

MORPHOLOGICAL AND CONSTITUENT ANALYSES OF AMERICAN
ALLIGATOR (*Alligator mississippiensis*) EGGSHELLS FROM CONTAMINATED
AND REFERENCE LAKES

By

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Abstract of Thesis Presented to the Graduate School
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Alligator eggs were collected from one polluted and two reference north-central Florida lakes. The eggs were artificially incubated and their shells collected after the neonates hatched. Thickness measurements were taken from multiple eggs and clutches within each lake over a four year period (1999, 2001, 2002, 2003). In 2003, eggshells from three eggs per clutch and five clutches per lake were also analyzed for constituent make-up, pore density, and basic morphology. Constituents were determined by both inductively-coupled plasma spectroscopy (ICP) and energy-dispersive spectral analysis (EDS). Pore density was assessed using light microscopy and the morphology described through the use of electron microscopy. Egg mass and neonate morphometrics were recorded.

We determined that shells from the reference lake were thinner than those from the contaminated lakes. Constituent analyses and morphology varied among the lakes. There

was no significant difference in pore density among the lakes. Neonates from the reference lake were larger than those from the polluted lakes.

We determined that eggshells from the reference lake were comparatively thinner, yielded less calcium, and lacked the fibrous portion of a region of the shell potentially to support the larger neonates. Comparatively increased calcium mobilization from the eggshell during incubation would be critical for proper skeletogenesis and other calcium-dependent functions in the larger hatchlings.

CHAPTER 1

A REVIEW OF REPRODUCTIVE BIOLOGY AND SHELL FORMATION IN REPTILES

Introduction

Modes of reproduction vary among vertebrates. In reptiles, there were initially three reproductive modes described: viviparity, the production of live offspring, ovoviparity, egg retention within the uterus and subsequent live birth, and the most common, oviparity, the laying of eggs containing relatively undeveloped embryos outside the body. More contemporary research suggests that ovoviparity is not a discrete reproductive mode, but instead an evolutionarily transient period between oviparity and viviparity (Guillette, 1993). Hence, species that retain eggs *in-utero* until late in embryonic development are considered oviparous, whereas species with a placenta are viviparous, thus abandoning the term “ovoviparous” in reptiles. Oviparous reptiles lay an amniotic egg that develops within the oviduct and it is this structure that creates an environment (the eggshell and its contents) capable of providing for the needs of the developing embryo. In viviparous species, the embryo is retained within the uterus until development is complete (Guillette, 1993). Therefore, the placenta must be present and provide the young with the ability to uptake nutrients, exchange gases and ions for respiration and waste removal, among other biological functions necessary for embryonic development. All vertebrate classes, excluding the Agnathans and the Aves, contain species that utilize this reproductive mode. Viviparity has evolved nearly 100 times in

reptiles, though only in lizards and snakes, and has not been reported in any species of turtles, crocodilians, or tuatara (Shine, 1983, 1985).

Oviducal Anatomy

As with all amniote female vertebrates, the Crocodilian oviduct (which includes the entire reproductive tract) is derived from the embryonic Müllerian duct (Austin, 1989). The oviduct undergoes regional specialization as the female sexually matures. At maturation, the alligator oviduct consists of seven anatomically distinct regions, starting anteriorly with the ampullae, followed by the infundibulum, uterine tube, uterotubular junction, anterior and posterior uterus, and vagina. Functionally, the oviduct is separated into three major regions, the uterine tube that secretes albumen (Palmer and Guillette, 1991), the anterior fiber-secreting region of the uterus, and the posterior calcium-secreting region of the uterus (Palmer and Guillette, 1992). Structurally and functionally, the anterior and posterior uterus of the alligator resembles those of birds and not of other reptiles. The anterior fiber-secreting uterus is similar to the avian isthmus, whereas the posterior calcium-secreting uterus is similar to the avian shell gland. Endometrial glands located at the anterior and posterior portions of the alligator uterus share similar cell types and secretory products with birds, and not of other reptiles (Palmer and Guillette, 1992). The glands of the anterior uterus produce the fibrous shell membrane, which lies proximal to the embryo, whereas the posterior uterine glands are hypothesized to secrete the large amount of calcium that forms the outer, rigid calcified shell. Unlike birds, however, alligators ovulate and “shell” a complete clutch of eggs simultaneously whereas birds ovulate and shell one egg at a time.

The Amniotic Egg

All amniotic eggs consist of yolk and albumen derived from the maternal parent. In addition, the eggs contain membranes derived from the embryo and in alligators, this includes the chorio-allantois (a fusion between the chorion and the allantois) and amnion (Ferguson, 1982). During the breeding season, circulating estrogens increase and vitellogenin (Vtg), the major egg yolk protein, is synthesized in the liver (Guillette et al., 1997). Vitellogenin circulates through the bloodstream and is taken up by the oocytes where it is cleaved into smaller yolk subunits (Berg et al., 2004). These subunits serve as the nutritional source for the developing embryo. In alligators, albumen is secreted by the uterine tube and serves as a water reservoir as well as a chemical and mechanical barrier to the embryo (Milnes et al., 2002). The amnion, a fluid-filled sac surrounding the embryo, is derived from an out-pocketing of the embryo's hindgut and also acts as a mechanical barrier while aiding in preventing dessication. Finally, the chorio-allantois, also derived from the embryo, surrounds the contents of the egg and serves as a waste repository (Pough et al., 2001).

The leathery shell membrane forms from the fiber-secreting region of the uterus and surrounds all of the egg contents. Around this fibrous membrane are four distinct layers comprising the eggshell; the innermost mamillary layer composed of calcium and organic material (20-29 μm thick), a fibrous organic layer distinguished by blebs that serve as attachment for calcite crystals (8-12 μm thick), a porous and heavily-fibered honeycomb layer (300-400 μm thick), and the outer densely calcified layer (100-200 μm thick) of numerous calcite crystals (Ferguson, 1982; Packard et al., 1982). Ferguson (1982) also documented constituent elements in 198 alligator eggshells through energy dispersive X-ray analysis. In all samples, the major constituents included calcium and

magnesium, the minor constituent was phosphorus, and trace elements included copper, silicon, sodium, aluminum, iron, zinc, and manganese.

Oviposition

The ancestral mode of reproduction in vertebrates is believed to involve oviparity and external fertilization. Extant forms still displaying this mode include all agnathan fishes, most teleost fishes, and many amphibians. Oocyte retention is short in duration (hours to days) and involves the secretion of a gelatinous coat onto the egg, the egg's transport through the reproductive tract, and finally, it exiting the maternal parent (Pough et al., 2002). The endocrine events related to ovulation, egg transport, and oviposition, are hypothesized to be closely associated (Guillette et al., 1991). However, in some extant oviparous reptiles displaying internal fertilization (turtles and some squamates), gestation length has increased, allowing time for shell calcification (Guillette et al., 1991). The further increase in gestation time in some squamates and mammals, is presumably ancestral to viviparity (Hogarth 1976). Because of the increasing temporal separation between ovulation and oviposition, its been suggested that hormones influence ovulation and oviposition differently between oviparous and egg-retaining/viviparous vertebrates. Previous research indicates that in birds, reptiles, and mammals, hormones, particularly prostaglandins (PGs) and arginine vasotocin (AVT) (or oxytocin for mammals) are important in stimulating the transport of eggs or embryos through the reproductive tract (Guillette et al., 1990), regardless of parity employed. It is hypothesized that oviposition in oviparous vertebrates is stimulated within the central nervous system (CNS) (Owman et al., 1986). Specifically, the CNS induces follicular rupture causing the release of PGF_{2α}, which stimulates oviducal contractions, induces egg-laying behavior, and stimulates arginine vasotocin (AVT) release from the

neurohypophysis (Guillette et al., 1990). In egg-retaining / viviparous vertebrates, it is hypothesized that a cervix has developed which does not respond to PGs or AVT during ovulation but does respond during oviposition due to a lack of AVT / PG receptors or β -adrenergic stimulated inhibition of oviducal contraction (Koob et al., 1984; Challis et al., 1988). During early-mid gestation, it is believed that β -adrenergic neurons (innervating the oviduct) overcome hormonal control of the oviduct, causing relaxation and inhibiting contractions. During late gestation and initial post-partum, β -adrenergic nerves begin degrading. In the viviparous lizard *Sceloporus jarrovi*, Rooney et al. (1997) observed the constant innervation of the uterus throughout pregnancy and subsequent denervation during vitellogenesis. This pregnancy-associated innervation supports the hypothesized mammalian role of oviductal innervation in maintaining myometrial quiescence (Rooney et al., 1997).

