DEVELOPMENTAL MORTALITY IN AMERICAN ALLIGATORS (*Alligator mississippiensis*) EXPOSED TO ORGANOCHLORINE PESTICIDES

By

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To Jesus, my personal Lord and Savior. John 14:6. “Jesus said to him, I am the way, the truth, and the life: no man comes to the Father, except by me.” Ephesians 2:8-9. “For by grace you have been saved through faith, and that not of yourselves; it is the gift of God, Not of works, lest anyone should boast.”

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Since the early 1900s, the lakes of the Ocklawaha Basin in central Florida have experienced ecological degradation due to anthropogenic development. One species affected by degradation has been the American alligator (*Alligator mississippiensis*). Decreased clutch viability (proportion of eggs in a nest that yield a live hatchling) was observed in the years after a chemical spill in which large amounts of sulfuric acid and dicofol, an organochlorine pesticide (OCP), flowed into Lake Apopka. Lake Apopka and other lakes in the Ocklawaha basin have also been contaminated by urban sewage and agricultural chemicals, with agricultural chemicals entering the lakes via rainfall run-off or back-pumping of water from agricultural lands). Decreased hatch rates are a problem at Lake Apopka, as well as at other OCP-contaminated sites in Florida. The purpose of my study was to determine the causes for decreased clutch viability, and to test the hypothesis that maternal exposure to OCPs is associated with embryonic mortality in alligators.

Field studies involved collecting and artificially incubating eggs from reference sites (Lake Lochloosa) and from OCP-contaminated sites (Lakes Apopka, Griffin, and Emeralda Marsh Restoration Area) to evaluate clutch viability as a function of egg and maternal OCP concentrations. Nutrient content of eggs and histopathology and morphometrics of embryos were also evaluated to identify potential factors associated with embryo mortality. In addition, a novel laboratory experiment exposed a captive population of adult alligators to an OCP mixture, and compared OCP burdens in eggs and clutch viability with a captive control group.

Results of field studies suggested that OCP concentrations (ng total OCP/g egg yolk, Mean ± SE) in reference site clutches (n = 19; 102 ± 16) were significantly (α = 0.05) lower than those of Apopka (n = 23; 7,582 ± 2,008), Griffin (n = 42; 1,169 ± 423), and Emeralda Marsh (n = 31; 15,480 ± 2,265). Clutches from reference sites also had significantly higher clutch viability (70 ± 4%) than those of Apopka (51 ± 6%), Griffin (44 ± 5%), and Emeralda Marsh (48 ± 6%). Furthermore, decreased thiamine concentrations in eggs may play a role in decreased clutch viability in wild clutches. Results of the captive study suggested that treated females produced eggs containing higher OCP concentrations (n = 7; 13,300 ± 2,666) than controls (n = 9; 50 ± 4). Eggs of treated females also exhibited decreased viability (9 ± 6%) as compared to controls (44 ± 11%). These field and laboratory studies support the hypothesis that maternal exposure to OCPs is associated with decreased clutch viability in American alligators, and that thiamine deficiency may also be a contributing factor in reduced clutch viability.

CHAPTER 1
INTRODUCTION

Habitat Degradation in the Ocklawaha Basin

In central Florida, several lakes within the Ocklawaha River Basin (Fig. 1-1) have experienced severe degradation of habitat quality since the early 1900s, as agricultural and urban development progressed. Indeed, Lake Apopka (headwaters of the Ocklawaha) was once renowned for its clear water and its excellent largemouth bass fishing. More recently, Lake Apopka has gained world-wide notoriety as the "poster child" for polluted lakes, because of highly publicized problems associated with environmental contamination. Initial degradation of Lake Apopka and other lakes within the Ocklawaha Basin occurred as the result of the loss of thousands of hectares of marsh habitat through the agricultural practice known as muck farming (which involves installing levees around an area of marsh, so the marsh can be drained; allowing the fertile peat to be farmed). This farming practice began in the 1940s and continued into the 1980s (Benton et al., 1991). In addition to sewer discharge from the city of Winter Garden entering the Lake Apopka, organochlorine pesticides (OCPs) were heavily and widely used to control crop-destroying insect pests.

Since the 1980s, use of most OCPs has been discontinued since they were determined to be persistent environmental contaminants that resist metabolic degradation and bioaccumulate in animal tissues, where they are potentially carcinogenic, immunotoxic, endocrine disrupting, and developmentally toxic (Fairbrother et al., 1999;

Ecobichon, 2001). Altered function of the reproductive and endocrine systems of wildlife and human populations have been suggested to occur after exposure to a variety of OCPs and OCP metabolites such as dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyltrichloroethylene (DDE), methoxychlor, dicofol, chlordane, dieldrin, and toxaphene (Colborn et al., 1993; Longnecker et al., 2002).

Further degradation and OCP contamination occurred in Lake Apopka in 1980. A chemical spill occurred when a highly acidic wastewater pond at the Tower Chemical Company's main facility overflowed into the Gourd Neck area of Lake Apopka (Fig. 1-1). Because of the large volume and acidity (sulfuric acid), and the high levels of DDT, dicofol, and related OCP contaminants that entered the relatively narrow area of the lake, aquatic vegetation and animals were severely affected. In 1983, the area was placed on the US Environmental Protection Agency's (EPA) National Priority Site List and became a part of the Superfund program; which was created by the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), later amended by the Superfund Amendments and Reauthorization Act (SARA). The CERCLA and SARA provide authority for the government to respond to the release and/or threat of release of hazardous wastes, and allow cleanup and enforcement actions. Lake Apopka is still listed and groundwater toxicity testing is ongoing (EPA, 2004).

Alligators as Potential OCP Receptors

The American alligator is an important member of Florida wetlands and plays important roles in the ecology, esthetics, and economy of Florida. Therefore, identifying physiological and ecological characteristics related to potential susceptibility to effects of contaminants, as well as potential exposure routes, is important in managing populations

for optimal human use. Especially important to consider, in regard to wildlife populations, are potential effects of OCPs on reproduction.

One of the first qualities that may be related to an alligator's susceptibility to reproductive effects of OCP contaminants is that alligators do not attain sexual maturity until approximately 6-10 years of age, which allows exposure and bioaccumulation of OCPs to occur before reproductive maturity. Potential implications are that, as females begin to mobilize body stores during vitellogenesis, the lipophilic OCPs that have accumulated in their fatty tissues during their lifespan would likely be deposited in what will later be the embryos' sole source of nutrition (egg yolk). Secondly, adults exhibit a long reproductive period (over 30 years), and a long life span (over 50 years) (Ferguson, 1985), and are higher order predators (which allows for increased OCP exposure and bioaccumulation, possibly leading to altered endocrine and reproductive function). Thirdly, alligators build nests that can be identified from considerable distances (which aids in egg collections), lay a large number of eggs (approximately 40 eggs per clutch), and have a long developmental period of 65-72 days (Ferguson, 1985), allowing extended exposure at a potentially critical stage of development. Thus, the propensity for OCPs to be bioaccumulated and biomagnified in biota (combined with the alligator's reproductive biology, longevity, ecological trophic level, and relatively long *in ovo* developmental period) suggests the potential for OCPs to alter reproductive function.

Developmental Biology of the American Alligator

Understanding normal embryonic development is an obvious necessity in determining the occurrence of abnormal embryonic development and identifying critical periods of development (e.g., organogenesis). Therefore, this brief review summarizes pre-ovipositional and post-ovipositional development of the alligator embryo.

Pre-ovipositional Development

Overall, when compared to other vertebrate species such as the domestic chicken and domestic pig, there is a paucity of data related to crocodilian development. Despite the relatively low number of publications, the quality of papers covering early embryonic development is fairly high, considering that much of the research took place approximately a century ago. The most appropriate place to begin discussing embryonic development would be the point when fertilization occurs. However, the precise timing and location of fertilization within a female alligator's oviduct is unknown and inadequately studied.

Pre-ovipositional development has been examined by sacrificing gravid females and collecting their eggs and embryos. Sacrifice of gravid females was required since alligator embryos are at a more advanced stage of development at the time of oviposition (Clarke, 1891). The earliest developmental stage examined in these pre-ovipositional studies were of Nile crocodile embryos (*Crocodylus niloticus*), in which all embryos exhibited body folds, a neural medullary groove, an embryonic shield, area opaca, early gut, and area pellucida (Voeltzkow, 1892).

After the appearance of the neural folds, the amniotic head fold is formed from an anterior fold in the blastoderm. The head fold is crescent shaped, because it begins to develop with its free ends pointing toward the posterior end of the embryo, and develops craniocaudally. The amniotic primordium develops in continuity with the head, and is derived from the somatopleure around the trunk.

Craniocaudal separation of the embryo from the blastoderm occurs partly as a result of the development of the dorsal amniotic fold, but separation is not complete until

post-ovipositional stage 3 (Day 3). The neural groove and blastopore become clearly demarcated as the ectoderm and endoderm of the blastoderm develop. The endoderm may form extensions that penetrate the underlying yolk. The blastopore goes through the entire blastoderm, with the primitive streak located posterior to the blastopore (Voeltzkow, 1892).

As the body folds develop, the border between embryonic and extra-embryonic tissues becomes visible. At this point, the beginning of the foregut is discernable, and the notochord stretches from the midline of the head fold to the anterior border of the blastopore. The primitive streak and primitive groove lie posterior to the blastopore, with the primitive groove being continuous at its posterior end. The primitive streak extends to a little less than halfway between the head fold and blastopore (Ferguson, 1985).

Neural folds have two origins. The first is a secondary fold located anteriorly in the head region, and growing posteriorly along the median dorsal line to form a V-shaped process, with the apex pointing toward the blastopore. The second is posterior folds that arise as ectodermal ridges extending forward from the blastopore, circumventing the neural groove. The apex of the V-shaped secondary head fold later disappears, and each of the separate arms becomes continuous with the corresponding posterior neural fold. Thus, the secondary head fold forms the anterior part of the neural folds. Closure of the folds occurs first in the middle region of the embryo closer to the anterior end of the neural groove in alligators (Ferguson, 1985) but closer to the posterior end in Nile crocodiles (Voeltzkow, 1892).

After the closure of the neural canal, the blastoporal or neurenteric canal is no longer visible. The neurenteric canal runs from its posterior cranioventral opening to

where it opens into the neural groove at its caudal limit. During this period, somites develop along the median axis, with the first pair developing halfway between the anterior and posterior ends. The peripheral somatic cells are compactly arranged, and contain small myocoels within the center of the somites. The mesodermal layers cleave and form the somatic and splanchnic components as the foregut develops.

The head fold of the embryo is positioned ventrally into the underlying yolk, which is accentuated by the bending of the anterior neural folds, and by the cranial flexure that occurs later. At this pre-ovipositional stage of development, the embryo has not yet attached to the inner surface of the eggshell membrane.

Because embryos are at an advanced stage of development at the time of oviposition (and because an entire clutch typically hatches within a 2-day period, with most hatchlings being similar in size), it appears that fertilization occurs over a short time period; and that embryos are actively developing during the next 2- to 3-week period in which the ova receive albumin, eggshell membrane, and eggshell depositions (Ferguson, 1985). Presently, little information exists about gaseous exchange and embryonic metabolism before oviposition, or about the processes that prevent the embryo from attaching to the top of the egg before oviposition.

Post-Ovipositional Development

Post-ovipositional development is better understood than pre-ovipositional development. Again, the amount of literature concerning crocodilian development is miniscule compared to the amount of literature dealing with human and chicken embryology.

