs is reduced, whereas in turtles and alligators, which have more abundant albumen, there is both a

secretory luminal epithelium and endometrial glands. These differences in tubal structure may be related to the quantity of albumen present in their eggs. Although the endometrial glands of birds secrete the bulk of the albumen proteins, it is unclear whether the luminal epithelium or endometrial glands of reptiles secrete their egg albumen proteins. Additionally, in birds different proteins are produced by different cell types, whereas ultrastructural studies in reptiles (Chapter II, IV) indicate that the endometrial glands of the tube are relatively uniform, suggesting the cells are more generalized in their structure and function.

CHAPTER VII LOCALIZATION OF EGG ALBUMEN SECRETING CELLS IN REPTILIAN OVIDUCTS

The tubal region (*tuba uterina*) of the reptilian oviduct has recently been shown to be the site of egg albumen syntheses and secretion (Palmer and Guillette, 1990b; Chapter VI). The reptilian tube is comparable to the magnum of avian oviducts in this regard, and may be structurally as well as functionally homologous (Palmer and Guillette, 1988, 1990a, b; Chapter VI). In birds, albumen proteins are synthesized and secreted within the magnum (Gilbert, 1979) where different populations of cells are specialized for the secretion of specific proteins. The secretory cells of the luminal epithelium secrete avidin and ovomucin (Kohler et al., 1968; Wyburn et al., 1970; Tuohimaa, 1975), although the bulk of albumen proteins are secreted by the tubular endometrial glands. These glands are composed of three types of cells; A, filled with electron dense granules, B, filled with low electron density secretory material and C, with prominent Golgi and extensive rough endoplasmic reticulum (Wyburn et al., 1970). Types A and C may reflect different phases of secretory activity of the same cells (Wyburn, 1970). Ovalbumin is secreted by A-cells, whereas lysozyme is released by B-cells (Kohler et al., 1968; Oka and Schimke, 1969; Wyburn et al., 1970), ovotransferrin and ovomucoid are also secreted by the endometrial glands (Schimke et al., 1977).

There may be differences in the secretion of albumen proteins between reptiles and birds. In the turtles *Sternotherus odoratus*, *Chrysemys picta*, and *Pseudemys scripta* (Chapter II) and the alligator (Chapter IV), the cells of the endometrial glands have characteristics of type A-cells of the avian magnum. Type A-cells secrete ovalbumin, which is similar to a major component of both turtle and alligator eggs. Two types of microvillous secretory cells have been described in the tubal luminal epithelium of turtles

and alligators (Chapters II and IV). One of these resembles avidin secreting mucus-cells in birds (Wyburn et al., 1970), whereas the other is a serous cell. In lizards, there are no endometrial glands in the tube (Guillette and Jones, 1985; Adams and Cooper, 1988; Uribe et al., 1988; Guillette et al., 1989; Kumari et al., 1990; Chapter II) and, not surprisingly, ovalbumin forms a very minor component of their albumen (Chapter V).

The following experiments are part of an ongoing investigation into the functional morphology and biochemistry of reptilian oviducts and their secretions. Immunocytochemistry (ICC), using antibodies specific for avian egg albumen proteins, was performed on the major secretory regions of the turtle and alligator oviduct. The purpose of these experiments was to identify and localize the cells that secrete albumen proteins, and particularly if different cell populations secrete different albumen protein constituents.

Methods and Materials

Specimens

The oviducts of the gopher tortoise (*Gopherus polyphemus*) and American alligator (*Alligator mississippiensis*) were examined during this study. The oviducts of 25 gopher tortoises were obtained from the Florida Museum of Natural History. These specimens were collected from Alachua, Putman, and Marion counties, Florida, and killed by ethanol injection into the brain within 48 hours of capture (Taylor, 1982). These specimens have been used for studies of reproductive anatomy by several authors (Taylor, 1982; Palmer, 1987; Palmer and Guillette 1988, 1990a). Additionally, thirteen alligators (*Alligator mississippiensis*) were collected (Permit #W88063) from throughout Florida. Within 24 hours of capture, the specimens were anesthetized with 20mg/kg sodium pentobarbital, and the oviducts surgically removed under sterile conditions. For each species, specimens of various reproductive conditions (vitellogenic, early gravid, late gravid, immediately post-oviposition and reproductively quiescent) were available.

Oviductal regions were initially distinguished as infundibulum, tube (*tuba uterina*), transition zone, fiber region (in alligators), uterus, and vagina. Representative tissues from each region were dissected free and prepared for histology and immunocytochemistry.

Histology and Immunocytochemistry

Tissues from both turtles (Taylor, 1982) and alligators were fixed in 10% neutral buffered formalin or Bouin's fixative, after which they were washed, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin (Humason, 1979). For histology, specimens were serial sectioned at $7\mu\text{m}$ on a rotary microtome, for a total of 10 slides per specimen, and stained with hematoxylin, eosin, and Alcian blue at pH 2.5 (Humason, 1979).