Environmental Perturbation to Shelling

Prior to the late 1940's, it was rare to find broken eggs in the nests of peregrine falcons (*Falco peregrinus*) and sparrowhawk (*Accipiter nisus*), but from 1950 on, shells from these species were commonly found broken or destroyed (Ratcliffe, 1973). Ratcliffe (1967) was the first to correlate eggshell breakage and thinning with the widespread use of synthetic organic chemicals used as pesticides and in industry. In particular, Ratcliffe noted that organochlorines were accumulating in the tissues of wild raptors concurrent with the post-war use of these compounds. Since these observations, several experimental studies demonstrated eggshell thinning in relation to exposure to the pesticide DDT (dichlorodiphenyltrichloroethane) and its metabolites by disturbing physiological mechanisms related to calcium (Anderson and Hickey, 1972; Ratcliffe, 1967; Laporte; 1982; Cooke, 1973), which makes up approximately 90% of the

eggshell (Tyler and Geake, 1953). Before 1950, British peregrines had a mean clutch size of 3.5 eggs, with an average of 2.5 fledglings per nest. After 1950, northern England and southern Scotland yielded 1.5 and 1.7 fledglings per nest, respectively, yet mean clutch size remained unchanged (Ratcliffe, 1973). Another parameter implemented to document eggshell changes over time was the ‘eggshell index,’ where the weight of the shell (mg) is divided by the product of the length and width of the shell. Not all birds studied incurred a decrease in eggshell index (table 1.1).

Table 1.1. Mean avian eggshell indices in Britain, prior to and after 1947 (modified from Ratcliffe, table 5, 1970).

Species	# eggs before 1947	# eggs 1947-	Mean index before 1947	Mean index after 1947	% change in index
Peregrine	509	211	1.836	1.485	19.12%
Sparrowhawk	298	279	1.423	1.178	17.22%
Golden Eagle	84	27	3.146	2.834	9.90%
Buzzard	83	96	4.97	4.92	0.46%
Raven	222	205	1.121	1.111	0.89%
Razorbill	46	33	2.339	2.350	0.90%

After Ratcliffe’s discoveries in Britain, Anderson and Hickey (1972) studied over 23,000 eggshells from 25 species in North America. Like Ratcliffe, Anderson and Hickey first determined if there was a significant decrease in eggshell indices before 1947. Unlike Ratcliffe, they combined data by decades and noted that eggshell changes were rare before 1940. The golden eagle was the only species that appeared to have no significant difference in shell index before 1940, which was attributed to low sample size. In addition to eggshell index, eggshell thickness was measured with a dial micrometer. Thickness measurements were compared with eggshell indices and statistically determined to be strongly correlated ($p<0.05$, Anderson and Hickey, 1972). All thickness

measurements were performed on shells that had thinned or broken during incubation, as opposed to fully incubated eggshells that hatched and produced viable offspring. It was then determined that 9 out of 25 North American species underwent shell thinning and shell weight decreases of more than 20%. These species included the peregrine falcon, marsh hawk, brown pelican, prairie falcon, cooper's hawk, double-crested cormorant, black-crowned night heron, bald eagle, and osprey.

The Role of Calcium

Embryonic alligators are thought to obtain calcium required for skeletogenesis from the eggshell primarily, with additional calcium obtained from yolk stores (Packard and Packard, 1984). Initially, alligators were thought to mobilize and deposit calcium similarly to other reptiles but conversely, the processes are more similar to those of birds. Both birds and alligators deposit calcium in the yolk prior to embryogenesis. At hatching, the neonate has a relatively substantial calcium yolk store for growth and development. Embryonic turtles and squamates, however, contain less yolk calcium at hatching than at oviposition (examples include *Chelydra serpentina*, Packard et al., 1984b; *Coluber constrictor*, Packard et al., 1984c; *Amphibolorus barbatus*, Packard et al., 1985). Due to this mechanistic parallel with birds, calcium mobilization and uptake is thought to resemble birds more closely than that of other reptiles. In either case, calcium must traverse cellular barriers including the yolk-sac and chorioallantoic membrane (CAM) to gain access to the embryo. Since alligators lay a highly calcified shell, and the amount of calcium in the shell decreases throughout incubation, it is believed that calcium metabolism can be regulated along the CAM interface, similarly to that in birds (Romanoff, 1960; Reider et al., 1980).

Calcium transport across the chorionic ectodermal cell membranes is believed to involve both mechanistic and regulatory events. Mechanistically, calcium translocation across the CAM can occur pericellularly or endocytotically. There is little evidence for the pericellular basis, leaving endocytosis as the likely transport model (Terepka et al., 1976; Tuan and Zrike, 1978). In the latter model, calcium binds to surface membrane calcium-binding protein (CaBP) and the ligand is encapsulated in pinocytic vesicles, which cross the ectoderm and fuse and empty into the serosal compartment. The function of CaBP and CAM calcium transport activity are probably linked and regulated by the presence of a transport substrate (the calcium rich eggshell) and vitamin K availability (Tuan 1987). In previous studies, the CAM of shell-less *in vitro* cultures of chicken embryos failed to demonstrate developmentally-specific expression of calcium transport activity or CaBP (Dunn et al., 1981; Tuan, 1980, 1984). In addition, vitamin K serves as a cofactor producing the active form of CaBP. A vitamin K deficiency results in a reduction in gamma-glutamyl carboxylation coincident with a decrease in CAM calcium transport activity. Interestingly, when CaBP mRNA is experimentally increased, CaBP level in ovo increases normally as well, suggesting disruption prior to protein translation and potentially during transcription. In various *in vitro* and *in vivo* studies (see review by Tuan, 1987), Ca^{2+} -ATPase and carbonic anhydrase are also implicated in CAM calcium transport function. CAM extracts revealed the presence of Ca^{2+} -ATPase, which was localized on the ectoderm through histochemistry (Tuan et al., 1984), and also aids in calcium transport. Chemically inhibiting Ca^{2+} -ATPase decreases calcium levels in the CAM (Akins and Tuan, 1993). Carbonic anhydrase (CA) is believed to provide local

acidification in the ectoderm and promote dissolution of eggshell calcite (CaCO_3) in vivo.

In doing so, CA can produce ionized calcium ready for uptake (Tuan and Zrike, 1978)

CHAPTER 2

PARAMETERS OF ALLIGATOR EGGSHELLS FROM REFERENCE AND CONTAMINATED NORTH-CENTRAL FLORIDA LAKES

Introduction

Although once classified as endangered, alligator populations in many wetland regions of Florida are stable or increasing (Woodward et al., 1999). Habitat degradation and changing land-use patterns, however, threaten some populations and a better understanding of the biology of this species is needed (Abercrombie, 1989). Population stability is based on reducing death and recruiting new individuals to the population each year. Variables affecting either of these characteristics will have a dramatic impact on the stability, growth, or decline of alligator populations in Florida's wetlands.