One important area to address when discussing post-ovipositional development is the staging scheme. Establishing a staging scheme or a normal table of development for

any species allows results of various studies to be compared (Billet et al., 1985). The currently accepted staging scheme for crocodilian embryology was proposed by Ferguson (1985). Before Ferguson's, the only other staging systems related to crocodilians came from Voeltzkow (1892), Reese (1912), and Webb et al. (1983). These works were impressive, considering the conditions these pioneers faced; but many stages were missing, and incubation conditions were poorly controlled.

Ferguson (1985) improved on their work by monitoring and controlling the temperature (30°C) and the relative humidity (95-100%) at which the eggs were incubated, allowing duplication of his experiment and standardization of the characteristics one should see in an embryo, given its stage. This accepted staging scheme is based on external morphological features, with limb and eye development being important diagnostic elements. With respect to craniofacial development, a fair amount of data exists, because of Ferguson's focus on the structure and development of the palate in the alligator, and on how its development relates to stage (Ferguson, 1981). Although the relationship between craniofacial development and developmental stage has been studied, information relating stage and development in other organ systems is somewhat lacking.

Alligator embryos are very sensitive to temperature. For example, 26-34°C is the optimum incubation temperature; anything above or below for an extended period will result in increased mortality (Ferguson, 1985). Furthermore, 0.5-1 C changes can mean the difference between an entire clutch of embryos being 100% females or 100% males, since crocodilians exhibit temperature-dependent sex determination (Lang & Andrews, 1994).

Ferguson's Post-Ovipositional Staging Scheme

Because our study used Ferguson's staging scheme, a summary description of Ferguson's (1985) staging scheme, it is summarized here. The normal table of development for crocodilians was based on examination of 1500 *Alligator mississippiensis* embryos, 300 *Crocodylus porosus* embryos, and 300 *Crocodylus johnsoni* embryos. One bias is that all of the alligator embryos used in developing this scheme originated from Rockefeller Wildlife Refuge, located in southern Louisiana. Alligator embryos from other geographic areas may develop at different rates, given the same incubation conditions. Alligators inhabiting Arkansas and North Carolina experience a shorter summer compared to populations inhabiting southern Louisiana or Florida. Shorter summers mean that optimal nest temperatures are maintained for a shorter period of time. Thus, embryos from more northerly latitudes may develop at an increased rate compared to embryos from southerly latitudes (given identical incubation conditions), since the northern embryos must complete development within a shorter time frame. This hypothesis is supported by evidence that crocodilian species (*Crocodylus porosus* and *C. johnsoni*) living along the equator have longer and more variable incubation periods and slower embryonic development than the (more northerly) Louisiana alligator (Deeming & Ferguson, 1990).

Setting aside the potential bias described above, developmental "stages" are determined by morphological characteristics alone, and are applicable to embryos regardless of incubation temperature. However, the developmental day(s) associated with each stage are only valid if the eggs are incubated at 30°C with a relative humidity of 95-100%. Temperatures lower than 30°C slow the rate of development, and temperatures above 30°C have been shown to increase the rate of development. Low

humidity within the nest has been shown to dehydrate eggs, causing embryonic mortality and alterations in growth patterns (Deeming & Ferguson, 1990).

Stage 1 covers the period from oviposition to the end of the first 24 hours, and is characterized by the embryo and blastoderm being not attached to the top of the inner eggshell membrane. The heart is a simple S-shaped tube. There are 16-18 pairs of somites along the trunk, and 3 pairs of somitomeres anterior to the otic vesicle. Although the brain has not yet regionalized, optic placodes and vesicles are present on the head. Body torsion has not begun. The notocord is evident, the gut is incomplete caudally and opens ventrally, and blood vessels are not present in the extraembryonic membranes.

Stage 2 (Day 2) embryos have 21-25 pairs of somites and a three-loop heart. However, one of the most notable characteristics is that the embryo attaches to the top of the egg, causing an opaque spot to form that is visible in an otherwise translucent egg, when the egg is candled. Blood vessels are now visible, and the hindbrain is discernable as a clear transparent region. The lens placode and optic cup are defined, and no body torsion has occurred.

Stage 3 (Day 3) embryos have 26-30 somites, and are completely delineated from blastoderm. Forebrain, midbrain, and hindbrain are now discernable, and the optic cup has an elongated horseshoe shape, extending below the lens vesicle to the primitive oronasal cavity. The head is positioned at a right angle to the body, but no body torsion has occurred.

Stage 4 (Day 4) embryos have 31-35 pairs of somites with the tail being distinct, straight, and unsegmented at the posterior end. Body torsion has started, with the cranial half rotated so that the right surface is contacting the shell membrane, while the left is

parallel with underlying yolk. The caudal half of the embryo remains at a right angle to the yolk. The heart is displaced from midline to the left side of the embryo. Three cranial arches are present; and cranial nerves to the cranial arches are visible, using oblique or transmitted illumination.

Stage 5 (Day 5) embryos have 36-40 pairs of somites, and the tail-tip bends ventrally at a right angle to the body, with 3-5 somites visible at its base. Body torsion is complete except for the tail. The otic pit is dorsal to the junction of the 2nd and 3rd brachial arches, and its external opening is closed.

Stage 6 (Day 6) embryos have visible nasal placodes, and the hindlimbs are barely discernable on each side; with the right hind limb slightly advanced over the left. Forelimb buds are not yet present, and body torsion is complete. The olfactory bulbs, forebrain, and midbrain are distinct. In the hindbrain, 4-6 neuromeres are discernible. Foregut and hindgut are formed, but midgut is incomplete ventrally. Major vitellogenic blood vessels emerge at the level of the 18th somite and smaller ones at the 6th and 11th somites.

Embryos at Stage 7 (Day 7) have distinct hind limb buds. In addition, forelimb buds are barely visible and extend over somites 12-15. The midbrain bulge is evident, and the tail-tip is curled at 90° to the rest of the tail. Three brachial arches are present; and at the level of the heart, the cranial end is bent at 90° to the rest of body.

Embryos at stage 8 (Day 8) have nasal pits external to the swellings of the olfactory bulbs, and distinct forelimb and hind limb buds that extend over somites 11-16 and 27-32, respectively. An apical ectodermal ridge is developing on the hind limb bud, and the tail is coiled through 2 turns and has 12-18 somites.

Stage 9 (Day 9) embryos have four brachial arches, and a visible maxillary process extending to the midpoint of the eye. The optic cup is large and round but unpigmented. A distinct apical ectodermal ridge is present on the hind limb, and the hind limb bud extends beyond the forelimb. The tail is curled through three 90° turns. The heart exhibits distinct atria and ventricles, and lung primordia are visible through the pericardial sac. Midgut and body walls are open ventrally from the caudal limit of the pericardial sac to 2/3 of the way down the body, and the liver and mesonephros are barely visible.

Stage 10 (Day 10-11) embryos have pigmented eyes (except for a central opaque lens) with the right eye developing pigmentation earlier and darker than the left eye. Five brachial arches are present, and medial and lateral processes are distinct elements on each side of the nasal pits. Maxillary processes delimit a distinct groove beneath the eye. The tail is coiled through four 90° turns, and the liver and mesonephros are clearly visible through the body walls.

Stage 11 (Day 12) embryos have a visible nasal pit slit forming between the medial and lateral processes. Forelimb and hind limb buds extend caudally from the body wall and exhibit distinct apical, ectodermal ridges. The forelimb has a distinct constriction that separates the distal and proximal elements, with constriction less obvious in the hind limb. A loop of midgut is visible at the umbilicus, the eye exhibits a distinct black pigment in the iris, and the chorioallantois extends 2/3 around the breadth of the shell membrane.

Embryos at stage 12 (Day 13-14) have a distinct notch in the midline of the face between the medial nasal processes. Forelimbs are starting to bend in the region of

constriction, so that they are positioned closer to the pleuron of the embryo. The elongated hind limb shows little differentiation into proximal and distal elements and, although there is a distinct apical ectodermal ridge, footplate formation is barely discernable.

Stage 13 (Day 15) embryos have distinct nasal pit slits, and forelimbs are now bent toward the pericardium. The distal portion of the hind limb is flattened and enlarged into a footplate primordium. The chorioallantois now extends as a ring around the inner circumference of the central eggshell membrane.

Embryos at Stage 14 (Day 16-17) have nasal pit slits that are closed due to the merging of the medial nasal, lateral nasal, and maxillary processes. Foot and hand plates are distinct, with the former more advanced than the latter. Lower jaw extends one-quarter beneath the upper jaw, the upper earflap is overgrowing the external ear opening, and the embryonic face rests on the large bulge of the thorax. A large loop of gut herniates through the narrow umbilical stalk and touches the yolk, and the abdominal viscera are visible through body walls. The tail is coiled and kinked at the tip, and contralateral reflexes occur.

Stage 15 (Day 18-20) embryos have lower jaws that extend one-third to one-half the length of the upper jaw. The anlage for the upper eyelid is an elevated rim of tissue above each eye. Distinct and proximal and distal regions, as well as hand and foot plates are present on both the fore and hind limb. There is a distinct hollow in the face beneath the anterior one-third of the eye.

Stage 16 (Day 21) embryos exhibit faint digital condensations in the footplate but not the hand plate. The lower jaw is now two-thirds the length of the upper jaw, with the

upper jaw being hook-shaped around the pericardial ridge. Caruncle development is observed, with two tiny widely spaced thickenings that are just discernable on the tip of the snout.

Embryos at stage 17 (Day 22-23) exhibit mesodermal condensations for the five forelimb digits and four hind limb digits, the head is extended off of the pericardial sac by neck elongation, and the external earflap is distinct.

Stage 18 (Day 24-26) embryos have discernable, distinct cartilaginous digital rays on the hand and foot. The margins of upper eyelid anlage extend over the superior rim of the iris, forming a distinct groove between the eyelids and the eye. Dorsal scalation is now evident, and the pericardial sac is starting to submerge into the ventral thoracic wall.

Stage 19 (Day 27-28) embryos have upper and lower eyelids, and the lower jaw lies behind the anterior margin of the upper jaw. Interdigital clefting has started, and slight marginal notches can be seen, particularly in the footplates. White flecks representing ossification are visible around the upper and lower jaws.

Stage 20 (Day 29-30) embryos have nail anlages starting to develop, first on the most medial digit of the foot, then on adjacent digits; followed by the most medial digit on the hand, and finally on the adjacent hand digits. Interdigital clefting now extends one-quarter the length of the digits, and the lower jaw is in adult relationship with the upper jaw. The pericardial sac is one-quarter withdrawn into the body, and ossification is evident in the proximal and distal elements of limbs. Scale formation is evident dorsally, and scutes (osteoderms) are beginning to appear in the neck region near the skull.

Stage 21 (Day 31-35) embryos have interdigital clefting now extending three-quarters down the digits, and phalanges can be distinguished. Scales are now visible on

the ventral body wall; and dorsally on the snout, neck, body, and tail. Scutes on neck are clearly defined. The pericardial sac is one-half withdrawn into the body, and a white ring in the iris surrounds the outline of the lens of the eye. Both upper and lower eyelids overlap the eye.

Stage 22 (Day 36-40) embryos have pigmented margins of the upper jaw, ventral flank, and proximal and distal elements of the limbs. Interdigital clefting is at the adult level, and the eyelids are typically closed from this point forward. The pericardial sac is two-thirds withdrawn.

Stage 23 (Day 41-45) embryos have more extensive pigmentation, with the embryos appearing light brown with dorsal stripes. Scales are present on distal and proximal elements, and nails have a slight distal elevation. The sensory papillae are present on lateral jaw margins, and scales are evident on gular skin. The midbrain is visible as a white bulge at the back of the cranium, and the pericardial sac is three-quarters withdrawn.

Stage 24 (Day 46-50) embryos are blacker. Nails on hands have elevations at their tips, and the nails are starting to form curves. The midbrain is covered by pigmented skin. The pericardial sac is fully withdrawn and the midline is closing. The volume of yolk outside the body cavity is large, and scales and scutes are evident all over embryo.