Vectastain (ABC kit PK-4001) and procedures were used for immunocytochemistry (ICC). Specimens for ICC were sectioned at $15\mu\text{m}$, deparaffinized in xylene and hydrated in graded alcohols. Slides were treated with 0.3% H_2O_2 in methanol (for 30 min) to reduce endogenous peroxidase activity, washed in Tris buffered saline (TBS) for 20 min, blocked with goat normal serum (20 min), and incubated with primary antibodies diluted 1:500 in TBS (30 min). Two different primary antibodies were used; either rabbit anti-whole-hen-egg-albumen (Sigma C6534), or affinity purified rabbit anti-hen-ovalbumin (Chapter V). Tissues were washed in TBS for 10 min, incubated with secondary (biotinylated goat anti-rabbit) antibodies (30 min), and washed in TBS (10 min). Sections were then incubated with Vectastain Elite ABC Reagent (30 min), washed in TBS (10 min), and incubated with substrate (5 min) in order to localize bound primary antibodies. The substrate solution consisted of 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 in 0.1M TBS, pH 7.2. Following development, slides were washed in tap water (5 min) and coverslips mounted.

Three different controls for nonspecific binding of antibodies were performed. Adjacent slides in a series were incubated with omission of primary antibodies, omission

of secondary biotinylated antibodies, or incubated with an excess of antigen (0.1%) mixed in with the primary antibody solution.

Results

Immunocytochemistry (ICC) exhibited binding of avian egg albumen antibodies within reptilian oviducts. Localization of biotinylated secondary antibodies demonstrated that antibodies specific for ovalbumin and whole-egg-albumen bound to the endometrial glands of the tube in both the turtle (Fig. 7-1) and alligator (Fig. 7-2). In addition, the whole-egg-albumen antibodies bound to the apical portion of luminal epithelial secretory cells. Staining of free secretory material within the oviductal lumen occurred with both primary antibodies. Other tissues of the tube, and all tissues of the fiber region in the alligator and uterus of both species showed only background staining.

All controls showed a dramatic decrease in the amount of binding present. However, slight binding continued to occur with α -whole-egg-albumen antibodies in the apical aspect of the luminal secretory cells.

Discussion

The tube of the reptilian oviduct has been hypothesized for many years to be the source of albumen protein, as first discussed by Gegenbaur (1878). The first experimental evidence supporting this hypothesis demonstrated the presence of avidin in the tubal tissues and their secretions (Botte et al., 1974; Botte and Granata, 1977). Although avidin is a minor component of avian eggs, further research has failed to detect avidin in reptilian albumen (Abrams et al. 1988, 1989; White, 1990). Only recently has it been demonstrated that reptilian egg albumen proteins are secreted by the oviductal tube (Chapter VI). These studies, however, did not specifically isolate which cells of the tube were producing the albumen.

Figure 7-1. Representative immunocytochemical localization of cells secreting ovalbumin-like proteins in the endometrial glands of the oviductal tube in the tortoise, *Gopherus polyphemus*, by the use of ovalbumin specific antibodies.
A: Localization of albumen secreting cells by immunocytochemistry (500X).
B: Controls for ICC in which the primary antibody has been omitted (500X). E, luminal epithelium; G, endometrial glands.

Figure 7-2. Representative immunocytochemical localization of cells secreting ovalbumin-like proteins in the endometrial glands of the oviductal tube in the alligator, *Alligator mississippiensis*, by the use of ovalbumin specific antibodies. A: Localization of albumen secreting cells by immunocytochemistry (625X). B: Controls for ICC in which the primary antibody has been omitted (625X). E, luminal epithelium; G, endometrial glands.

It has been demonstrated that polyclonal antibodies to avian albumen proteins cross-react with reptilian albumen proteins (Chapter V). Immunocytochemistry (ICC) using these antibodies has localized cells secreting albumen proteins within the tube of both turtles and alligators. No binding was found in other regions of the oviduct, such as the fiber region in alligators or the uterus of either species. Additionally, the secretory material within the tubal lumen stained similarly to the endometrial glands. Cells of the endometrial glands bound both α -ovalbumin and α -whole-egg-albumen antibodies in a fairly uniform manner. There were few instances of cells staining darker or lighter than surrounding cells, suggesting that ovalbumin is secreted by most or all of the endometrial cells. In the endometrial glands of avian oviducts, there are three distinct types of cells (Wyburn, 1970). Only type A-cells have been shown to secrete ovalbumin, whereas type B-cells secrete lysozyme (Kohler et al., 1968; Oka and Schimke, 1969; Wyburn et al., 1970). A protein similar in molecular weight and cross-reacting with ovalbumin antibodies has been identified in the eggs and oviductal secretions of reptiles (Chapter V, VI). Further, the cells of the endometrial glands of the tube in turtles and alligators have characteristics of ovalbumin-secreting cells of the avian oviduct (Chapter II, IV). The ultrastructure and ICC of turtle and alligator oviducts suggest that the cells of the tubal endometrial glands are not specialized for secretion of a few specific proteins, as in birds, but may produce a complex mixture of proteins. Many or all of the cells synthesize and presumably secrete ovalbumin. Antibodies specific for other reptilian albumen proteins should be used to test if particular cells are involved in their production.