For over two decades, the Florida Fish and Wildlife Conservation Commission (FWC) has documented the hatching success of alligators from various Florida lakes. Since 1993, alligators from Lake Woodruff, part of a National Wildlife Refuge, repeatedly exhibit the highest hatch rates (77%-86%), when compared with rates documented from other Florida lakes, particularly Lakes Griffin (30%-32%) and Apopka (32%-52%) (Woodward et al., 1999). Lake Apopka has been subject to agricultural and municipal run-off, extensive herbicide and pesticide use, and a major pesticide spill in 1980 that contained dicofol and DDT (dichlorodiphenyltrichloroethane), among other compounds (Schelske and Brezonik, 1992).

DDT degrades into two primary metabolites, DDE and DDD (USEPA 1996). Of the metabolites, p, p'-DDE (specifically) has been associated with altering the

physiological process of eggshell formation that subsequently led to eggshell thinning and population declines of numerous avian species, particularly raptors and shorebirds (see review by Lundholm, 1997). Approximately four years after the pesticide spill in 1980, a sharp decrease in alligator egg viability on Lake Apopka was documented by the FWC. This decline was hypothesized to be in response to the contamination event (Woodward et al., 1993). Conversely, no major point source of pollution, such as a pesticide spill, has been reported on Lake Griffin, but this lake has received extensive agricultural runoff, municipal storm water runoff, and currently exchanges water with former agricultural fields that are part of a restoration program to create emergent wetlands (see Marburger et al., 2002). Clutches of alligator eggs obtained from Lake Griffin have also exhibited decreased hatch rates along with relatively recent increased adult alligator mortality (Ross 2000; Schoeb et al., 2002). Hatch rates for Lakes Apopka and Griffin still remain lower than those of Lake Woodruff, particularly throughout the duration of this study (Table 2.1), though the mechanism(s) by which this occurs remains unknown.

Table 2-1 Mean alligator egg viability rates and sample size of clutches collected from three study areas and incubated under 32°C in an artificial incubator, 1999, 2001-2003 (Woodward, pers. com.).

Year	Study Areas					
	Lake Woodruff		Lake Apopka		Lake Griffin	
	Mean	N	Mean	N	Mean	N
1999	0.85	18	0.56	9	0.41	21
2001	0.79	16	0.53	15	0.55	19
2002	0.74	11	0.27	7	0.56	20
2003	0.90	7	0.31	11	0.72	17

A number of factors could contribute to embryonic mortality. One of these factors could be a change in eggshell structure or composition leading to altered eggshell

function during incubation. The crocodilian oviduct more closely resembles birds than that of other oviparous reptiles (Palmer and Guillette, 1992). It is a heterogeneous structure consisting of seven regions. Functionally and ultrastructurally, the anterior uterus of the alligator resembles the avian isthmus where eggshell membrane formation takes place. Endometrial glands located in this region produce proteinaceous fibers that are hypothesized to compose the eggshell membrane. The posterior uterus is similar to the avian shell gland, which secretes the calcareous eggshell. Unlike birds, however, alligators ‘shell’ eggs of a clutch simultaneously whereas birds ovulate and ‘shell’ each egg of a clutch individually.

To date, several studies indicate that the supply of calcium to the eggshell gland in ducks is not impeded by p,p'-DDE, but rather, this organochlorine contaminant disrupts calcium transport within the eggshell gland (Lundholm, 1997, 1990a, 1990b; Kolaja, 1977). In addition, p,p'-DDE inhibits Ca^{2+} -ATPase or Ca^{2+} - Mg^{2+} -ATPase activity in the eggshell gland suggesting one mechanism for DDE-induced eggshell thinning. Calcium transport across the eggshell gland mucosa is coupled with both sodium and bicarbonate (Eastin and Spaziani, 1978; Pearson and Goldner, 1973) and accordingly, the impaired movement of one of these ions can influence the movement of others (particularly calcium). Additionally, the prostaglandins $\text{PGF}_{2\alpha}$ and PGE_2 have been implicated in eggshell thinning. DDE disrupts the synthesis of these two prostaglandins, which reduces bicarbonate transport in the duck shell gland lumen, in turn reducing calcium transport. Both $\text{PGF}_{2\alpha}$ and PGE_2 have previously been closely associated with eggshell formation in birds (Hammond et al., 1980).

Alligators from Lake Apopka lay eggs with elevated concentrations of p,p'-DDE, and juveniles and adults in the population have elevated concentrations of this contaminant in their blood and eggs (Heinz et al., 1991; Guillette et al., 1999) presumably due to eating p,p'-DDE-contaminated food items (Rauschenberger et al., 2004). The application of the pesticide DDT to croplands began in 1945 in the United States, and continued until the Environmental Protection Agency (EPA) restricted its use in 1972. This pesticide and its metabolites are persistent, bioaccumulating, and biomagnifying in the food chain (see review, Guillette et al., 2005). Due to the previous affiliation between DDT and its metabolites with avian eggshell thinning (Anderson and Hickey, 1972; Henny and Bennett, 1990; Hickey 1969; Ratcliffe 1967), as well as the phylogenetic relationship (Gower 2002) and similarity in egg shell formation (Palmer and Guillette, 1991, 1992) between birds and crocodilians, we hypothesized that alligator eggs could undergo eggshell thinning as a result of p,p'-DDE exposure.

To begin to test the hypothesis that organochlorines, such as p,p'-DDE, could contribute to higher mortality of alligator embryos in several Florida wetlands through altered shell function, we examined alligator eggshell structure and composition from clutches laid on three Florida lakes. Morphometric data were collected as well as microscopic structural data (light and scanning electron microscopy).

Materials and Methods

Alligator eggs were collected from three north-central Florida freshwater lakes (lakes Woodruff, Griffin, Apopka) in June of 1999, 2001, 2002, and 2003. The eggs were transported to the University of Florida and incubated until hatching. The shells of eggs that produced viable neonates were collected after hatching, stored in whirlpacks,

and frozen at –20 °C for processing at a later date. Those eggs that did not produce viable neonates were not included in any analyses.

Thickness Measurements

Alligator eggshells are composed of an inner tough fibrous layer composed of protein fibers covered by an outer calcium rich crystalline layer. The hard outer layer was used to determine eggshell thickness and hereafter is referred to as the ‘eggshell’. Eggshells were rinsed of organic matter with tap water, air-dried at 20 °C for 72 hours, and oven dried at 42 °C for 90 minutes. Thickness measurements were taken with a dial caliper to the nearest millimeter at five different regions on each egg. Three measurements were obtained from the shell that would have covered the equatorial region of the egg and two measurements were obtained from the polar ends of each egg. Three eggs were measured from each clutch so that a clutch mean could be determined (Table 2.2). The clutch mean was then used in all statistical analyses.

Table 2-2 Number of clutches from which eggshell thickness was examined each year of the study period from 3 lakes. (Note: 3 eggs/clutch were measured).

Year	Lake Woodruff	Lake Apopka	Lake Griffin
1999	12	12	12
2001	11	8	10
2002	10	5	15
2003	9	5	10

In 2003, five clutches (shell fragments from 3 different eggs /clutch) from each lake were further analyzed for pore density and elemental composition (including heavy metals) and were examined by scanning electron microscopy (SEM).

Pore Density

Eggshell fragments were dyed with 0.15 ml of fast green in 30 mls of dH₂O for 2 minutes and allowed to air-dry in the laboratory overnight. A 2.4 cm² circular region of the egg was isolated from the equatorial region of each egg. Pores were counted with a dissection microscope and final values were converted to pores/cm².

Elemental Analyses (EDS)

Energy Dispersive Spectral Analysis (EDS) was performed to detect constituent elements in each eggshell sample. Briefly, EDS operates by submitting the sample to electron bombardment producing a range of X-rays of different energy levels that correspond with a particular element. Carbon, calcium, and oxygen values were averaged for each clutch and within each lake and were compared using ANOVA. (Elements detected below 0.10% are not included in analyses, as this represents the lowest reliable quantitative detection limit for this analysis).