Stage 25 (Day 51-60) embryos look identical to hatchlings, except smaller. The external yolk is beginning to be withdrawn, and few gross morphological changes are evident at this and later stages. Growth relationships (head length: total length ratio) and the amount of external yolk present are the major observable differences.

Stage 26 is not present in alligators. This stage was established using tooth eruption sequences and is useful only for saltwater crocodiles (*Crocodylus porosus*) and freshwater crocodiles (*Crocodylus johnsoni*).

Stage 27 (Day 61-63) embryos have withdrawn the yolk sack into the body, ending with skin forming across the umbilical scar. The last stage before hatching (Stage 28, Day 64-70) ends with the umbilical scar being diminished in length and width.

Overall, the first 35 days are a period of rapid organogenesis, and the second 35 days are characterized by embryo growth. Since organogenesis has been shown to be a sensitive period in regard to effects of developmental toxicants (Schmidt & Johnson, 1997), the first 35 days of incubation appear to be the most susceptible time points for toxicant-induced mortality.

In summary, the established staging scheme provides a way to estimate the age of the clutch at the time of collection, and allows one to later determine if a clutch is undergoing normal development. One can determining if a clutch is undergoing normal development by examining embryos at pre-selected time points and comparing their morphological age to their calendar stage (i.e., does an embryo exhibit the normal morphological characteristics that it should exhibit, given its calendar age?). In addition, embryonic development may be compared among clutches and among populations, by collecting embryos at pre-determined stages of development.

Organochlorine Pesticide Toxicity in Vertebrates

Classification, Mode of Action, and Pathology

Organochlorine pesticides (also known as chlorinated hydrocarbon insecticides) may be separated into five classes of compounds. These classes are DDT and its analogs, cyclodienes and similar compounds, toxaphene (composed of several congeners), mirex

and chlordecone (which have cage-like structures), and benzene hexachloride (BHC). In rodent models, studies suggest that OCPs can adversely affect the function of neurons and cause cellular damage to the liver and kidneys (Smith, 1991). Organochlorine pesticides affect neural transmission by altering enzyme activity (Ca^{2+}-ATPase, phospokinase) and the electrophysical properties (K^+, Na^+ ion exchange) of nerve cell membranes. Different analytes may elicit similar effects (neuronal hyperactivity), but by different mechanisms. For example, studies suggest DDT and its analogs affect the nerve axon by keeping Na^+ channels open longer than normal. Cyclodienes, alternatively, may affect neural transmission at presynaptic terminals and may affect the γ-aminobutyric acid (GABA)-regulated chloride channel. Although they can cause severe neural dysfunction, little morphological changes are evident in neural tissue, even at lethal doses (Smith, 1991).

Morphological changes are evident in the liver and include hepatocellular hypertrophy and focal necrosis. Hypertrophy is due to enlargement of the smooth endoplasmic reticulum (SER) and formation of a lipid droplet in the center of the SER (caused by OCP-induced expression of microsomal enzymes within the SER). Functional alterations may also occur in hepatocytes, with disruption of intercellular communication (by hindering transfer of growth inhibitors) (Smith, 1991).

Morphological changes have also been found in the liver and kidney of fish chronically exposed to organochlorine pesticides. For example, chronic exposure to OCPs induce hepatic lesions, such as foci of vacuolated hepatocytes and spongiosis hepatic (lesions of hepatic parenchyma). Renal lesions induced by chronic OCP

exposure include dilation of tubular lumina, and vacuolization (degeneration) and necrosis of tubular epithelium (Metcalfe, 1998).

In addition to morphological changes, organochlorine pesticides may adversely affect endocrine and reproductive function in laboratory models and wildlife populations. Mechanisms include direct toxicity on endocrine glands (such as o,p'-DDD's ability to permanently inactivate the adrenals), competitive binding of steroid hormone receptors, increased expression of steroid-metabolizing hepatic microsomal enzymes, and inhibition of hormone synthesis (such as DDE-induced inhibition of proglandin synthesis, leading to eggshell thinning in raptors) (Gross et al., 2003).

Exposure and Effects of OCPs in Crocodilians

Current knowledge on the effects of environmental contaminants on crocodilian reproductive physiology is important in understanding the likelihood of developmental alterations occurring as a result of exposure; and understanding which mechanisms may be involved.

Campbell (2003) reviewed the effects of organic and inorganic contaminants on crocodilians. Campbell reported only 26 studies related to the bioaccumulation of organic contaminants, with just 35% (8/23) of crocodilian species being represented. Of the 26 studies, 38% involved American alligators (*Alligator mississippiensis*), 26% involved Nile crocodiles (*Crocodylus niloticus*), 13% involved American crocodiles (*Crocodylus acutus*), and 13% involved Morolet's crocodile (*Crocodylus moreletii*). Slightly more studies were found that investigated effects of organic contaminants. With respect to these 39 studies, only 13% (3/23) of crocodilian species were represented, consisting of the American alligator (91% of studies), the Nile crocodile (5%), and the African dwarf crocodile (*Osteolaemus tetraspis*, 4%). Of these studies, American

alligators are the only species in which an effort has been made to determine the relationship between OCPs and depressed hatch rates, with most of this work involving populations in central Florida.

Reproductive Problems in Florida Alligators

In the early to mid 1980s, studies showed that the population of juvenile alligators inhabiting the aquatic ecosystem of Lake Apopka, Florida, declined by 90%. This decline was preceded by a 1980 chemical spill and decades of OCP contamination via anthropogenic activities described earlier. The loss of juveniles was attributed primarily to a dramatic decrease in clutch viability (the proportion of eggs in a clutch that produce a live hatchling) (Woodward et al., 1993).

Alterations in sexual differentiation, sex steroid hormone concentrations, and metabolism were also documented among Lake Apopka alligators. For example, testosterone was lower in male alligators from Lake Apopka as compared to those of control sites. Ovaries of female juvenile alligators from Lake Apopka showed abnormalities, suggesting that reproductive alterations were occurring in both sexes (Gross et al., 1994; Guillette et al., 1994; Gross, 1997). In addition, high concentrations of OCPs were measured in egg yolk, but concentrations were not clearly associated with increased mortality (Heinz et al., 1991).

Later studies suggested that the cause for the population decline was potentially more complex than previously suggested. First, poor egg viability for Lake Apopka alligators was more closely associated with muck farm reclamation (wetland restoration) sites than with tissue and egg concentrations of the predominant pesticide residue (DDE) (Giroux, 1998). Second, altered endocrine function and decreased egg viability were documented among alligators at another site, Lake Griffin, where tissue and egg

concentrations of residues such as DDE are modest or intermediate compared with those of Lake Apopka. However, like Lake Apopka, Lake Griffin is highly eutrophic and has adjacent muck farms and muck farm reclamation areas (Marburger et al., 1999). Third, poor reproductive success among Lake Apopka alligators appeared to result from both decreased proportions of fertile eggs that produce a live hatchling and decreased proportions of hatchlings that survive through the first 20 days of life (which is the toxicant-sensitive organogenesis period); and decreased proportions of unbanded eggs (i.e., eggs that are nonviable on initial examination) (Masson, 1995; Wiebe et al., 2001).

Unbanded eggs show no evidence of embryo-eggshell attachment (as indicated by the presence of an opaque spot or band that results from fusion of extraembryonic membranes to the dorsal portion of the inner eggshell membrane). Unbanded eggs may result from very early embryo mortality (fertilization has been confirmed in many cases by the presence of paternal DNA, via DNA microsatellite analysis); or may result from infertile eggs (Rotstein, 2000).

The last similarity between alterations in alligator populations of Lake Griffin and Lake Apopka is increased mortality among adult Lake Griffin alligators (Schoeb et al., 2002), which is similar to increased adult mortality on Lake Apopka in the early 1980s. These data indicate that alligator populations are adversely affected at each of several life stages. Although anatomic and endocrinologic effects of exposure to endocrine-disrupting OCPs could account for many of these effects, additional underlying mechanisms are almost certainly involved. Overall, these data point to a complex process involving the introduction of OCPs into these aquatic ecosystems from

chemical spillage or from muck farming and reclamation activities; possibly leading to developmental toxicity, in addition to endocrine disruption.

Specific Aims

The overall objective of our study was to determine the causes of decreased hatch rates among alligators in contaminated sites, and to determine if causal links could be established between specific adverse effects and exposure to individual OCPs or combinations of OCPs. The project consisted of epidemiological field studies, which evaluated embryonic development and mortality as a function of maternal and environmental exposure to OCPs and egg nutrient composition; and controlled laboratory experiments to test hypothesized links between decreased hatch rates, altered egg composition, and exposure to selected OCPs.

Specific aim 1: Conduct field epidemiological studies to determine the relative contributions of unbanded eggs, embryonic mortality in banded eggs, and decreased perinatal mortality to the overall decreased reproductive success in alligators at OCP-contaminated sites, to determine which OCPs or combinations of OCPs are most closely associated with adverse effects at each life stage, and to examine the relationship between OCP burdens in maternal tissues and eggs. For Specific Aim 1, the hypotheses were

H1a: Adverse effects at early life stages are associated with muck farm environments, exposure to specific OCPs or OCP combinations, or both;

H1b: Specific OCPs found in maternal tissues are highly correlated to those present in eggs indicating maternal transfer of OCPs and that maternal size is correlated with OCP burdens and hatch rates;

H1c: Eggs in which embryonic and perinatal mortality occur result from developmental abnormalities, altered structure or composition of the egg, or both.

Specific aim 2: Conduct controlled *in ovo* and *in vivo* experiments with alligators to confirm causal links between decreased hatch rates and affected life stages as a

function of exposure to selected OCPs or altered egg qualities, or both. For Specific Aim 2, the hypotheses were

H2a: Exposing a captive breeding population of adult alligators to an environmentally relevant mixture of OCPs will elicit OCP concentrations in eggs and developmental effects similar to those observed in wild eggs from OCP-contaminated field sites;

H2b: Exogenous in ovo alteration of egg nutrients based on data from field studies will alter embryonic development.