Antibodies to whole-egg-albumen also showed cross-reactivity to the apical margin of secretory cells in the luminal epithelium. Two types of luminal epithelial cells have been identified in reptiles, one of which is ultrastructurally similar to albumen secreting (avidin and ovomucin) cells of the avian magnum (Chapter II). Although it is unknown if avidin is incorporated into the egg albumen of reptiles (Abrams, et al. 1988,

1989; White, 1990), it is known to exist in the tubal tissues and secretions of lizards (Botte et al., 1974; Botte and Granata, 1977). This may account for the binding of antibodies to the apical portion of luminal epithelial cells. Ovomucin, another protein secreted by avian luminal epithelial cells, is largely responsible for the structural integrity of bird albumen. Reptilian albumen exhibits significant structural organization, being layered in concentric sheaths surrounding the yolk (Chapter V). However, there is only limited chemical evidence for the presence of ovomucin in reptilian eggs (Burley et al., 1987). It would be constructive to identify what is responsible for the structural integrity of reptilian albumen, and if ovomucin is involved.

CHAPTER VIII IMPLICATIONS FOR THE EVOLUTION OF REPRODUCTIVE MODES IN TETRAPOD VERTEBRATES

The shelled amniote egg is considered to have been a major factor in the radiation of vertebrates into terrestrial habitats, but the evolution of eggshell formation has received little scientific investigation. However, the process of eggshell formation has been extensively studied in birds and egg-laying mammals, the monotremes. Until recently, even basic information on gross oviductal anatomy was limited in reptiles. Even fewer studies had examined the process of reptilian albumen and eggshell formation. This lack of information concerning reptilian oviductal anatomy, and how it functions in formation of egg coats, presented a barrier to the understanding of the evolution of reproductive modes not only in reptiles, but also in more derived amniotes, the birds and mammals.

Oviparous Reproductive Modes in Amniotes

Birds are sequential ovulators, so only one egg of a clutch is invested with albumen and eggshell at a time by the single oviduct. The albumen consists of a complex mixture of proteins which are secreted around the yolk in a structured fashion. There are three types of endometrial gland cells that secrete the bulk of the albumen, although the luminal epithelium also is involved in albumen secretion. Each cell type forms only a few specific albumen proteins, and there is a sequence of cell types along the albumen secreting oviductal region (magnum), which correlates with the sequential layers of albumen proteins surrounding the yolk.

In birds, the fibrous and calcareous layers of the eggshell are produced in separate regions of the oviduct (Solomon, 1983). After an individual egg is coated with

albumen in the magnum, it enters the isthmus where the proteinaceous fibers of the eggshell membrane are deposited. The egg then travels into the shell gland where calcium deposition occurs. The avian oviduct is essentially an assembly line, with each region of the oviduct involved in only one specialized function, and only one egg is dealt with at a time. The entire process from ovulation to oviposition requires about 24 hours, and is complete before the next egg of a clutch is ovulated.

The egg-forming process in monotremes, the egg-laying mammals, is much different from that in birds. The tube produces a thin layer of albumen on both eggs of the clutch virtually simultaneously (Hughes, 1974). The uterus produces all layers of the eggshell (Hughes and Shorey, 1972; Hughes and Carrick, 1978), which are somewhat different than in birds and reptiles. The eggshell membrane is composed of proteinaceous particles instead of fibers. Additionally, the calcareous layer is greatly reduced, but it is secreted following membrane formation. As in reptiles, all eggs of a clutch are ovulated simultaneously. In monotremes, therefore, the regions of the oviduct are multifunctional, coating multiple eggs at a time, and the uterus secretes both eggshell layers.