Elemental Analyses (ICP)

Inductively coupled plasma-optical emission spectrophotometry (ICP-OES, Perkin Elmer 6100) was used to detect heavy metals. ICP-OES uses an argon plasma to generate measurable atomic emission. The emission lines for each atom are monitored at specific wavelengths. Standard metal solutions in 5% nitric acid were obtained (Fisher Scientific Inc.) and diluted to appropriate concentrations in plastic volumetric labware to avoid the problem of metals adhering to glass. The analyses of these standard solutions were used to generate calibration curves. Alligator eggshells were dissolved in 5% nitric acid (metal analysis grade). The resulting solution was analyzed under identical conditions as the standards and the shell concentrations calculated using the calibration curves. All standards and samples were analyzed in triplicate. Both analysts were blind

to the sample status of each eggshell. Sufficient concentrations of aluminum and magnesium were detected and statistically analyzed (ANOVA) to determine possible differences among lakes.

Scanning Electron Microscopy (SEM)

Two fragments from the equatorial region of each shell were desiccated, mounted on SEM stubs and sputter coated with gold. One fragment was viewed in cross section (200X and 5000X), and the other fragment was oriented to view the internal surface of the shell (250X, 450X, 4000X, 8000X). The shells were qualitatively characterized by their crystalline structure, shell membrane structure, relative fiber and node presence, pore morphology, and layer density.

Statistics

Analysis of Variance (ANOVA) was used to compare data among lakes (Statview for Windows, version 5.0©). Statistical significance was determined if $p \leq 0.05$. If a significant difference was determined, Fisher's post-hoc analyses were used for pair-wise comparisons.

Results

Eggshell Thickness

Regarding shell thickness, there was no interaction among lakes and years combined (2-way ANOVA, $p=0.5117$). There was a significant difference in shell thickness among lakes in 1999 and 2003, but no differences were detected in 2001 and 2002. This variation could be due to small sample sizes for a given year and lake. In support of this concept, if data from all years were combined for each lake, a significant difference among lakes was observed ($p = 0.02$; Fig. 1). As hypothesized, eggshells obtained from the more contaminated Lakes Apopka and Griffin, exhibited similar shell

thickness, but contrary to our predictions, they had thicker eggshells than those obtained from Lake Woodruff (Apopka vs. Woodruff, $p = 0.0003$; Griffin vs. Woodruff, $p = 0.0021$; Fig. 1). It must be noted that these thickness measurements were obtained from egg shells post hatch, and represent the thickness remaining after embryonic development was complete.

Pore Density

There was no significant difference in eggshell pore density among lakes ($p = 0.55$; Fig 2). Further, we detected no difference in pore density among clutches from Lakes Woodruff and Griffin, whereas there was a significant difference among clutches from Lake Apopka ($p = 0.01$; Fig. 3).

Elemental Analyses (EDS)

There was a significant difference in calcium, carbon, and oxygen concentrations in the eggshells obtained from the various lakes of this study ($p = 0.005$, $p = 0.0006$, $p = 0.004$, respectively). That is, we observed that shell samples from Lake Woodruff were similar in percent calcium to those from Lake Apopka but significantly greater than those from Lake Griffin ($p = 0.0047$; Fig. 4). Lake Woodruff eggshells contained significantly lower percent carbon than both Lakes Apopka ($p = 0.0129$) and Griffin ($p = 0.0002$), which were similar (Fig. 4). Eggshells from Lakes Woodruff and Apopka contained similar percentages of oxygen and both exhibited significantly higher levels than those recorded from shells from Lake Griffin ($p = 0.001$, $p = 0.0250$, respectively; Fig. 4).

Elemental Analyses (ICP)

Lead, arsenic, iron, aluminum, zinc, nickel, manganese, chromium, calcium, magnesium, and cadmium were examined in each sample. Only calcium, aluminum, and magnesium were detectable (mgs/liter). There was a significant difference in aluminum

and magnesium levels detected in the shells from the three lakes ($p = 0.025$, $p = 0.020$, respectively). Eggshells obtained from Lake Woodruff contained significantly more aluminum than shells from Lakes Apopka ($p = 0.009$) and Griffin ($p = 0.047$), which were similar (Fig. 5). Shells obtained from Lake Griffin contained significantly less magnesium when compared to shells from either of the other two lakes, Lake Woodruff ($p = 0.014$) or Lake Apopka ($p = 0.016$, Fig. 4).

Scanning Electron Microscopy (SEM)

A qualitative analysis of the structure of the eggshells using scanning electron microscopy revealed several interesting differences when shells were compared among lakes. An irregular network of fibers is present on the inner surface of the calcareous eggshells from all three lakes, although their relative abundance varies (Fig 6 & 7). Eggs from Lake Woodruff can be categorized as having *several*, *intermediate*, *few*, and *no fibers* at all (Fig. 6). They are randomly orientated and overlap extensively. The majority of eggs from Lakes Griffin and Apopka contained *few* or *no* fibers at all. Several nodes are present and give each fiber a coated appearance (Fig. 8). Little is known about the make up of these fibers; however, it has been suggested that these nodes are made of glycosaminoglycans (GAG) (Ferguson 1982). Functionally, they can represent a template for calcified crystalline growth similar to that in mammals where hydroxyapatite crystals give rise to bone and dentin (Brown 1975; Jenkins 1978). The fibrous arrangement and size and amount of nodes appear more similar between samples from Lakes Apopka and Griffin (Fig. 7). Shells from Lake Woodruff, however, appear to have fewer and larger nodes (Fig. 8).

There were also similarities in pore-structure of shells obtained from the three lakes. Pores were characterized by crater-like concentric rings that develop on the outer-

surface of the eggshell (Fig. 9a,d,g). The pore orifice leads to an irregular shaped cavity that extends through the calcareous shell (Fig. 9b,e,h). Within each pore cavity, the crystalline structure is apparent, though the patterns can vary among lakes (9c,f,i).

SEM cross-sectional views revealed the honeycomb layer previously described for alligator eggshells (see Ferguson, 1982). Interestingly, we observed that all eggshells from Lake Woodruff lacked the organic portion of the honeycomb region and contained a smoother, denser region of crystallized calcium (Fig. 10), whereas eggshells from lakes Griffin and Apopka displayed a region similar to that described by Ferguson (Fig. 10). This difference in eggshell structure was unexpected. Finally, the structural make-up of the inner eggshells appeared more dense and similar in Lakes Woodruff and Griffin when compared to Lake Apopka (Fig. 11).

Discussion and Conclusions

An analysis of eggshell structure and composition revealed that variation existed among alligator populations from central Florida lakes. Some of the variation noted appears to be correlated to contaminant exposure but other aspects are not explained by the environmental data collected during this study. We observed that post hatching eggshell thickness is significantly less from eggshells from Lake Woodruff versus shells from Lakes Griffin and Apopka. Additionally, we observed a major difference in the post hatching shell, in that shells from Lake Woodruff lacked the organic portion of the honeycomb region that lies below the dense outer cortical region composed of tightly fitted calcium crystals. Finally, we observed that shells from Lake Woodruff had higher concentrations of aluminum when compared to eggshells from other lakes. Previous studies of birds exposed to organochlorine contaminants reported thinner eggshells (for review, see Lundholm, 1997). Unlike our study, previous studies have examined

eggshells that either had non-viable embryos or were obtained during early incubation. We have not been able, to date, to identify studies that were performed similar to ours, where eggshells were obtained after successful incubation.