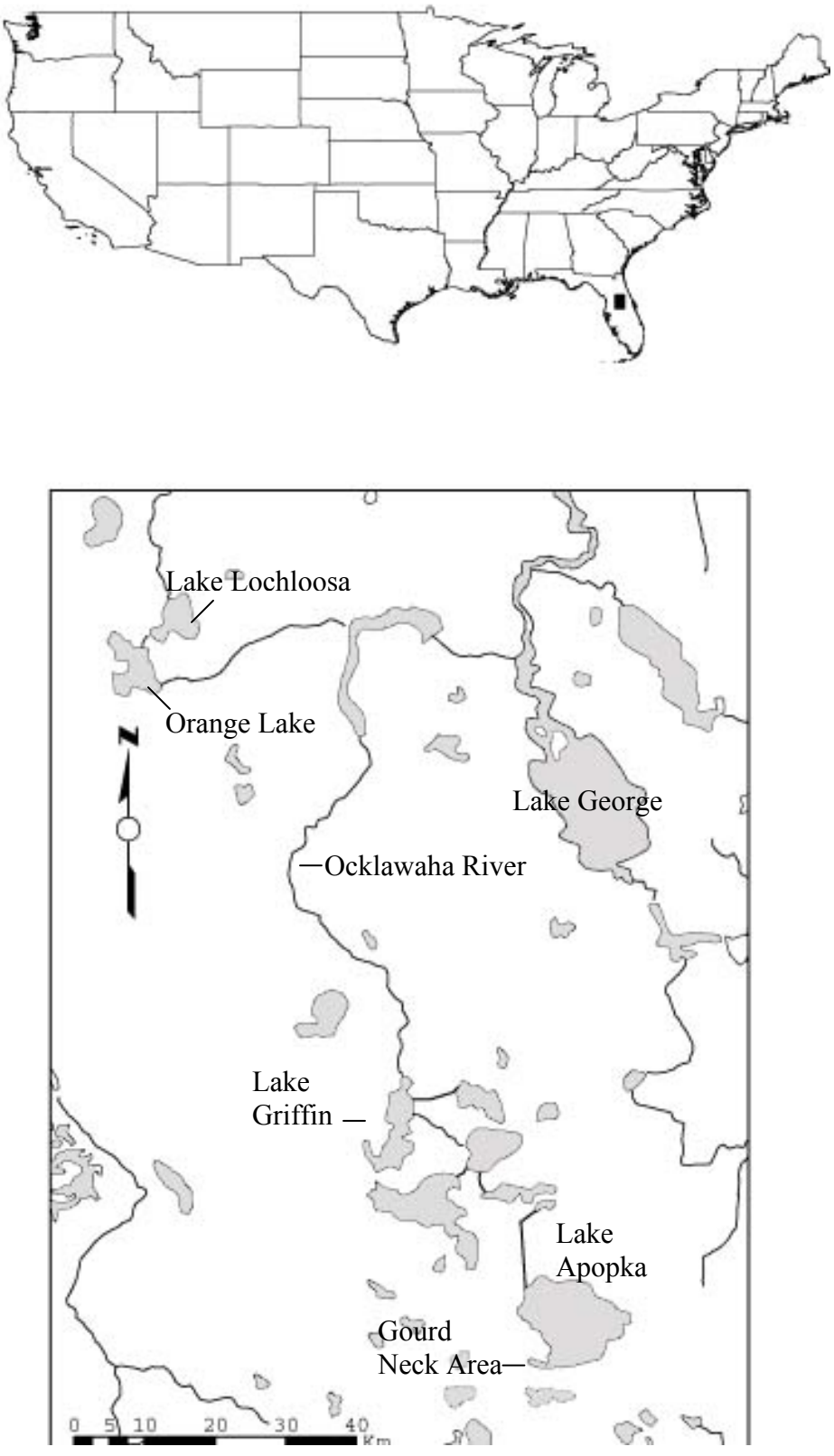

Figure 1-1. Map of Ocklawaha Basin.

CHAPTER 2
EGG AND EMBRYO QUALITY OF ALLIGATORS FROM REFERENCE AND ORGANOCHLORINE CONTAMINTED HABITATS

In the southeastern US, aquatic ecosystems have experienced habitat degradation, alterations in water quality, and in some cases, important declines in biodiversity due to increases in land development and associated anthropogenic impacts. A case-in-point is the Ocklawaha River Basin in central Florida. Within this basin, American alligators (*Alligator mississippiensis*) from impacted lakes have exhibited poor clutch viability (number eggs that yield a live hatchling / total number of eggs found in clutch) (Masson, 1995), abnormal reproductive hormone concentrations (Gross et al., 1994), and unexplained adult mortality (Schoeb et al., 2002). During the mid 1980s, clutches from alligators on Lake Apopka experienced severe declines in clutch viability (declined from 50% to 4%), and alligator clutches from other impacted lakes had only moderate viabilities (range of 40 to 60%). These rates were below those observed in other less impacted Florida lakes (reference sites), including Lake Woodruff National Wildlife Refuge (79%), Orange Lake (82%), and the Everglades Water Conservation Areas (65-75%) (Woodward et al., 1993; Masson, 1995; Rice, 1996).

Possible causal factors for reduced hatch rates in alligator populations within the impacted sites within the Ocklawaha River Basin include pesticides, algal toxins, nutritional changes, density-related stress, and diseases. In one case, a chemical spill from a chemical manufacturing plant in 1980 near Lake Apopka (EPA, 2004) was temporally associated with the decline in reproductive success and consequent alligator

population decline on Lake Apopka during the early 1980s. However, decreases in clutch viability for Lake Apopka appeared to be more related to proximity to muck farm restoration areas as compared to yolk concentrations (Giroux, 1998), which is consistent with decreases in clutch viability on Lake Griffin and Emeralda Marsh, Griffin's adjacent muck farm restoration area (Sepúlveda et al., 2001).

Poor reproductive success threatens the long-term conservation of alligators, potentially altering the ecology of affected ecosystems, and substantially reducing the aesthetic and economic values of alligators in affected areas. Understanding and characterizing poor reproductive performance and determining associated factors is needed so that efficacious mitigation strategies may be developed. Thus, the overall objective of the present study was to determine the relative contributions of losses during *in ovo* development in American alligators at impacted sites in central Florida, and to evaluate whether organochlorine pesticides (OCPs) are associated with adverse developmental effects and altered clutch characteristics.

Materials and Methods

Egg Collections and Incubation

Lakes Apopka (N 28° 35', W 81° 39'), Griffin (N 28° 53', W 81° 46'), Emeralda Marsh Conservation Area ((N 28° 55', W 81° 47'), and Lochloosa (N 29° 30', W 82° 09') in Florida were selected as collection sites because prior studies indicate vastly different levels of OCP exposure across these sites (Gross unpublished data, (Masson, 1995).

Alligator nests were located via aerial (helicopter) and ground surveys (airboat), and clutches were subsequently collected by ground crews. The top of each egg was marked before eggs were removed from the nest to ensure proper orientation; thus,

preventing embryo mortality due to inversion. Embryo mortality due to inversion occurs if an embryo has attached to the top of the egg, inversion may either break embryonic attachment or cause the yolk mass to settle on top of the embryo, crushing it.

After marking each egg and placing about 5 cm of nest substrate in a uniquely numbered polypropylene pan (43 cm x 33 cm x 18 cm), all eggs found in each clutch were placed in the pan in five rows with six eggs per row. If a clutch contained more than 30 eggs, a second layer of nest substrate was added and the additional eggs were set. The top layer of eggs was covered with nest substrate so that there was no space left between the top of the pan and the top of the eggs (approximately 10 cm). Clutches were transported to the US Geological Survey's Center for Aquatic Resources Studies, Gainesville, Florida (CARS). Upon arrival, clutches were evaluated for embryonic viability using a bright light candling procedure. Viable eggs (i.e. having a visible band) were nested in pans containing moist sphagnum moss and incubated at 30.5ºC and ~98% humidity, in an incubation building (7.3 m x 3.7 m). This intermediate incubation temperature will normally result in a 1:1 male/female sex ratio, since alligators have temperature dependent sex (or gender) differentiation. One or two eggs were opened from each clutch to identify the embryonic stage of development at the time of collection, and to collect yolk samples for later measurement of OCP burdens. From each clutch, information on the following parameters was collected: total number of eggs found per nest (fecundity); number of unbanded eggs, number of damaged eggs, number of dead banded eggs, number of live banded eggs, total clutch mass, and average egg mass of clutch.

For years 2001 and 2002, some clutches were involved in an embryo development study. For these clutches, each clutch was evenly divided between two pans, with half of the clutch left relatively undisturbed (except for weekly monitoring of embryo mortality) to determine clutch viability (the number of live hatchlings / fecundity), and the other half of the clutch used to study embryo development and morphometry (Chapter 5).

Analysis of OCPs in Yolk

Analytical grade standards for the following compounds were purchased from the sources indicated: aldrin, alpha-benzene hexachloride (α-BHC), β-BHC, lindane, δ-BHC, *p,p'*-dichlorodiphenyldichloroethane (*p,p'*-DDD), *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), dichlorodiphenyltrichloroethane (*p,p'*-DDT), dieldrin, endosulfan, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor epoxide, hexachlorobenzene, kepone, methoxychlor, mirex, *cis*-nonachlor, and *trans*-nonachlor from Ultra Scientific (Kingstown, RI, USA); *cis*-chlordane, *trans*-chlordane, and the 525, 525.1 polychlorinated biphenyl (PCB) Mix from Supelco (Bellefonte, PA, USA); oxychlordane from Chem Service (West Chester, PA); *o,p'*-DDD, *o,p'*-DDE, *o,p'*-DDT from Accustandard (New Haven, CT, USA); and toxaphene from Restek (Bellefonte, PA, USA). All reagents were analytical grade unless otherwise indicated. Water was doubly distilled and deionized.

Egg yolk samples were analyzed for OCP content using methods modified from Holstege et al. (1994) and Schenck et al. (1994). For extraction, a 2 g tissue sample was homogenized with ~1 g of sodium sulfate and 8 mL of ethyl acetate. The supernatant was decanted and filtered though a Büchner funnel lined with Whatman #4 filter paper (Fisher Scientific, Hampton, NH, USA) and filled to a depth of 1.25 cm with sodium sulfate. The homogenate was extracted twice with the filtrates collected together. The

combined filtrate was concentrated to ~2 mL by rotary evaporation, and then further concentrated until solvent-free under a stream of dry nitrogen. The residue was reconstituted in 2 mL of acetonitrile. After vortexing (30 s), the supernatant was applied to a C18 solid phase extraction (SPE) cartridge (pre-conditioned with 3 mL of acetonitrile; Agilent Technologies, Wilmington, DE, USA) and was allowed to pass under gravity. This procedure was repeated twice with the combined eluent collected in a culture tube. After the last addition, the cartridge was rinsed with 1 mL of acetonitrile which was also collected. The eluent was then applied to a 0.5 g NH2 SPE cartridge (Varian, Harbor City, CA, USA), was allowed to pass under gravity, and collected in a graduated conical tube. The cartridge was rinsed with an additional 1 mL portion of acetonitrile which was also collected. The combined eluents were concentrated under a stream of dry nitrogen, to a volume of 300 μL, and transferred to a gas chromatography (GC) vial for analysis.

GC/MS Analysis

Analysis of all samples was performed using a Hewlett Packard HP-6890 gas chromatograph (Wilmington, DE, USA) with a split/splitless inlet operated in splitless mode. The analytes were introduced in a 1 μL injection and separated across the HP-5MS column (30 m x 0.25 mm; 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA) under a temperature program that began at 60º C, increased at 10º C/min to 270º C, was held for 5 min, then increased at 25º C/min to 300º C and was held for 5 min. Detection utilized an HP 5973 mass spectrometer in electron impact mode. Identification for all analytes and quantitation for toxaphene was conducted in full scan mode, where all ions are monitored. To improve sensitivity, selected ion monitoring was used for the

quantitation for all other analytes, except kepone. The above program was used as a screening tool for kepone which does not optimally extract with most organochlorines. Samples found to contain kepone would be reextracted and analyzed specifically for this compound.

For quantitation, a five-point standard curve was prepared for each analyte ($r^2 \geq 0.995$). Fresh curves were analyzed with each set of twenty samples. Each standard and sample was fortified to contain a deuterated internal standard, 5 µL of US-108 (120 µg/mL; Ultra Scientific), added just prior to analysis. All samples also contained a surrogate, 2 µg/mL of tetrachloroxylene (Ultra Scientific) added after homogenization. Duplicate quality control samples were prepared and analyzed with every twenty samples (typically at a level of 1.00 or 2.50 µg/mL of γ-BHC, heptachlor, aldrin, dieldrin, endrin, and *p,p'*-DDT) with an acceptable recovery ranging from 70 – 130%. Limit of detection ranged from 0.1-1.5 ng/g for all OCP analytes, except toxaphene (120-236 ng/g), and limit of quantitation was 1.5 ng/g for all analytes, except toxaphene (1500 ng/g). Repeated analyses were conducted as allowed by matrix interferences and sample availability.

Data Analysis

Specific OCP analytes were removed from analysis if measurable concentrations were found in < 5% of all clutches. Numerical data were log-transformed [ln(x)], while proportional data were arcsine square root transformed to meet statistical assumptions.