The function of the oviduct in albumen and eggshell formation in reptiles has only recently been examined in detail using modern techniques. In all reptiles examined to date, the albumen is formed in the upper tubal region (*tuba uterina*) of the oviduct. Using explant tissue culture, the tubal region of the reptilian oviduct was shown to synthesize and secrete albumen proteins (Chapter V). In turtles and alligators, the endometrial glands have characteristics of type A-cells of the avian magnum (Chapters II, IV), which are known to secrete ovalbumin (Wyburn et al., 1970). The luminal epithelium of the tube is composed of three cell types; ciliated cells and two types of microvillous secretory cells, one of which resembled albumen producing cells of the avian oviduct (Chapters II, IV). Immunocytochemistry, using polyclonal antibodies to avian albumen proteins, has demonstrated that the endometrial glands of the tube secrete an

ovalbumin-like protein (Chapter VII), one of the components of reptilian albumen (Chapter V). Additionally, although most of the proteins are secreted by the endometrial glands, some may be produced by the luminal epithelium (Chapter VII). This homogeneity in glandular ultrastructure and secretory nature indicates that the tube of reptiles is less specialized than the magnum of birds, which exhibits specialized cell types for producing specific proteins, and structural organization of cell types along the length of the magnum. In lizards, the tube is aglandular, so that the little albumen found in their eggs (Chapter V) is probably produced by the luminal epithelial cells (Chapter III).

Formation of all layers of the eggshell occurs within a single region in turtles and lizards (Chapters II, III). The endometrial glands ultrastructurally resemble the fiber producing glands of the avian isthmus (Chapters II, III), and extrude intact fibers that are wrapped around the egg to form the eggshell membrane (Chapter III). This is in contrast to previous theories, which concluded that the fibers polymerized directly on the eggs surface (Giersberg, 1923; Weekes, 1927). The production of fibers is controlled to form layers within the membrane (Chapter III), and is complete within 24 hours. Following formation of the membranous eggshell, calcium secretion begins on all eggs while they are still retained in the uterus (Chapters II, III), and continues throughout the rest of gravidity. The uterus in turtles and lizards is, therefore, dualistic in function, producing both the eggshell membranes and calcareous shell on an entire clutch simultaneously. The control of this complex function remains unknown, although luteal hormones, such as progesterone, may be involved.

The anatomy of alligator oviducts is substantially different from that of other reptiles, possessing a distinct secretory region, the fiber region, between the tube and uterus. The fiber region histochemically and ultrastructurally resembles the eggshell fiber forming region in birds, the isthmus (Chapter IV). Further, the extrusion of eggshell fibers from the endometrial glands confirms that this region of the alligator

oviduct produces the proteinaceous fibers of the eggshell membrane (Chapter IV). It is unclear if eggs are coated with all fiber layers by a single portion of the fiber region, or if each egg is coated with the different layers as it passes along the length of the fiber region. The alligator uterus secretes the calcareous eggshell and is ultrastructurally similar to the shell gland of birds (Chapter V), but distinct from the uterus of other reptiles. The calcareous shell is formed on all eggs of a clutch simultaneously. Although the oviduct of alligators resembles that of birds in having separate regions for the formation of each egg coat, it still handles all eggs of a clutch simultaneously, as in other reptiles.

The tuatara (*Sphenodon punctatus*) is the most anatomically ancient of extant reptiles, and the only living member of its order (Benton, 1985). In the tuatara, only two oviductal regions have been identified in production of albumen (tube) and eggshell components (uterus) (Cree, personal communication). The albumen is greatly reduced in quantity, and all layers of the eggshell are produced in the uterus. However, the fibrous and calcareous eggshell appear to be secreted simultaneously by the uterus (as determined by the appearance of calcium crystals that extend through the fibrous membrane; Packard et al., 1988), rather than sequentially, as in turtles and lizards (Chapters II, III). This represents the simplest oviductal anatomy and physiology observed in any reptile studied to date.

Evolution of Amniotic Oviparity

In order to make inferences regarding the evolution of amniote oviparity, two assumptions must be made. First, that the simplest anatomy and physiology is ancestral, whereas more complex features are derived, and second, that structures with more generalized functions are ancestral to those which are more specialized. These assumptions are made unless there is evidence that derived characteristics have been secondarily lost. Following these assumptions, comparisons of oviductal functional

morphology and eggshell formation among reptilian taxa, birds, and mammals reveal a progression of reproductive anatomy and physiology from simplest to most complex. The arrangement produced by this is shown in Fig. 8-1. This is not intended to represent the evolutionary relationships among these taxa, but rather to use those characteristics of extant groups to decipher the evolution of oviparous reproductive modes among amniotes.

Not surprisingly, the simplest and most generalized reptilian oviductal anatomy and physiology occurs in the tuatara, (*Sphenodon punctatus*), the most anatomically ancient of extant reptiles. In the tuatara, both layers of the eggshell membrane are secreted by the uterus simultaneously, indicating there is little physiological control for the separation of uterine functions. The amount of albumen is greatly reduced, as is the thickness of the calcareous eggshell layer.

Turtles and squamates demonstrate more advanced anatomy and physiology than the tuatara. Although both eggshell layers are produced in a single region, the uterus, there is sufficient physiological control to separate eggshell membrane formation and calcareous shell secretion temporally. The amount of albumen present, as well as the thickness of the calcareous shell, is highly variable among species.