We hypothesize that Lake Woodruff embryos exhibit elevated gas exchange producing larger neonates. Elevated gas exchange would presumably generate greater CO₂ levels at the shell interface with the pores and honeycomb region maximizing calcium mobilization via the generation of carbonic acid. The lack of the organic portion in the honeycomb region can be a result of increased carbonic acid production and its subsequent dissolution of the eggshell. Milnes et al., (submitted) observed that mean egg mass was greater for eggs from Lake Woodruff ($86.9 \pm 0.68\text{g}$) compared to Lake Apopka ($77.7 \pm 0.72\text{g}$) in 1999. When adjusted for egg mass, hatchling body mass and SVL varied between lakes, with Woodruff hatchlings having greater mean body mass when incubated at 32°C and 33.5 °C, and greater SVL when incubated at 32°C (Milnes et al., submitted). These data suggest that even with differences in egg mass accounted for, neonates from Lake Woodruff are larger. We have previously observed that Lake Woodruff neonates are more likely to survive compared to neonates from Lake Apopka (Guillette et. al., 1994). We suggest that vigorous, healthy neonates come from healthy embryos that utilize more calcium in the eggshell and could have larger chorioallantoic membranes. Future studies need to examine the hypothesis that embryos from Lake Woodruff generate more CO₂ and greater carbonic acid levels that etch the inner surface of the eggshell more effectively.

The lack of a honeycomb region in the outer calcium eggshell obtained from Lake Woodruff was unexpected. Again, it should be noted that these shells were obtained after

successful incubation so the lack of this region may be a post-oviposition modification, not an original structural difference. The honeycomb region of the eggshell lies between the outer densely calcified layer and the organic layer and is composed of both a fibrous organic matrix and calcite crystals (see Packard and Packard 1984). This honeycomb region would be expected to trap CO₂ and water released from the chorioallantoic membrane, which lines the inner surface of the proteinaceous shell. We note above that the eggshells obtained from Lake Woodruff neonates were thinner, possibly due to the calcified portion of the honeycomb region being eroded (and mobilized to the embryo) to a greater extent than that of the same region in eggshells from other lakes. Additionally, Woodruff eggshells contained less carbon, as shown through energy dispersive analysis, which may also contribute to their respective thinner shells.

Eggshells from Lake Woodruff also had greater concentrations of aluminum, a metal not required by living systems. Since aluminum is the most abundant metal in the earth's crust (Rengal 2002), it is possible that relatively higher aluminum levels are natural since Lake Woodruff has undergone less anthropogenic influences than the other lakes. Potentially, lower aluminum levels may impede normal biological functions in the biota underexposed. Lake Griffin eggshells contained more magnesium than shells from other lakes. This mineral is necessary for calcium metabolism and could be elevated in Lake Griffin shells due to a lack of proper calcium metabolism by the embryo. The concentrations of these minerals could have biological impact but future studies would be required to determine if the levels measured influence alligator embryonic viability and eggshell development.

It's important to note that all of the samples of alligator eggshells were from post-hatching and viable neonates, whereas bird eggshells had to be cracked or show evidence of housing a dead embryo to be included in analyses. It is possible that bird eggshells were collected at different stages of embryonic development, and would therefore be expected to have different amounts of calcium mobilized from the shell, thereby influencing shell thickness. This could explain why not all species were seemingly influenced by p,p'-DDE. There can also be interspecific and/or intraspecific variation in when, gestationally, shells begin cracking. Perhaps species that incur comparatively increased gestation times or larger clutches are more impacted by compromised calcium transport than those with shorter gestation times and smaller clutches. The same could occur within species, depending on the maturity and/or overall health of the female parent. Some species shells' may be more prone to swelling (from ambient humidity) or dessication, which influences embryonic viability and ultimately biases the sample collected. To avoid these biases, future studies could focus on shells from post-hatching and viable avian neonates. Lastly, hormones are known to disrupt transport channels indirectly related to calcium mobilization and can be another source of altered calcium transport. These potential calcium transport offenders may not be mutually exclusive of one another, thus compounding calcium transport further.

Both bird and alligator eggshells could serve as potential biomarkers of healthy neonates since the embryos completed development within them. Conversely, it would be interesting to compare alligator shells from early incubation to bird shells of early incubation. Shells sampled from relatively parallel incubation windows could be directly compared to determine relative constituents, their mobilization, and the influence of

environmental factors on these parameters. Regardless if the shells are from early or late incubation, it could be worthy to examine the effects of other abiotic factors such as substrate, temperature, or altitude on eggshell integrity of DDE exposed and unexposed species.

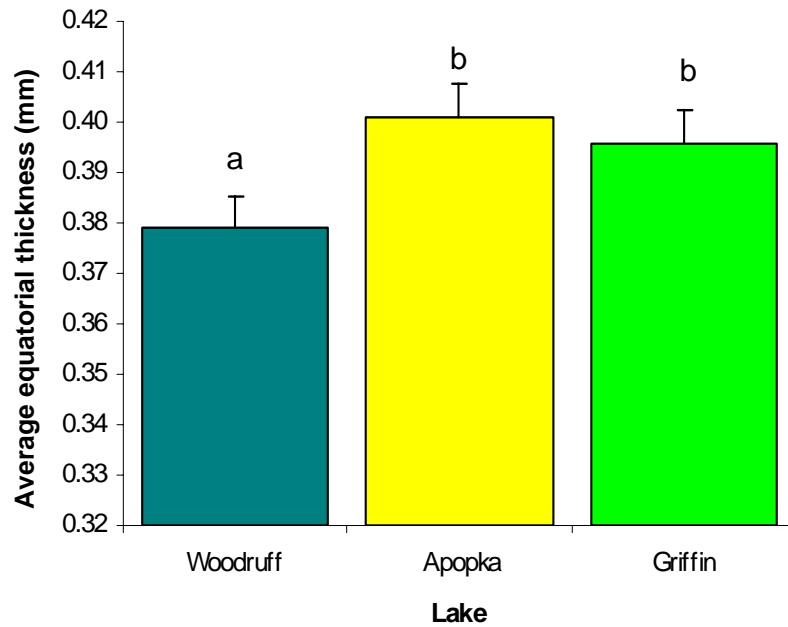


Figure 2-1 The mean (+/- 1s.e.) equatorial eggshell thickness among lakes and years (1999,2001,2002,2003). (Different superscripts indicate significant differences between lakes).

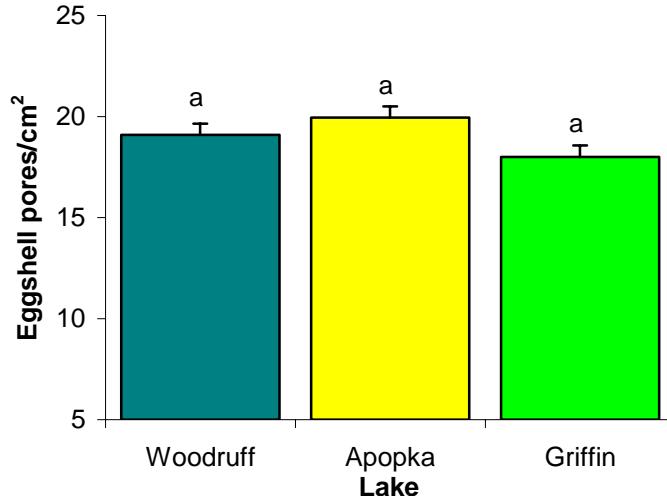


Figure 2-2 The mean (+/- 1s.e.) number of eggshell pores among lakes in 2003. (similar superscripts denote no significant difference among lakes).

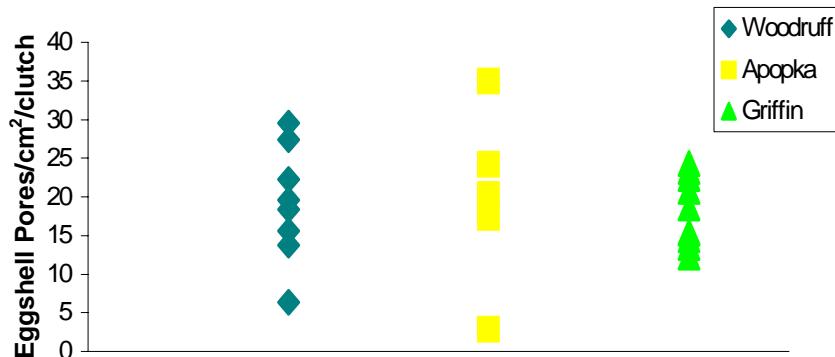


Figure 2-3 The mean (+/- 1s.e.) number of alligator eggshell pores per clutch, per cm², among lakes in 2003. Note the variation about the means, particularly from Lake Apopka.