ANOVA (PROC GLM; SAS Institute Inc., 2002) was used for inter-site comparisons of adult female and clutch characteristics, and the Tukey test was used for multiple comparisons among sites ($\alpha = 0.05$). Because relationships between response variables and explanatory variables (Table 2-1) in ecological studies are often complex

with interactions occurring, an indirect gradient multivariate analysis method, Detrended Correspondence Analysis (DCA) (ter Braak, 1986) was used to initially evaluate data structure. Two matrices were constructed for DCA, with the first representing the response variables (clutch number x clutch parameters) and the second representing the explanatory variables (clutch number x OCP burdens) (Table 2-2). DCA results indicated that a direct gradient, multivariate linear analysis, redundancy analysis (RDA) (Rao, 1964), was appropriate since the gradient lengths of the DCA ordination axes were equal to or less than 2 standard deviations (ter Braak, 1995).

RDA is the canonical form of principal components analysis (PCA). In RDA, as in PCA, a straight line is fitted to each the response variable (clutch survival parameters) in an attempt to explain the data of all response variables. Similar to PCA, the lower the residual sum of squares, the better the environmental variable is at explaining the variation in response variables. RDA, unlike PCA, restricts the clutch scores (from the response variables measured on each clutch) to a linear combination of the environmental (explanatory variables). Because clutch scores are constrained to a linear combination of environmental variables, RDA explains slightly less variance compared to PCA (ter Braak & Tongeren, 1995; ter Braak, 1994). For RDA involving compositional data (i.e., clutch viability rates or percentages) and quantitative environmental variables, compositional data is log-transformed (ln (x + 1)) with correlation biplots being centered by the response variables (i.e., unbanded egg percentage) and by the samples (i.e., clutches) (ter Braak, 1994). These correlation biplots provide a way to examine relationships among a number of response variables and explanatory factors with response variable arrows forming a biplot of correlations with each other, environmental

arrows forming a biplot among each other, and response variable arrows and environmental arrows forming a biplot of correlations with each other (ter Braak, 1995).

For the RDA, separate matrices were constructed for response variables measured as a percentage (i.e., clutch viability) and response variables measured as a number (i.e., clutch mass) because percentage data were ln(x+1) transformed and not standardized, while continuous data were ln(x) transformed and standardized(ter Braak & Smilauer, 2002). Automatic forward selection of the best four explanatory variables was conducted for both sets of RDA analyses and tested for significance by Monte Carlo permutation tests. DCA and RDA were conducted using the program CANOCO (ter Braak & Smilauer, 2002). Biplots of environmental variables and response variables were then constructed to interpret relationship between clutch parameters (response variables) and explanatory factors.

Results

Inter-Site Comparisons of Clutch Characteristics

From 2000-2002, 168 clutches were collected from Lakes Lochloosa (n = 44), Apopka (n = 31), Griffin (n = 47), and Emeralda Marsh (n = 46). No significant differences were determined among sites with respect to clutch mass (overall mean ± standard error: 3.7 ± 0.08 kg), egg mass (83 ± 1.4 g), or percentage of unbanded eggs (15 ± 1.7%) (Table 2-3).

In contrast, significant differences were determined among sites with respect to fecundity, clutch viability, percentage of damaged eggs, percentage of early embryo mortality, and percentage of late embryo mortality. Clutches from Lochloosa had lower fecundity and late embryo mortality rates compared to all other sites. In addition, Lochloosa clutches had greater clutch viability rates than all other sites and lower early

embryo mortality rates than all other sites, except for Apopka. Clutches from Emeralda Marsh had greater incidence of damaged eggs than all other sites, except for those of Lake Griffin (Table 2-3).

Organochlorine Pesticides Burdens and Clutch Characteristics

From 2000-2002, clutch characteristics and OCP burdens were measured on 115 clutches collected from Lakes Lochloosa (n = 19), Apopka (n = 23), Griffin (n = 42), and Emeralda Marsh (n = 31). No significant differences were determined among sites with respect to clutch mass (overall mean ± standard error: 3.8 ± 0.09), clutch viability (50 ± 3.1), percentage of damaged eggs (4 ± 1), unbanded eggs (13 ± 1.6), early embryo mortality (21 ± 2.3), and late embryo mortality (11 ± 1.7) (Table 2-4). However, significant differences were determined for fecundity and egg mass, with Lochloosa clutches having lower fecundity than all other sites, and greater average egg mass compared to those of all other sites, except for Lake Apopka. Furthermore, significant differences were detected among sites with respect to individual OCP concentrations in egg yolks, total OCP concentrations in egg yolks, and number of OCPs detected at measurable levels. For total OCP concentrations and number of analytes detected at measurable levels, egg yolks of Lake Lochloosa clutches had significantly lower total concentrations and a lower number of analytes detected at measurable levels (Table 2-4).

Individual OCP analyte concentrations in egg yolks of Lochloosa clutches were significantly less than those of the other sites, except for Lake Griffin with respect to aldrin and *trans*-nonachlor. Aldrin and *trans*-nonachlor egg yolk concentrations of Lochloosa clutches did not significantly differ from Lake Griffin, but egg burdens of these analytes of both sites were significantly less than those of Lake Apopka and Emeralda Marsh (Table 2-4).

Clutch Survival and OCP Burdens in Egg Yolks

Because a number of site specific factors may potentially affect clutch survival parameters and since OCP burdens varied greatly among sites, relationships between OCP egg yolk variables and clutch survival were evaluated on a site-by-site basis.

For Lake Lochloosa, redundancy analysis with Monte Carlo permutation tests for significance indicated that none of the four extracted OCP variables (Table 2-5) were found to be significantly correlated with the clutch survival variables. Number of OCPs detected (NOC) approached significance ($P = 0.07$), was negatively associated with clutch viability, positively correlated with percentage unbanded eggs and late embryo mortality, and accounted for 11% of the variation in clutch survival parameters (Fig. 2-1).

For Lake Griffin, redundancy analysis with Monte Carlo permutation tests for significance indicated that three of the four extracted OCP variables were found to be significantly correlated with the clutch survival variables and together accounted for 21% of the variance in clutch survival parameters. The extracted OCP variables were concentration of *p,p'*-DDE, toxaphene, and *p,p'*-DDT, accounting for 8, 7, and 6%, respectively, of variation in clutch survival variables (Table 2-5). Clutch viability was positively associated with toxaphene and *p,p'*-DDE egg yolk concentrations, but had little to no correlation with *p,p'*-DDT yolk burdens. Early embryo mortality rates were negatively associated with *p,p'*-DDE and toxaphene. Late embryo mortality rates were positively associated with toxaphene, and negatively associated with *p,p'*-DDT, and *p,p'*-DDE. Unbanded egg rates were positively associated with *p,p'*-DDT and *p,p'*-DDE, but negatively associated with toxaphene (Fig. 2-2).

For Lake Apopka, redundancy analysis with Monte Carlo permutation tests for significance also indicated that three of the four extracted OCP variables were found to

be significantly correlated with the clutch survival variables. These OCP variables were percentage dieldrin (lambda A = 17%), percentage trans-chlordane (12%), and percentage aldrin (10%), and together accounted for 3% (∑ lambda A's) of the variance in the clutch survival parameters (Table 2-5). Clutch viability was positively associated with aldrin, weakly associated with *trans*-chlordane, and negatively associated with dieldrin. Early embryo mortality and unbanded egg rates were positively associated with dieldrin and *trans*-chlordane, and negatively associated with aldrin. Late embryo mortality rates were negatively with all three OCP variables (Fig. 2-3).

For Emeralda Marsh, redundancy analysis with Monte Carlo permutation tests for significance also indicated that only percentage toxaphene was found to be significantly correlated with the clutch survival variables, and it accounted for 9% of the variance in the clutch survival parameters (Table 2-5). Percentage toxaphene was positively associated with clutch viability, weakly associated with late embryo mortality, and negatively associated with early embryo mortality and unbanded egg rates (Fig. 2-4). Percentage of heptachlor epoxide showed a near significant association ($P = 0.09$) with clutch parameters, being negatively correlated with clutch viability and positively correlated with early and late embryo mortality rates.

Average Egg Mass, Clutch Size and OCP Burdens

For Lochloosa clutches, three of four OCP variables were determined (via RDA analysis) to be significantly associated with egg and clutch size parameters and accounted for 64% of the variation in egg and clutch size parameters. These OCP variables included number of OCPs detected at measurable levels (NOC) (lambda A = 31%), *p,p'*-DDT concentrations (20%), and *trans*-nonachlor concentrations (13%) (Table 2-6).

NOC and *trans*-nonachlor concentrations were negatively associated with average egg mass but positively associated with fecundity and clutch mass. In contrast, *p,p'*-DDT concentrations were positively associated with egg mass, negatively associated with fecundity, and had little to no association with clutch mass (Fig. 2-5).

For Lake Griffin clutches, however, no significant associations were found between OCP variables and egg and clutch size variables. In contrast, percentage *o,p'*-DDT in Emeralda Marsh clutches was found to be positively associated with increasing egg and clutch mass but negatively associated with fecundity. Lastly, Lake Apopka clutches were somewhat similar to Emeralda clutches in that one OCP variable (*p,p'*-DDD concentration) was found to be positively associated with egg and clutch mass and negatively associated with fecundity (Table 2-6).

Discussion

Inter-Site Comparisons of Clutch Characteristics

The results of the present study suggested that the relative contributions of losses during in ovo development in alligators at impacted sites in Florida are lower clutch viability, higher rates of damaged eggs, higher rates of early embryo mortality, and higher rates of late embryo mortality. Although not significantly different among sites, infertility and/or embryo mortality before embryo attachment (unbanded eggs) also appears to be a major constituent of reduced clutch viability among all sites. In order of importance, major constituents of reduced clutch viability for all sites include early embryo mortality, unbanded eggs, late embryo mortality, and damaged eggs. In addition, clutches from OCP-contaminated sites had an average of 10 more eggs per clutch as compared to the reference site, but average clutch mass was not significantly different,

making average egg mass of reference site clutches greater than that of clutches of OCP-contaminated sites.

The reduced clutch viability, increased rates of unbanded eggs and embryo mortality, and concurrent increase in fecundity without proportional increase in clutch mass observed in clutches from OCP-contaminated sites, as compared to the reference site (Lochloosa), suggest that females and their embryos from contaminated sites may be responding to one or more environmental factors common to the three OCP-contaminated sites. Although measurement of all environmental factors is impractical, the large differences in OCP concentrations in alligator eggs between reference and OCP-contaminated sites were found. Specifically, total OCP egg yolk burdens and number of OCPs detected at measurable levels in Lake Lochloosa were significantly less than those of Lake Griffin clutches, and OCP burdens in Lake Griffin clutches were, in turn, significantly less than those of Lake Apopka and Emeralda Marsh.

Although Lake Apopka and Emeralda Marsh were not determined to be significantly different with respect to total OCP concentrations in egg yolks, significant differences were determined between these two high OCP exposure sites in regard to certain analytes, as well as the total number of OCPs detected at measurable levels. Clutches from Emeralda Marsh had a greater number of OCP analytes in their egg yolks and contained higher concentrations of cis-chlordane, p,p'-DDD, o,p-DDD, trans-chlordane, and toxaphene compared to those from Lake Apopka. Conversely, clutches from Lake Apopka had higher concentrations of aldrin, dieldrin, heptachlor epoxide, and oxychlordane compared to those of Emeralda Marsh.