The oviparous mammals, the monotremes, have a unique egg structure. A thin layer of albumen is secreted in the tube, and both layers of the eggshell are secreted sequentially within the uterus (Hughes and Carrick, 1978). There is only a single type of albumen secreting cell within the tube (Hughes, 1974), similar to the tubal structure of reptiles (Chapters II, IV), which also lack specialized cells for albumen synthesis and secretion typical of the avian magnum (Gilbert, 1979; Solomon, 1983). Although the eggshell membranes are made from proteinaceous secretions of the endometrial uterine glands, they are formed from particles, rather than fibers as found in both birds and reptiles (J. P. Hill, 1933; Hughes, 1974). This may be similar to the outer layers of eggshell membrane in many reptilian species, which consists of a thin layer of

Figure 8-1. Theoretical sequence of adaptations in the evolution of eggshell formation within the amniotes, based upon extant characteristics. Two assumptions have been made; first, that the simplest anatomy and physiology is ancestral, whereas more complex features are derived, and second, that structures with more generalized functions are ancestral to those which are more specialized.

MODE OF EGGSHELL FORMATION

REPRESENTATIVE
GROUPSSPATIAL SEPARATION PER EGG

Eggshell layers formed sequentially
on individual eggs of a clutch in
separate oviductal regions

Birds

SPATIAL SEPARATION PER CLUTCH

Eggshell layers formed sequentially
on an entire clutch in separate
oviductal regions

Crocodylians

TEMPORAL SEPARATION PER CLUTCH

Eggshell layers formed sequentially
on an entire clutch within a single
oviductal region

Chelonians
Squamates
Mammals

NO SEPARATION

Eggshell layers formed simultaneously
on an entire clutch within a single
oviductal region

Tuatara

proteinaceous particles (Schleich and Kästle, 1988; Packard and DeMarco, 1990). The calcareous shell is greatly reduced in monotremes (J. P. Hill, 1933; Hughes and Carrick, 1978), as in the tuatara and many squamates. This may allow for the extensive swelling of the eggs due to fluid uptake within the uterus (Hughes and Carrick, 1978). These unique characteristics make placement of monotremes within the progressive series difficult, although they may be closest to turtles and squamates based on temporal separation of eggshell secretion within the uterus.

A more advanced reproductive mode than that of other reptiles occurs in the crocodilians, which spatially separate formation of eggshell components along the length of the oviduct. The eggshell is thick and rigid in all species, and the albumen layer well developed. There are still some typical reptilian features, such as simultaneous ovulation of a clutch of eggs, which are all coated concurrently within the oviducts.

In birds, the process became even more advanced with sequential ovulation and further specialization of cells and tissues for albumen and eggshell formation. Different populations of cell types occur along the magnum for albumen secretion and even within the shell glands for shell formation. This represents the most anatomically complex and structurally specialized oviduct of oviparous amniotes.

This sequence of reproductive anatomy and physiology exhibited by extant oviparous vertebrates shows what may approach the evolutionary sequence leading from ancient stem reptiles to advanced forms. Initially, simple anatomical structures may have exhibited generalized functions that were poorly controlled, as in the uterus of the tuatara, which secretes all layers of the eggshell simultaneously for an entire clutch of eggs. The oviductal anatomy gradually became more specialized leading up to the avian condition, in which only one egg of a clutch is passed through the oviduct at a time, and each region, as well as cells within that region, are specialized for the task they perform.

Phylogeny of Amniotic Vertebrates

Mammals and birds both evolved from ancient reptilian stock. Most authorities agree that archosaurs (birds and crocodilians) share a common ancestor (Romer, 1966), but the origin of mammals has been more controversial. Traditional phylogenetic trees based on the fossil record (Fig. 8-2), have indicated that mammals diverged early from ancestral amniotes (Gauthier et al., 1988). Yet, recent studies examining many extant characters have hypothesized that mammals and birds share a common ancestor with crocodilians, the Thecodontia (Gardiner, 1982; Løvtrup, 1985: Fig. 8-3).

Oviductal anatomy may provide important insight into the evolution of the modern avian and mammalian modes of oviparous reproduction from its ancestral reptilian origins. A split leading to the oviparous reproductive anatomy and physiology of birds occurs within the reptiles (Fig. 8-4), and not between vertebrate classes as previously suggested. The reproductive mode and oviductal morphology exhibited in monotremes (Hughes and Storey, 1972) resembles the tuatara, turtles, and squamates, with all layers of the eggshell being deposited in the uterus, whereas the oviduct of birds is more similar to that of crocodilians. Additionally, the structure of the monotreme eggshell supports the hypothesis that mammals diverged early from other amniotes. These data support the hypothesis that crocodilians and birds share a common ancestor, but that mammalian origins are closer to other reptilian stocks.