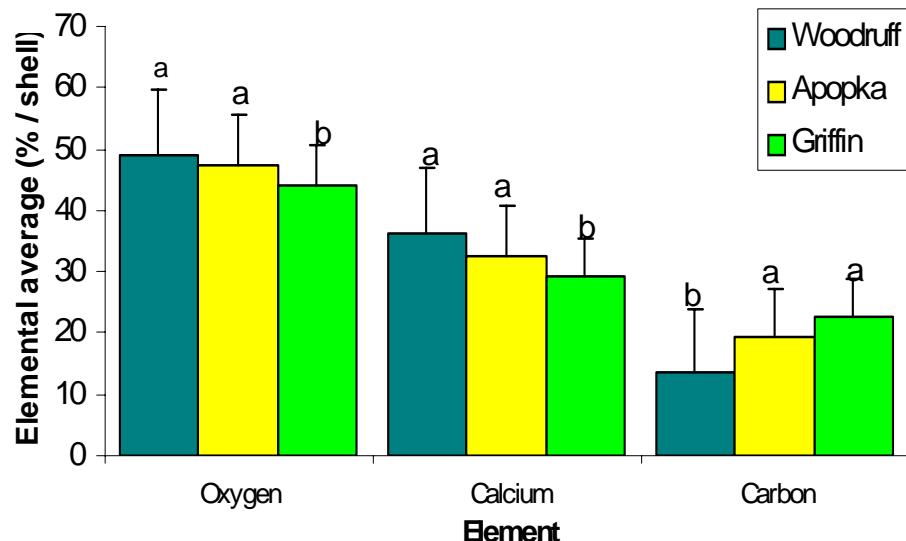


Figure 2-4 The mean (+/- 1s.e.) percent of the three most abundant elements in alligator eggshells detected by Energy Dispersive Analysis (EDS), (n=3 shells/clutch, 5 clutches/lake; Different superscripts denote significant differences among lakes).

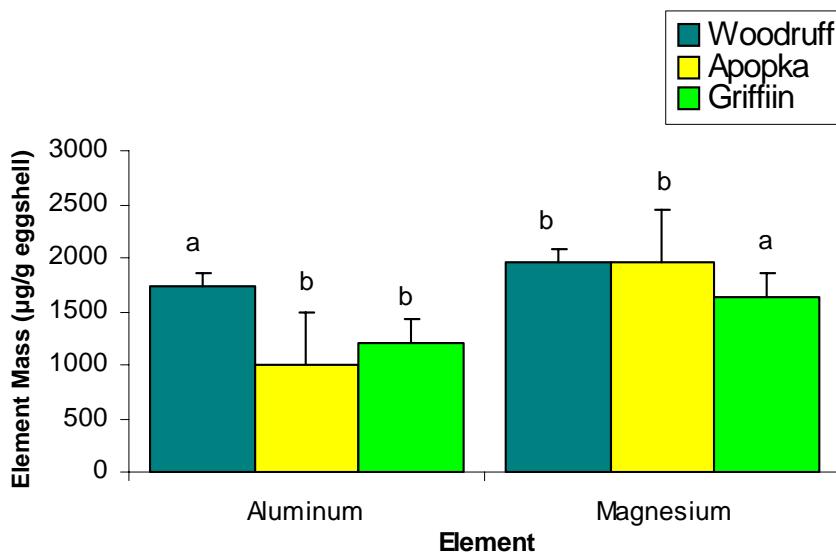


Figure 2-5 The mean (+/- 1s.e.) percent of the three most abundant elements in alligator eggshells detected by Energy Dispersive Analysis (EDS),(n=3 shells/clutch, 5 clutches/lake; Different superscripts denote significant differences among lakes).

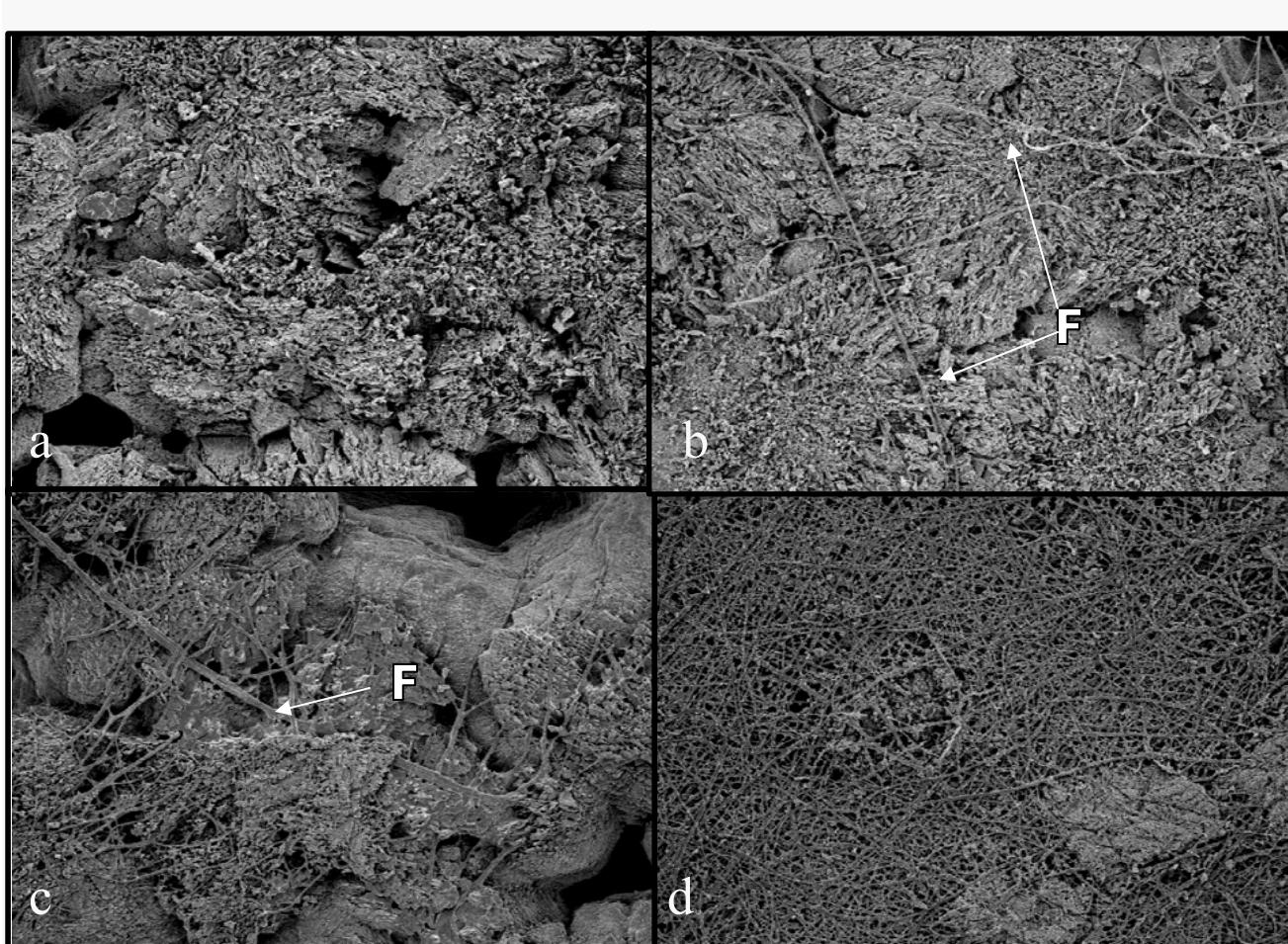


Figure 2-6 Scanning electron micrographs of the inner surface of Lake Woodruff alligator eggshells (250X). Note the variation in relative fiber presence, a) no fibers b) few c) intermediate d) several.