The differences in OCP exposure profiles among sites likely reflect the differences in historic land-use and OCP applications, as opposed to differences in xenobiotic biotransformation among the different alligator populations inhabiting the respective sites. Importantly, although Emeralda Marsh is separated from Lake Griffin by only a levee easily traversed by alligators, large differences in OCP egg burdens were noted between the two sites. Such differences in exposure suggest that the highly exposed adult females which oviposite within Emeralda Marsh likely have established territories and reside year round within Emeralda Marsh (a former agricultural property). Furthermore, the relatively high egg burdens in clutches of Emeralda Marsh likely occurred over a relatively short period since this 2,630 ha area was not flooded until the early 1990s (Marburger et al., 1999).

In summary, the differences in OCP egg burdens between the reference site and the contaminated sites support the hypothesis that OCP contaminants may be associated with reduced clutch viability, given that OCPs have been causally linked to reduced reproductive success in other oviparous species (Donaldson & Fites, 1970; Fry, 1995).

Clutch Survival Parameters and OCP Burdens

Results of redundancy analyses more directly addressed the question of whether OCPs are associated with reduced clutch viability by relationships on a site-by-site basis to control for potential site-associated confounding factors. For Lake Lochloosa, no significant correlations were determined although significance might have been detected given a greater sample size. The positive but insignificant correlations between increases in unbanded egg and late embryo mortality percentage and number of OCPs may suggest that increased OCP burdens in eggs play a role in clutch viability or it may simply indicate that older females have increased levels of OCPs due to increased exposure time

and that decreased clutch viability is due to decreased egg quality associated with senescence.

For Emeralda Marsh, the weak associations between OCP variables and clutch survival variables suggests that other factors may be involved in reduced embryo survival and increased rates of unbanded eggs. The weak associations for Emeralda Marsh are surprising given that relatively stronger associations were determined for the other high exposure site (Lake Apopka; Table 2-5), as well as the intermediate exposure site (Lake Griffin, Table 2-5), with Emeralda Marsh being separated from Lake Griffin by only a non-fenced levee.

Stronger associations were noted for Lake Apopka in contrast to the weak, associations noted for Emeralda Marsh. The positive association between early embryo mortality and unbanded egg rates and extracted OCP variables for Lake Apopka clutches suggests that the percentages of dieldrin and *trans*-chlordane in eggs may play an important role in altered egg fertility and/or early embryo survival Interestingly, the percentage of aldrin, (dieldrin's parent compound) had a negative association with late embryo mortality, a positive association with clutch viability, and near-zero correlations with percentage unbanded eggs and early embryo mortality. However, dieldrin (a metabolite formed from aldrin) had strong, positive correlations with percentage unbanded eggs and early embryo mortality, and a negative correlation with clutch viability, suggesting this metabolite has greater efficacy than its parent compound in affecting embryo survival. The potential consequence exists that increasing a female alligator's ability to biotransform aldrin to dieldrin may increase the risk of early embryo mortality. Another important note is that the level of dieldrin in Apopka clutches was

two-fold greater than those of Emeralda Marsh, suggesting that OCP mixture composition may be more important than sum OCP concentrations.

For Lake Griffin, the negative to near-zero association between early embryo mortality rates and extracted OCP variables suggests that OCP burdens in eggs may not play an important role in early embryo mortality. However, the positive association between toxaphene burdens and late embryo mortality suggests that as toxaphene burdens increase, so does the risk for increased embryo death during the last 35 days of development. Furthermore, the positive association between *p,p'*-DDT concentrations and unbanded egg rates suggests that these analytes may be involved in altered egg fertility and/or embryo survival (prior to eggshell membrane attachment) (Fig. 2-2).

Egg and Clutch Size and OCP Burdens

For Lochloosa, the strong associations between OCP burdens and egg and clutch size parameters suggest that, although a low OCP exposure site, certain patterns of OCP exposure are strongly associated with egg and clutch size characteristics. The positive associations *p,p'*-DDT concentrations have with clutch weight and average egg weight and *p,p'*-DDT's negative association with fecundity may be potentially related to senescent females, since older females have been reported to lay smaller clutches of larger eggs (Ferguson, 1985) and would likely have higher OCP burdens due to extended exposure period. In contrast, the positive associations that NOC and trans-nonachlor have with fecundity and clutch mass, and the negative associations these OCP variables have with egg mass, suggests that increased OCP exposure may have altered clutch and egg size characteristics, as opposed to female age. Although these speculations are interesting from a low exposure effect standpoint, they are irrelevant at the population-effect level since clutch viability rates were unrelated.

Since the low exposure site had stronger associations between OCP variables and egg and clutch size variables than intermediate and high exposure sites, one might initially conclude that other factors are more important than OCP burdens in influencing egg and clutch size characteristics. While this may be the case, the fact that the intermediate and high exposure sites have significantly greater fecundity (averaging 10 more eggs per clutch compared to the low exposure site), significantly less average egg mass, and similar clutch mass suggest that females attaining their maximum physiological response in regard to number of eggs ovulated. These intermediate and highly exposed females appear to be producing more ova but are unable to sequester additional egg components (i.e., lighter eggs), in effect decreasing the amount of energy and structural supplies available to each embryo and resulting in lighter eggs and higher embryo mortality rates.

In summary, our results suggest that, over all sampled clutches, clutch survival parameters and egg and clutch size parameters vary between the low OCP exposure site (Lochloosa) and the intermediate-high OCP exposure sites. Furthermore, OCP burdens do not appear to be related to clutch survival for the low exposure site but are associated with clutch survival for the intermediate-high OCP contaminated sites. In contrast, egg and clutch size parameters appear to be a sensitive endpoint in OCP response in alligators due to the strong associations noted between OCP and clutch size variables for the low exposure site and the lack thereof for the intermediate-high exposure sites, suggesting attainment of maximum response. In order to better determine the role of OCPs in the reduced reproductive efficiency of OCP-exposed alligator populations, suggested future studies should examine the relationship between maternal OCP burdens and respective

egg burdens, presence of other environmental contaminants, maternal factors associated with clutch survival and OCP burdens, and how egg composition relates to clutch survival and OCP burdens.

Table 2-1. Reproductive, morphometric, and contaminant parameters measured on clutches of alligator eggs collected during summer 2000, 2001, and 2002.

Clutch Parameter	Definition	Measured as
Response variables		
Fecundity	Total No. of eggs in one clutch	*n*
Clutch mass	Total mass of eggs in one clutch	kg
Ave. Egg Weight	Clutch mass / Fecundity	g
Unbanded eggs% [a]	No. of unbanded eggs / fecundity x 100	Percentage
Early embryo mortality%	No. of deaths < dev. Day 35 / fecundity x 100	Percentage
Late embryo mortality%	No. of deaths ≥ dev. Day 35 / fecundity x 100	Percentage
Clutch Viability	No. eggs yielding live hatchling / fecundity x 100	Percentage
Explanatory variables		
[OCP analyte] in egg yolk	ng OCP analyte / g egg yolk wet weight	ppb
% OCP analyte	[OCP analyte] / ∑ [OCP] x 100	Percentage

[a]An egg with no evidence of embryonic attachment

Table 2-2. Explanatory variables included in RDA with forward selection of four best variables ($\alpha = 0.05$).

Variable	Code
Age	Age
No. OCPs at measurable levels	NOC
∑ [OCP]	TOC
% Aldrin	ALD%
[Aldrin]	[ALD]
% *cis*-Chlordane	CC%
[*cis*-Chlordane]	[CC]
% *cis*-Nonachlor	CN%
[*cis*-Nonachlor]	[CN]
% Dieldrin	DL%
[Dieldrin]	[DL]
% Heptochlor epoxide	HE%
[Heptachlor epoxide]	[HE]
%Lipid content	LPC%
% Mirex	MX%
[Mirex]	[MX]
% *o,p*-DDT	ODDT%
[*o,p*-DDT]	[ODDT]
% *o,p*-DDD	ODDD%
[*o,p*-DDD]	[ODDD]
% Oxychlordane	OX%
[Oxychlordane]	[OX]
% *p,p*'-DDE	PDDE%
[*p,p*'-DDE]	[PDDE]
% *p,p*'-DDD	PDDD%
[*p,p*'-DDD]	[PDDD]
% *p,p*'-DDT	PDDT%
[*p,p*'-DDT]	[PDDT]
% *trans*-Chlordane	TC%
trans-Chlordane	[TC]
% *trans*-Nonachlor	TN%
[*trans*-Nonachlor]	[TN]
% Toxaphene	TX%
[Toxaphene]	[TX]

Table 2-3. Summary of clutch parameters and site comparisons for clutches of American alligator eggs collected during 2000-2002.

Parameter[a]	Lochloosa	Apopka	Emeralda	Griffin	Summary
N$^{o.}$ Clutches	44	31	46	47	168
Fecundity (*n*)	36 ± 1.2 B	46 ± 1.3 A	46 ± 1.1 A	45 ± 1.2 A	43 ± 0.7
	(22–56)	(28–56)	(27–64)	(19–58)	(19–64)
Clutch mass (kg)	3.4 ± 0.15	4 ± 0.13	3.8 ± 0.21	3.6 ± 0.13	3.7 ± 0.08
	(1.6–4.8)	(2.4–5.1)	(1.9–9.2)	(1.5–5.2)	(1.5–9.2)
Egg mass (g)	87 ± 2.2	86 ± 2	83 ± 4	80 ± 1.6	83 ± 1.4
	(61–139)	(62–120)	(58–180)	(46–113)	(46–180)
Clutch viability (%)	70 ± 3.9 A	51 ± 5.8 B	48 ± 5.5 B	44 ± 4.9 B	53 ± 2.6
	(0–100)	(0–98)	(0–97)	(0–92)	(0–100)
Damaged eggs (%)	2 ± 1.4 B	2 ± 0.6 B	5 ± 1.3 A	4 ± 1.8 AB	3 ± 0.7
	(0–60)	(0–16)	(0–33)	(0–63)	(0–63)
Unbanded eggs (%)	11 ± 2.2	21 ± 4.9	14 ± 3.7	17 ± 3.2	15 ± 1.7
	(0–84)	(0–100)	(0–100)	(0–100)	(0–100)
Early Emb. Mort. (%)	12 ± 2.7 B	15 ± 4.2 AB	23 ± 3.9 A	22 ± 3.9 A	19 ± 2
	(0–69)	(0–94)	(0–95)	(0–100)	(0–100)
Late Emb. Mort. (%)	6 ± 1.7 B	12 ± 3.5 A	10 ± 2.4 A	13 ± 3.1 A	11 ± 1.4
	(0–34)	(0–77)	(0–82)	(0–89)	(0–89)

[a]Values indicate mean ± standard error of mean with ranges in parentheses. Values with different letters (A-B) indicate significant differences ($\alpha = 0.05$); same letters indicate significant differences were not detected. Clutch viability = No. of eggs yielding a live hatchling / Fecundity x 100, Damaged eggs = No. damaged eggs / fecundity x 100, Unbanded eggs = No. of unbanded eggs / fecundity x 100, Early Emb. Mort. = No. of embryonic deaths on or before developmental Day 35 / fecundity x 100, and Late Emb. Mort. = No. of embryonic deaths post dev. Day 35 / fecundity x 100).

Table 2-4. Organochlorine pesticide burdens and clutch parameters and site comparisons for clutches of American alligator eggs collected during 2000-2002.