Evolution of Shelled Eggs

The evolution of shelled amniotic eggs from the jelly coated eggs of amphibians has proven difficult to decipher due to lack of intermediate forms. However, it is possible to form hypotheses that are testable using extant species that will shed light on the evolution of shelled eggs. This has important implications for the study of vertebrate evolution in general, as the shelled amniotic egg was critical in the radiation of vertebrates into terrestrial habitats.

Figure 8-2. Dendrogram of the traditional phylogenies of the Amniota, which is based largely upon the fossil record (adapted from Carroll, 1988).

Figure 8-3. Revised classification of the Amniota based upon characteristics of extant species (adapted from Gardiner, 1982; and Løvtrup, 1985).

Figure 8-4. Oviductal functional morphology, egg structure, and mode of eggshell formation in extant vertebrates is consistent with the phylogeny of amniotes based upon the fossil record.

¹C. J. Hill, 1933, 1941; Hughes and Shorey, 1972; Hughes, 1974; Hughes et al., 1975; Hughes and Carrick, 1978.

²Fox, 1977; Fox, 1984; Palmer and Guillette, 1988, 1990a, b; Guillette et al., 1989; Chapters II, III.

³Gilbert, 1979; Solomon, 1983; Chapter IV.

⁴J. P. Hill, 1933; Hughes and Carrick, 1978.

⁵Packard et al., 1977; Ewert, 1979; Tracy and Snell, 1985.

⁶Powrie and Nakai, 1986; Manolis et al., 1987.

⁷Packard et al., 1977; Hirsch, 1983; Packard et al., 1982; Packard and Hirsch, 1986; Packard et al., 1988; Schleich and Käastle, 1988; Packard and DeMarco, 1990.

⁸Simkiss, 1968; Simkiss and Taylor, 1971; Board, 1982; Ferguson, 1982; Manolis et al., 1987; Tullett, 1987

Eggshell layers
formed in single
oviductal region¹

Sparse albumen⁴

Particulate
eggshell membrane⁴

Sparse calcium⁴

Eggshell layers
formed in single
oviductal region²

Variable albumen⁵

Fibrous eggshell
membrane⁷

Variable calcium⁷

Eggshell layers
formed in separate
oviductal regions³

Abundant albumen⁶

Fibrous eggshell
membrane⁸

Abundant calcium⁸

Figure 8-5. Diagrammatic representation of theories concerning the evolution of the shelled eggs of amniotes from the jelly coated eggs of amphibians (adapted from Smith, 1960; Tihen, 1960; and Needham 1963).

ANCESTRAL AMNIOTE

Terrestrial eggs
Outer protein layer further
toughened and calcified
Internal fertilization

DERIVED AMPHIBIAN

Semi-terrestrial eggs
Outer jelly layer toughened
to reduce water loss and
protect egg
Internal fertilization

INTERMEDIATE AMPHIBIAN

Aquatic (brackish) eggs
Jellies create osmotic
barrier to water flux
Fertilization (?)

ANCESTRAL AMPHIBIAN

Aquatic (freshwater) eggs
Jellies create osmotic
barrier to water influx
External fertilization

A hypothetical sequence for the evolution of shelled eggs is presented in Fig. 8-5 (Smith, 1960; Tihen, 1960; Needham, 1963). It is clear that the eggs of ancestral amphibians were fully aquatic and externally fertilized. Surrounding aquatic amphibian eggs are thick layers of "jellies", actually a complex mixture of glycosaminoglycans (GAGs) and some glycoproteins (Needham, 1963). This mixture is secreted around the egg in concentric layers as it passes down the *pars convoluta* of the oviduct. The bulk of the jellies are secreted by the endometrial glands of the *pars convoluta*, the luminal epithelium consisting of mostly nonsecretory ciliated cells. The other regions of the amphibian oviduct are nonsecretory, such as the infundibulum which receives the eggs from the ovaries, and the uterus which retains eggs briefly, if at all.

There are several functions described for amphibian jellies. Most important for the evolution of shelled, terrestrial eggs is their function in water balance. The jellies form a series of osmotic barriers surrounding the yolk and developing embryo, preventing the influx of water which would inundate the embryo (Needham, 1963).

As some eggs evolved to be semiterrestrial (being able to withstand short periods of desiccating conditions), the jellies took on the opposite role by preventing water loss from osmosis and evaporation. Water bound by the jellies may have initially served as a temporary reservoir during periods of exposure to the atmosphere. However, water loss was reduced in terrestrial amphibian eggs by transforming the outer jellies into a toughened, rubbery layer, while the inner jellies remained hydrated to serve as a water reservoir.