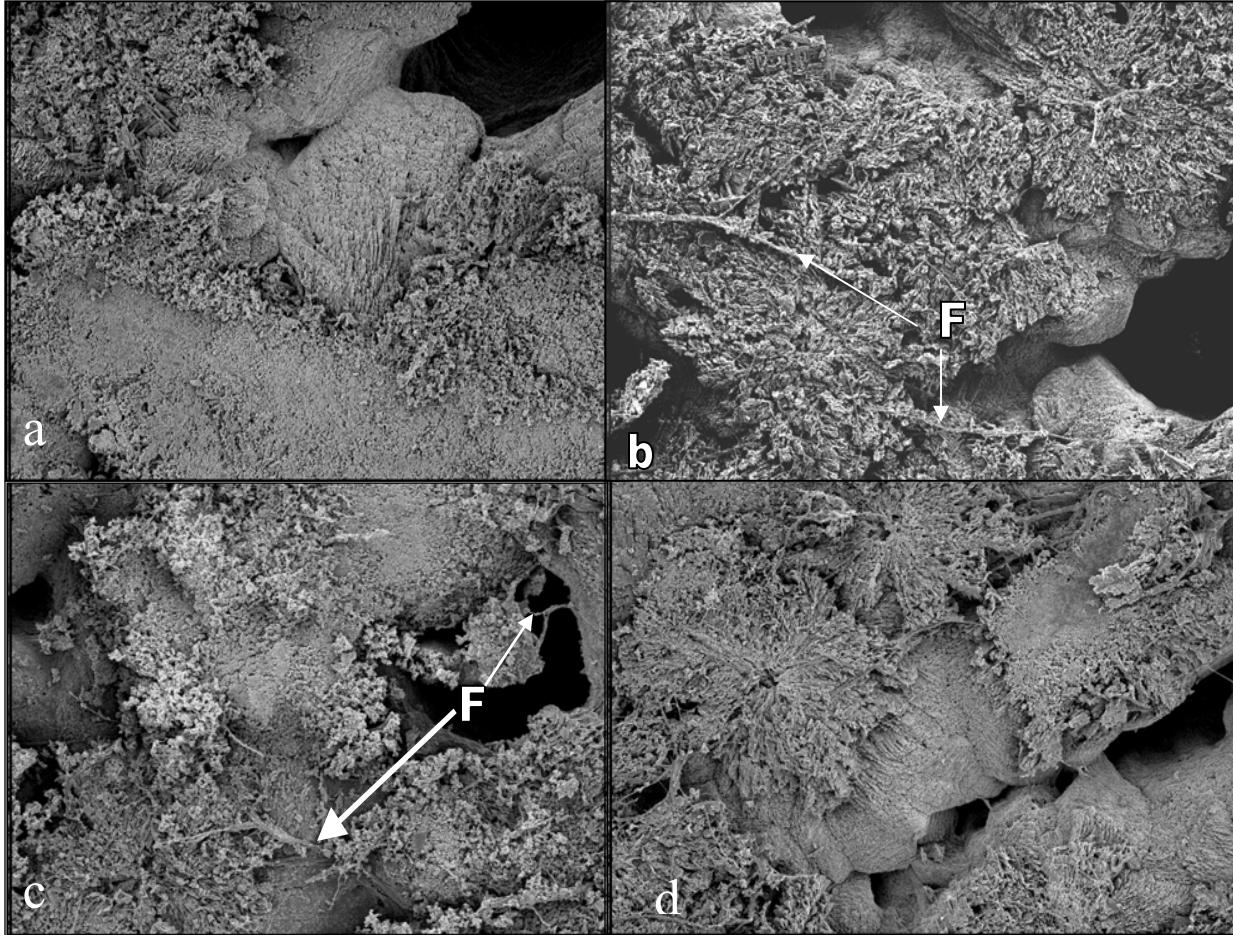


Figure 2-7 Scanning electron micrographs of the relative amount of shell fibers (F) in Apopka (a,b) and Griffin (c,d). Shells from both lakes displayed few to no fibers (250X, n = 3 shells/clutch, 5 clutches / lake), relative to some Woodruff shells.

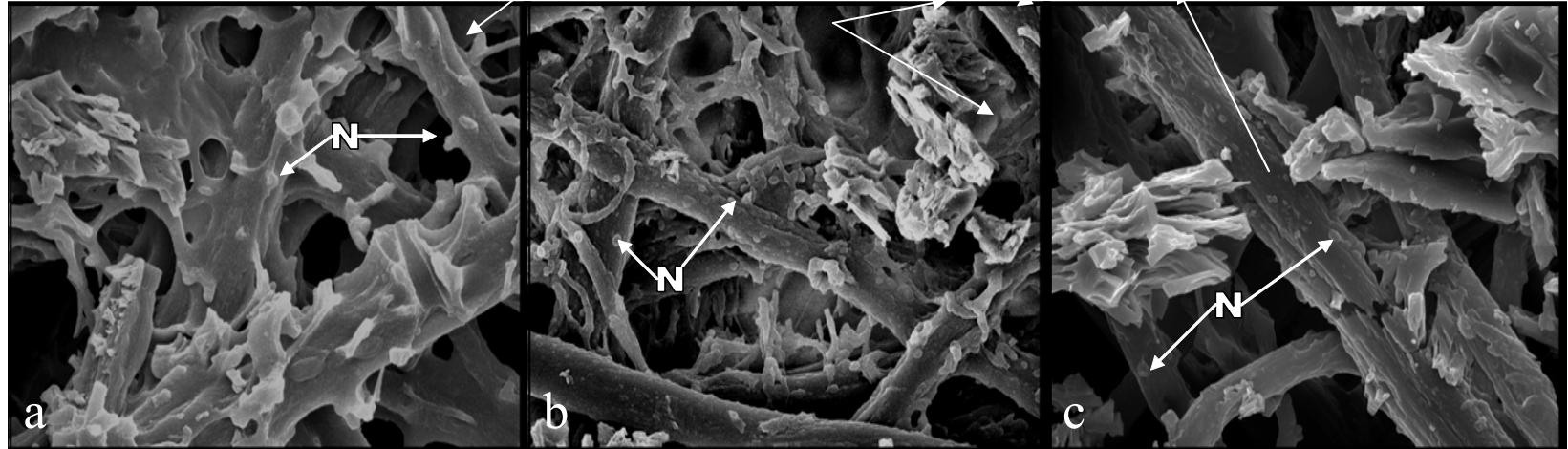


Figure 2-8 SEM images of shell membranes from a) Woodruff, b) Apopka, c) Griffin in 2003. Note the numerous fibers and the relative size of the nodes (N) on Woodruff vs. Apopka and Griffin (8000x) ($n= 3$ shells/clutch, 5 clutches/lake).