Parameter[a]	Loch.	Apopka	Emeralda	Griffin	Summary
N$^{o.}$ Clutches	19	23	31	42	115
Fecundity (*n*)	40 ± 1.7 B	47 ± 1.4 A	46 ± 1.3 A	46 ± 1.2 A	45 ± 0.7 A
	(26–56)	(31–56)	(27–64)	(24–58)	(24–64)
Clutch mass (kg)	3.6 ± 0.17	4 ± 0.16	3.8 ± 0.25	3.7 ± 0.13	3.8 ± 0.09
	(2.2–4.8)	(2.5–5.1)	(2.1–9.2)	(1.5–5.2)	(1.5–9.2)
Egg mass (g)	90 ± 2.9 A	86 ± 2.5 AB	82 ± 4.8 B	79 ± 1.5 B	83 ± 1.6
	(78–139)	(62–120)	(58–180)	(46–105)	(46–180)
Clutch viability (%)	65 ± 5.5	52 ± 6.4	50 ± 6.9	43 ± 5.1	50 ± 3.1
	(0–95)	(0–98)	(0–97)	(0–92)	(0–98)
Damaged eggs (%)	4 ± 3.1	2 ± 0.8	6 ± 1.6	5 ± 2	4 ± 1
	(0–60)	(0–16)	(0–32)	(0–63)	(0–63)
Unbanded eggs (%)	11 ± 2	17 ± 4.2	10 ± 2.3	15 ± 3.2	13 ± 1.6
	(0–33)	(0–81)	(0–58)	(0–100)	(0–100)
Early Emb. Mort. (%)	13 ± 3	15 ± 4.2	26 ± 5.1	24 ± 4.3	21 ± 2.3
	(0–36)	(0–90)	(0–95)	(0–100)	(0–100)
Late Emb. Mort. (%)	8 ± 2.5	14 ± 4.6	10 ± 2.5	13 ± 3.3	11 ± 1.7
	(0–34)	(0–77)	(0–61)	(0–89)	(0–89)
Aldrin (ng/g)	0 ± 0 C	4 ± 0.3 A	2 ± 0.3 B	0 ± 0 C	3 ± 0.3
	(0–0)	(2.9–5.2)	(1.5–4.3)	(0–0)	(1.5–5.2)
Methoxychlor (ng/g)	0 ± 0 C	8 ± 1 B	9 ± 1 B	17 ± 0.3 A	9 ± 0.8
	(0–0)	(5.7–16.4)	(5.8–18.4)	(16.9–17.5)	(5.7–18.4)

Table. 2-4. Continued.

Parameter[a]	Orange/Loch	Apopka	Emeralda	Griffin	Summary
Mirex (ng/g)	2 ± 0.4 B	6 ± 1.1 A	3 ± 0.5 AB	3 ± 0.2 AB	4 ± 0.4
	(1.2–2.7)	(1.1–17.2)	(0.1–10.3)	(1.1–4.5)	(0.1–17.2)
Dieldrin (ng/g)	4 ± 0.5 D	344 ± 80.9 A	142 ± 20.4 B	23 ± 3.8 C	118 ± 20.9
	(1.3–8.2)	(12.5–1783.2)	(8.7–386.7)	(2.9–124)	(1.3–1783.2)
Hep. Epoxide (ng/g)	3 ± 0.8 C	17 ± 5.6 A	7 ± 1.4 B	7 ± 1 B	8 ± 1.4
	(1.2–9.7)	(1.2–135.5)	(0.1–32.1)	(1.1–29.6)	(0.1–135.5)
cis-Chlordane (ng/g)	2 ± 0.2 D	43 ± 7.6 B	90 ± 13 A	11 ± 0.9 C	37 ± 5
	(1.2–4.1)	(6.6–179.2)	(8.9–281)	(4.3–31.8)	(1.2–281)
cis-Nonachlor (ng/g)	5 ± 0.6 C	88 ± 27.3 A	66 ± 9.7 A	18 ± 1.6 B	43 ± 6.7
	(2.4–12.5)	(10.5–656.2)	(11.6–232.2)	(6.5–54.2)	(2.4–656.2)
Oxychlordane (ng/g)	4 ± 1 D	51 ± 14.6 A	23 ± 3.8 B	10 ± 1.3 C	21 ± 3.4
	(1.2–17.8)	(3.9–353.8)	(3.2–109.5)	(1.1–41.9)	(1.1–353.8)
p,p'-DDE (ng/g)	74 ± 11.7 C	5794 ± 1794.7 A	8069 ± 1402 A	271 ± 31.3 B	3445 ± 610.6
	(28–231)	(18.3–42653.4)	(36.2–33554.8)	(62.9–979.1)	(18.3–42653.4)
p,p'-DDD (ng/g)	2 ± 0.2 D	42 ± 8.5 B	1289 ± 196.1 A	7 ± 0.9 C	382 ± 78.7
	(1.2–3)	(10.6–192.8)	(10.3–2962.8)	(2.7–28.9)	(1.2–2962.8)
p,p'-DDT (ng/g)	1 ± 0 C	9 ± 2.1 AB	12 ± 1.2 A	5 ± 0.8 B	10 ± 1
	(1.2–1.3)	(1.2–45.6)	(5.8–25.5)	(1.1–7.2)	(1.1–45.6)
o,p'-DDD (ng/g)	0 ± 0 C	5 ± 0.7 B	37 ± 5.1 A	1 ± 0 B	29 ± 4.5
	(0–0)	(3.1–9.2)	(0.1–104)	(1.3–1.3)	(0.1–104)
o,p'-DDT (ng/g)	1 ± 0 C	11 ± 1.9 A	170 ± 161.6 A	4 ± 0.3 B	48 ± 42
	(1.2–1.4)	(1.2–38.5)	(4.2–4372.8)	(1.1–7.4)	(1.1–4372.8)

Table. 2-4. Continued.

Parameter[a]	Orange/Loch	Apopka	Emeralda	Griffin	Summary
trans-Chlordane (ng/g)	3 ± 0.7 C	8 ± 1.5 B	25 ± 3.3 A	2 ± 0.2 C	11 ± 1.5
	(1.2–3.7)	(1.3–27.4)	(2.9–58.2)	(1.1–8.7)	(1.1–58.2)
Toxaphene (ng/g)	0 ± 0 C	2738 ± 224.5 B	6865 ± 552.4 A	3043 ± 425.9 B	5456 ± 483
	(0–0)	(1896.1–3809.1)	(2300.6–12975.4)	(1927.9–4533.2)	(1896.1–12975.4)
trans-Nonachlor (ng/g)	8 ± 1.6 C	212 ± 66.9 A	191 ± 30.5 A	36 ± 4.7 B	108 ± 17.5
	(2.5–24.6)	(10.5–1569.2)	(14.2–718.6)	(8.6–155.2)	(2.5–1569.2)
∑OCPs (ng/g)	102 ± 15.5 C	7582 ± 2008.2 A	15480 ± 2265.4 A	1169 ± 422.8 B	6133 ± 940.8
	(42.7–289.4)	(472.5–47333.8)	(269.6–53559.7)	(101.5–16795.4)	(42.7–53559.7)
N$^{o.}$ OCPs	9 ± 0.3 D	13 ± 0.3 B	14 ± 0.2 A	11 ± 0.1 C	12 ± 0.2
	(7–11)	(10–16)	(13–17)	(9–13)	(7–17)

Table 2-5. Results of RDA evaluating associations between clutch survival parameters and OCP variables.

Site	Variable[a]	LambdaA	*P*	*F*
Lochloosa	NOC	0.11	0.074	2.25
	[DL]	0.09	0.194	1.59
	PDDT%	0.08	0.166	1.72
	PDDE%	0.11	0.104	2.45
Apopka	DL%	0.17	0.004	4.25
	TC%	0.12	0.024	3.32
	ALD%	0.10	0.042	3.16
	LPC%	0.06	0.16	1.85
Emeralda Marsh	TX%	0.09	0.044	2.99
	HE%	0.06	0.09	2.27
	ME%	0.06	0.15	1.85
	[HE]	0.06	0.15	1.89
Griffin	[PDDE]	0.08	0.024	3.67
	[TX]	0.07	0.016	3.16
	[PDDT]	0.06	0.04	2.71
	[ODDD]	0.04	0.09	1.96

[a]See Table 2-2 for definition of variable codes.

Table 2-6. Results of RDA evaluating associations between egg and clutch size parameters and OCP variables.

Site	Variable	LambdaA	*P*	*F*
Lochloosa	NOC	0.31	0.004	10.15
	[PDDT]	0.20	0.042	4.29
	[TN]	0.13	0.006	6.77
	OX%	0.08	0.088	2.8
Griffin	PDDD%	0.05	0.134	2.32
	[ODDT]	0.03	0.406	0.91
	[PDDT]	0.02	0.236	0.95
	[CC]	0.01	0.54	0.33
Emeralda	[ODDT]	0.22	0.01	8.07
	CC%	0.05	0.146	2
	ODDT%	0.05	0.182	1.82
	LPC%	0.04	0.21	1.7
Apopka	[PDDD]	0.24	0.01	6.51
	[ME]	0.08	0.112	2.54
	[PDDT]	0.05	0.218	1.5
	PDDE%	0.05	0.294	1.29

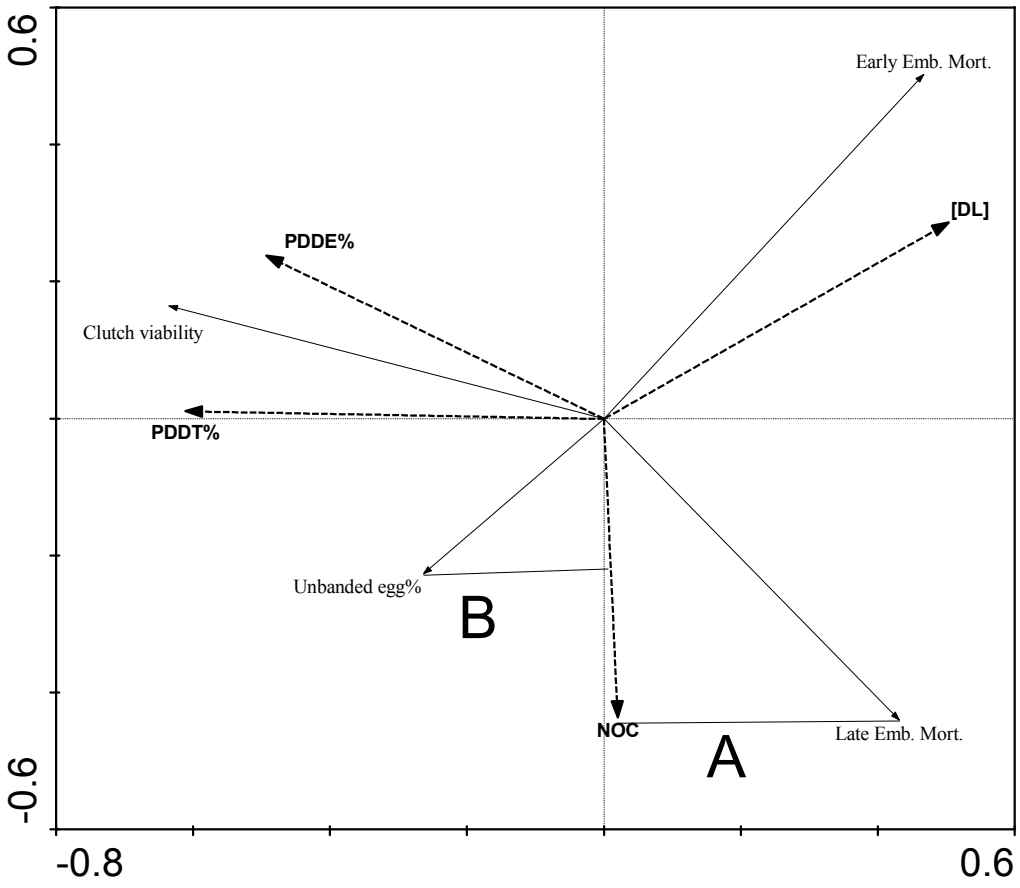

Figure 2-1. Biplot of clutch survival parameters (solid lines) and organochlorine pesticide variables (dashed lines) for clutches of alligator eggs collected from Lake Lochloosa during summer 2001-2002. Arrows pointing in the same direction indicate a positive correlation (e.g., clutch viability and PDDE%), arrows that are approximately perpendicular indicate near-zero correlation (e.g., late emb. mort. and [DL]), and arrows pointing in opposite directions indicate negative correlations (e.g., clutch viability and [DL]. Arrow lengths indicate rank order of correlations. For example, late emb. mort. has higher positive correlation with NOC (A) compared to unbanded egg% (B). Cosine of angle formed between individual clutch variables and individual OCP variables (see Table 2-2 for code definitions) equals correlation coefficient (r) (ter Braak, 1995). For example, arrows pointing in exactly opposite directions have an angle of 180°, and since cos(180) = -1.0, the arrows are perfectly, negatively correlated (r) (ter Braak, 1995).