It has been hypothesized that the inner, hydrated layers of terrestrial amphibian eggs evolved to become the albumen of reptilian eggs, whereas the toughened outer layer became the eggshell membrane (Smith, 1960; Tihen, 1960; Needham, 1963). This suggests the following hypotheses.

First, the jellies of amphibian eggs evolved to become the albumen of reptilian eggs. Both contain glycoproteins and GAGs, and have many properties in common,

such as water balance and antibacterial defense (Heatwole, 1961; Palmer and Guillette, 1990b). It is likely that if reptilian albumen has evolved from amphibian egg jellies, they may have some components in common. Recent biochemical evidence (Palmer, unpublished data) suggests that one component of amniote egg albumen also occurs in amphibian jellies. Clearly, this needs to be investigated further.

Second, the eggshell membranes of reptilian eggs is derived from the outer, toughened jelly layer of terrestrial amphibian eggs. The protein constituents of the fibrous eggshell membrane have yet to be determined. In fact, the proteins of the membrane have proven extremely difficult to study, being highly insoluble. Further studies are required to investigate these proteins and their possible relationships with amphibian egg proteins.

There are a couple of hypotheses for the origin of the calcareous eggshell of amniotic eggs. Mineral crystals have been reported in the outer jellies of some primitive amphibians (Salthe, 1963). The function of these minerals is unknown, but may have been a source of calcium or other minerals for the embryo. Particularly, reptiles use their eggshell as a source of calcium for embryonic development. This ability to secrete minerals into the jellies (particularly from the posterior regions of the oviduct, which secrete the outer jelly layers) may have been the precursor of the calcareous reptilian shell. Alternatively, the calcareous shell may have evolved secondarily among amniotes. This needs to be further investigated before their possible implications for the evolution of shelled eggs can be determined.

The origin of shelled reptilian eggs remains obscure. The above experiments would be a first step to the unraveling of this important evolutionary event. Although it is possible that the different modes of egg-shelling evolved from a common ancestor, it is equally possible that the formation of shelled eggs has evolved in amniotes more than once. Obviously, much work needs to be done before the evolution of amniotic oviparity can be understood.

CHAPTER IX SUMMARY AND CONCLUSIONS

These studies investigated the structure and function of reptilian oviducts, particularly in regards to formation of a shelled egg. The ultrastructure and biochemistry of oviducts and eggs were examined and compared with known structures and proteins in other vertebrates. The conclusions from these studies have important implications for understanding the evolution of reproductive modes in tetrapod vertebrates.

The oviductal ultrastructure of the turtles *Pseudemys scripta*, *Sternotherus odoratus*, and *Chrysemys picta* was examined to determine structural homologies with avian and mammalian oviducts. The endometrial glands of the tube contained numerous irregularly shaped secretory granules of various electron densities, whereas those of the uterus were spherical and electron dense. These structures correspond to those of the endometrial glands of the avian magnum and isthmus, respectively. This supports the hypothesis that the reptilian tube secretes albumen whereas the uterus is the site of formation of both the fibrous and calcareous eggshell layers.

The oviductal changes associated with eggshelling were examined in the lizard *Sceloporus woodi*. It was determined that the eggshell fibers are secreted within the uterus during the first 24 hours of gravidity. By day 3 following ovulation the secretory cells of the luminal epithelium become extensively hypertrophied, and calcification of the eggshell begins. The functional morphology of the uterine endometrial glands resembles that in chelonians. This demonstrates that the uterus in lizards, and probably in other squamates and chelonians, is dualistic in function, secreting both layers of the eggshell membrane.

The oviductal functional morphology of alligators is unlike other reptiles, but exhibits a morphology and ultrastructure similar to birds. The tube ultrastructurally resembles the avian magnum. The endometrial glands of the fiber region have spherical, electron dense secretory granules, like the avian isthmus, and was shown to secrete the eggshell fibers. The uterine endometrial glands have cuboidal cells with electron light secretory granules, as in the avian shell gland, which secretes the calcareous eggshell layer. Thus, there is a split in the reproductive functional morphology of reptiles, with alligators resembling the birds, whereas chelonians and squamates are similar to monotremes in having only one oviductal region for eggshell formation.

The proteins of the egg albumen from a wide variety of reptiles were examined using electrophoresis and immunodetection. The results indicated for the first time that ovalbumin is a major component of alligator and turtle eggs, but occurs in small quantities in squamates. Alligator and turtle albumen share many similar proteins with each other and with birds, whereas squamate albumen tends to have unique protein constituents. This may have important ecological implications, since albumen proteins are known to possess many biologically important functions. The differences in protein composition among reptilian taxa may partially determine suitable nest site selection for the mother, and developmental constraints on the embryo.