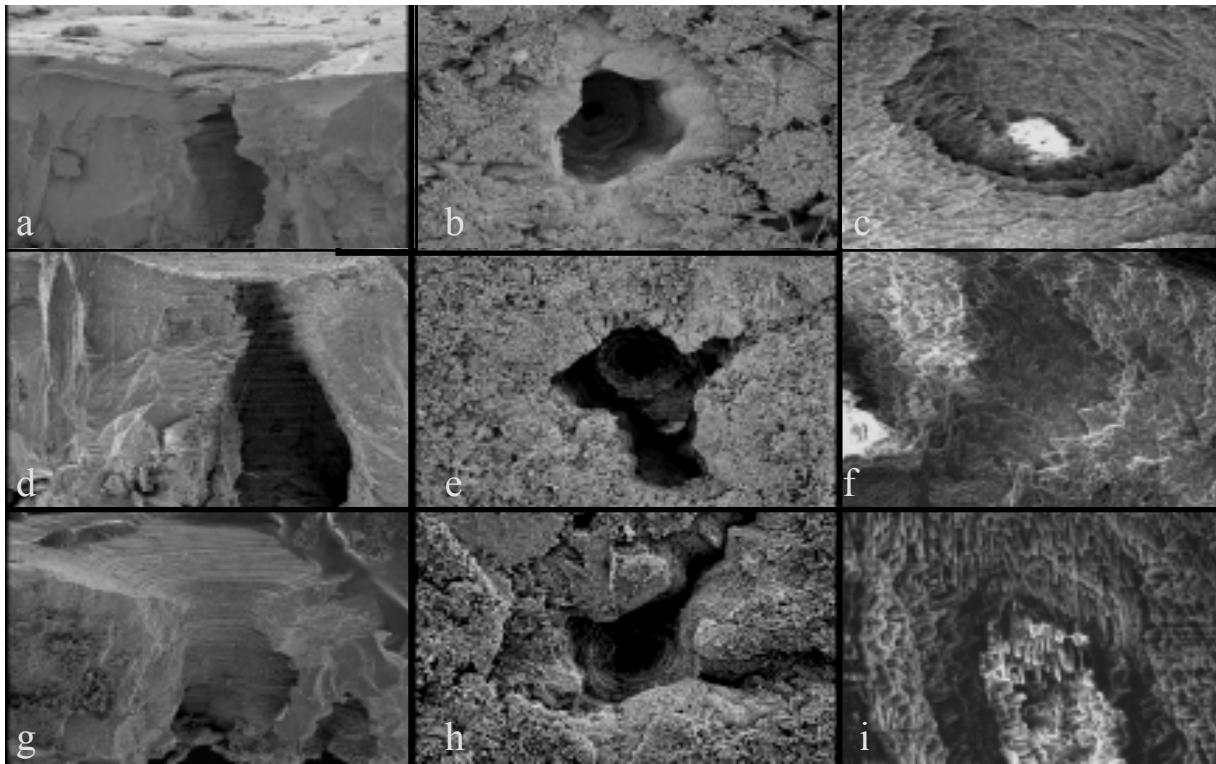


Figure 2-9 SEM images of eggshell pore cavities from 2003 eggshells. Woodruff (a,b,c), a= cross section (200x), b= outer shell surface (200x), c= internal view of pore. Apopka (d,e,f): d=cross section (200x), e= outer shell surface (200x), f=internal view of pore (8000x). Griffin (g,h,i,): g=cross section (200x), h=outer shell surface (200x), I=internal view of pore, (n= 3 shells/clutch, 5 clutches/lake).

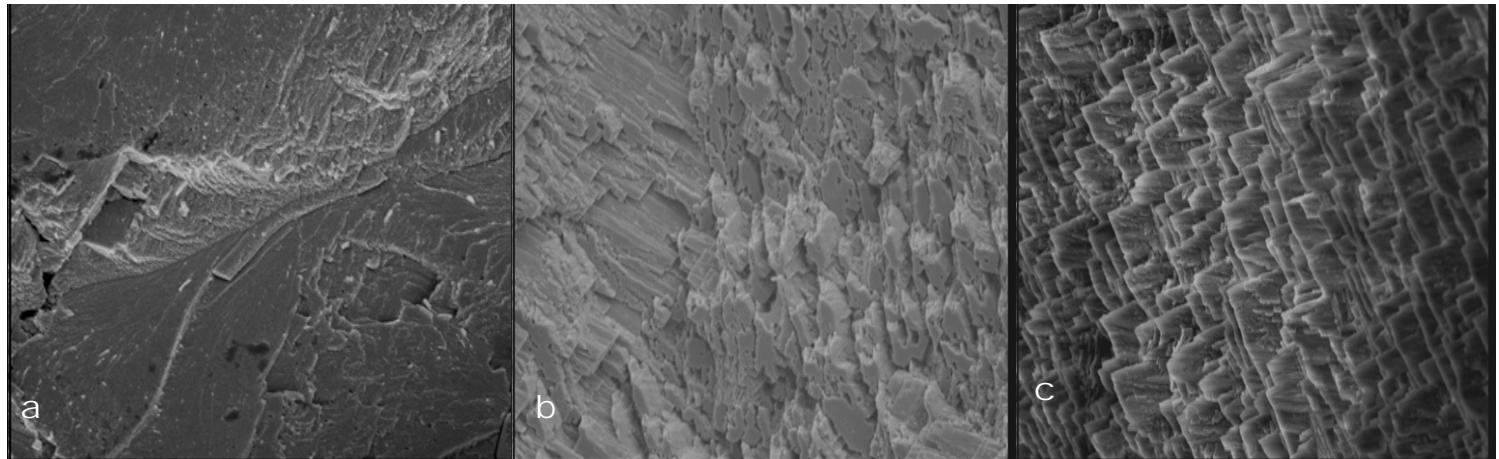


Figure 2-10 SEM Cross-sectional views at 5000X from lakes a) Woodruff, b) Apopka, c) Griffin (2003). Note the smooth surface layer (a) versus jagged and multi-edged surfaces (b & c) ($n=3$ shells/clutch, 5 clutches per lake).

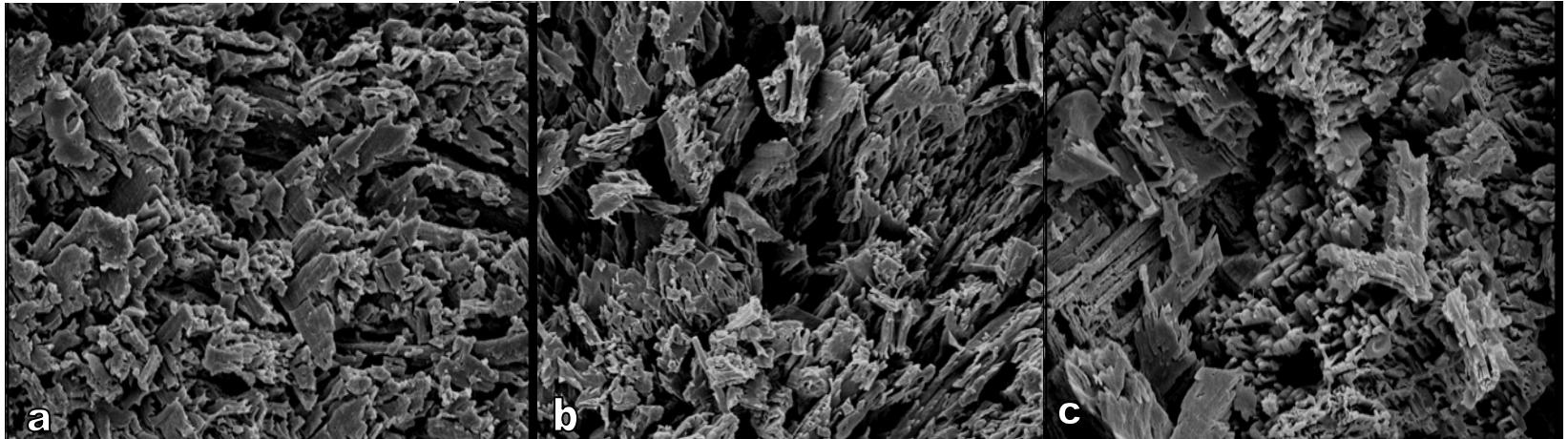


Figure 2-11 SEM image of the calcium crystalline structure on the inner eggshell surface, a) Woodruff, b) Apopka, c) Griffin
4000X, n = 3 shells/clutch, 5 clutches/lake).

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BIOGRAPHICAL SKETCH

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