The site of albumen synthesis and secretion in the turtle oviduct was examined using explant tissue culture. It was determined that the tubal region is active in protein synthesis, and that one of the proteins secreted is similar to ovalbumin in avian eggs and ovalbumin-like proteins in reptilian eggs. This suggests that the albumen proteins of reptilian eggs are synthesized and secreted by the tube of the reptilian oviduct.

The endometrial glands were determined to be the major site of protein secretion within the tube of turtles and alligators. Ovalbumin-like proteins were localized in the endometrial gland cells using immunocytochemistry. Most of the cells

contained ovalbumin-like proteins, suggesting that the cells of reptilian tube are less specialized than those of the avian magnum. Some cross-reactivity with antibodies to whole-hen-albumen occurred within the luminal epithelia, suggesting that some proteins may be produced there as well.

The functional morphology and biochemistry of reptilian oviducts and eggs has important implications for the evolution of reproductive modes in tetrapod vertebrates. In extant species, a progressional series of oviductal functional morphology and mode of eggshell formation is exhibited, from extremely simple oviducts that have generalized anatomy and physiology (tuatara), to anatomically complex oviducts that are highly specialized with extensive physiological controls (birds), with several intermediate conditions clearly evident. For the first time the divergence between avian and mammalian reproductive modes has been traced to extant reptiles. These data dispute recent phylogenetic schemes that have linked mammals and birds to a common archosaurian ancestor (Gardiner, 1982; Løvtrup, 1985).

Comparative Terminology

The terminology of oviductal anatomy in reptiles, birds, and mammals has become very confusing. Although the term infundibulum is used in all groups, most other regions have different names. The tube (*tuba uterina*) of reptiles, since it secretes albumen, is homologous with the magnum of birds, and the Fallopian tube of monotremes. In squamates, the tube is greatly reduced and aglandular and may represent a secondary loss of complexity. The short, restricted, and translucent aglandular region posterior to the tube in turtles and alligators has been called the isthmus or transition zone. The term isthmus may be confusing since it does not secrete the eggshell membrane as does the avian isthmus, and is therefore not homologous. Further, it is not homologous with the mammalian isthmus, which is part of the Fallopian tube.

The eggshell forming regions of the alligator and avian oviduct appear to be homologous, with the anterior region forming the eggshell membranes and the posterior region secreting the calcareous layer. The eggshell membrane forming region has been termed the fiber region in reptiles and the isthmus in birds. The alligator uterus and avian shell gland are likewise homologous in structure and function, both secreting the calcareous eggshell. In mammals, turtles, squamates, and the tuatara, both functions of eggshell formation occur within a single region, the uterus. This primitive condition probably predates the separation of functions, and even in alligators and birds there is not a distinct demarcation between the fiber and calcium secreting regions. It is likely that the separate fiber and calcium secreting regions are therefore both derivatives of the more primitive uterus found in some reptilian groups and in monotremes.

The vagina in reptiles and birds serves as a birth canal rather than a receptacle for an intromittent organ, as is found in mammals. In many reptiles, the vaginal canal is greatly reduced with an muscular sphincter (Fox, 1977), and may serve more as a cervix in retaining eggs than as birth canal, although its endometrial histology is very similar to the vagina of birds. This suggests more study is needed into the structure and function of this region in reptiles.

Future Research Directions

The anatomy and physiology of reptilian oviducts in producing albumen and eggshell components is an area requiring indepth investigation. Particularly, the endocrine control of oviductal functions remains largely unknown in reptiles. The composition of reptilian egg albumen, including protein characterization, isolation, and determination of biological properties, requires extensive investigation. This may not only shed light on the physiological development of the embryo, but also on the evolution of proteins and the ecology of eggs in general. Although the genetics of albumen transcription has been studied intensely in birds, it remains to be examined in

reptiles. The mechanism of protein release in birds and reptiles is still unclear. The biochemical nature of eggshell membrane proteins continues to be debated, and may prove to be a unique class of proteins, as has been previously suggested. The protein composition of amphibian jellies, and their possible correlation with reptilian egg albumen, remains to be determined. The comparative study of oviductal anatomy and its hormonal control, as well as the study of egg proteins, including their secretion, function in embryonic development, and role in ecological interactions of eggs with their nest environment, may shed new light on the evolution of amniotic eggs.

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BIOGRAPHICAL SKETCH

Brent David Palmer was born May 13, 1959, in Burbank, California. He attended public and private schools in the Los Angeles area and graduated with honors from U. S. Grant High School in 1978. He studied at Los Angeles Valley College from 1978 until 1982, receiving the Chancellor's Distinguished Honor Award and graduating summa cum laude with an Associate of Arts degree. From 1982 until 1985 he attended California State University, Northridge, studying with Dr. James Dole, and graduating magna cum laude with a Bachelor of Arts in Biology.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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