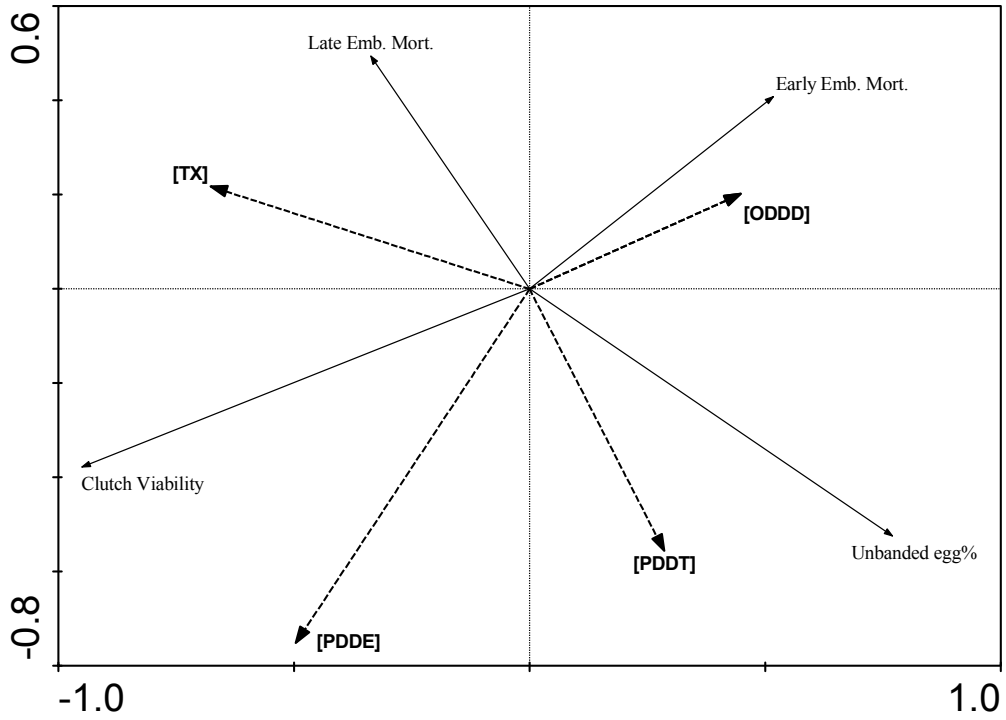

Figure 2-2. Biplot of clutch survival parameters (solid lines) and organochlorine pesticide variables (dashed lines) for clutches of alligator eggs collected from Lake Griffin during summer 2000-2002.

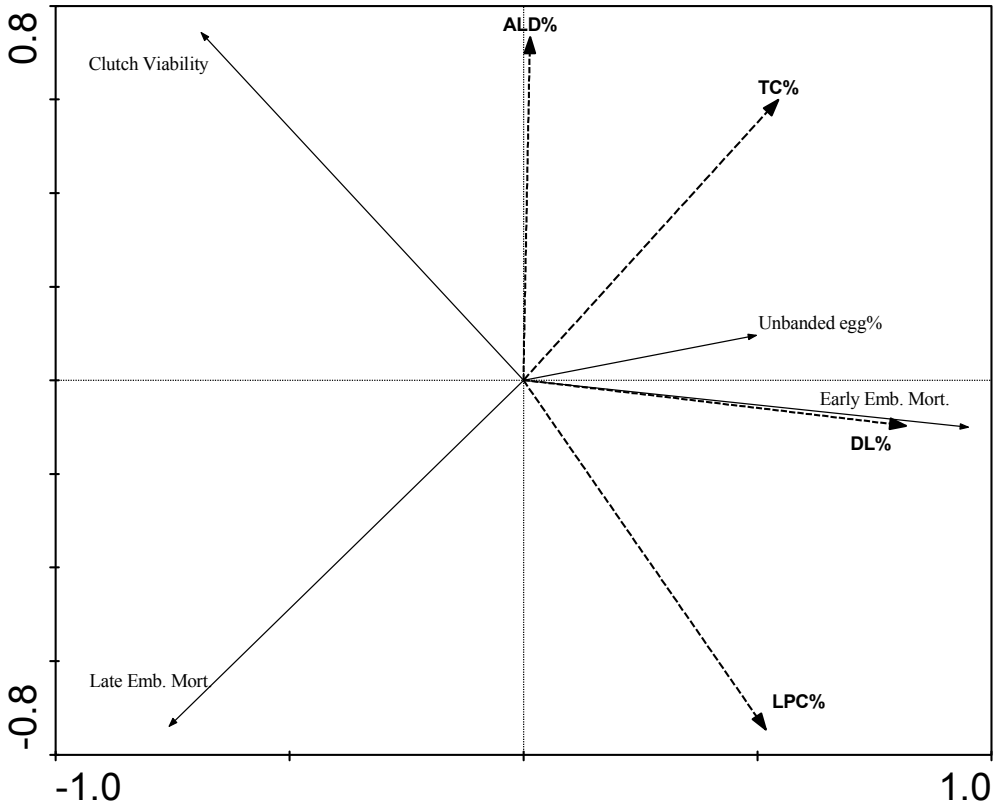

Figure 2-3. Biplot of clutch survival parameters (solid lines) and organochlorine pesticide variables (dashed lines) for clutches of alligator eggs collected from Lake Apopka during summer 2000-2002.

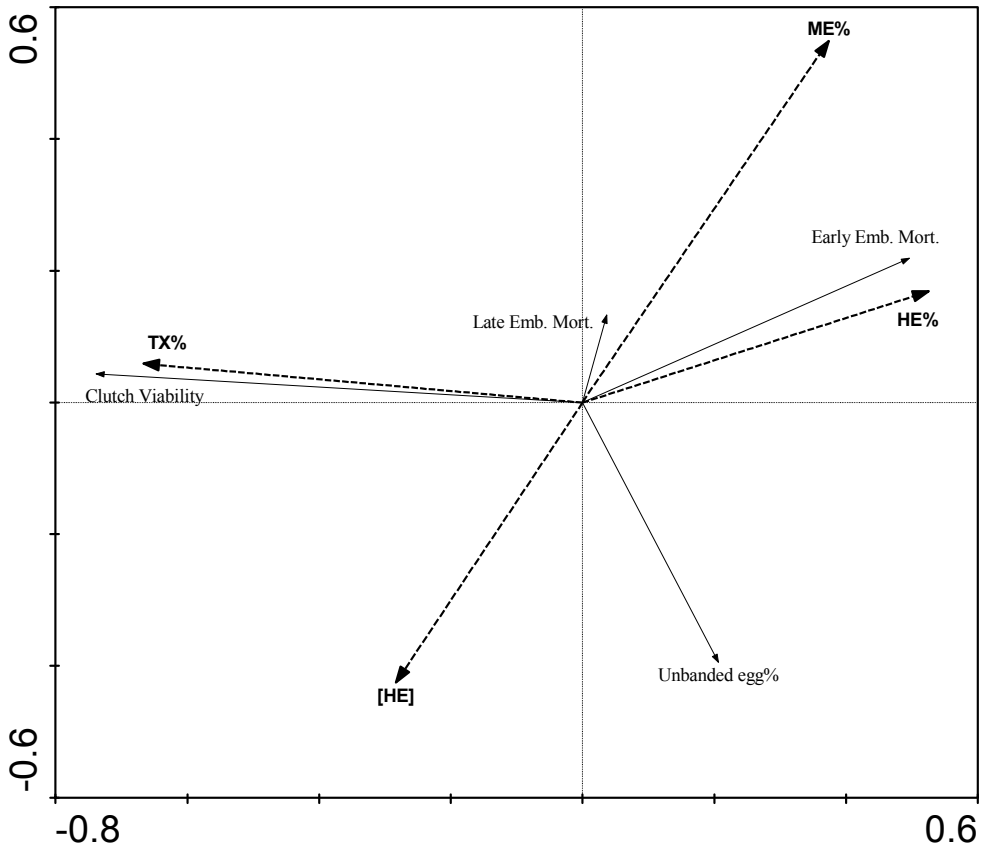

Figure 2-4. Biplot of clutch survival parameters (solid lines) and organochlorine pesticide variables (dashed lines) for clutches of alligator eggs collected from Emeralda Marsh during summer 2000-2002.

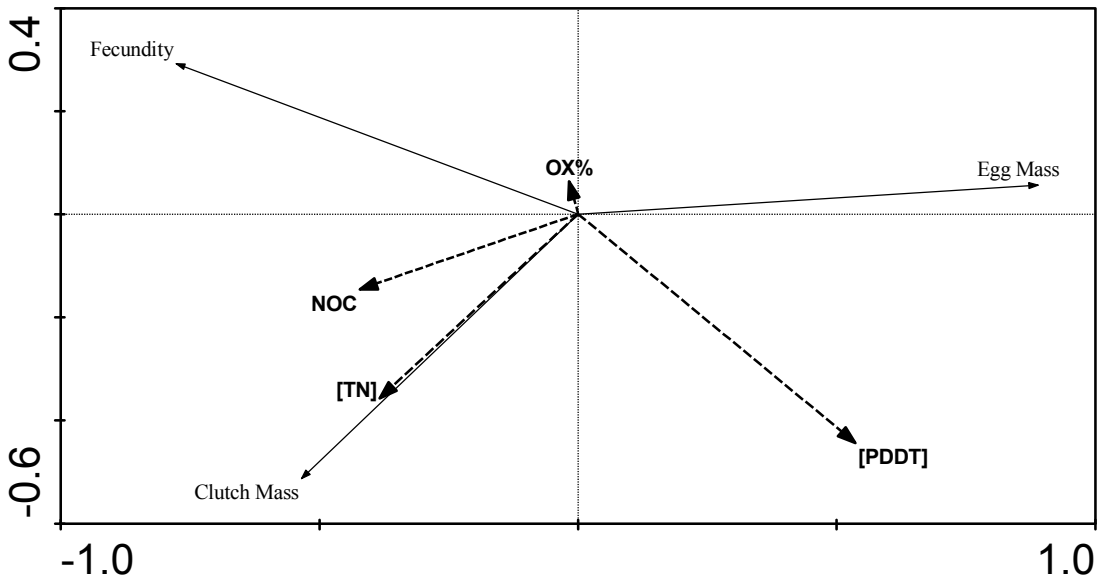

Figure 2-5. Biplot of egg and clutch size parameters (solid lines) and organochlorine pesticide variables (dashed lines) for clutches of alligator eggs collected from Lake Lochloosa during summer 2001 and 2002.

CHAPTER 3
MATERNAL TRANSFER OF ORGANOCHLORINE PESTICIDES

Studies have documented organochlorine pesticide (OCP residues) in eggs and/or somatic tissues of several crocodilian species including the American alligator, *Alligator mississippiensis* (Heinz et al., 1991), Morelet's crocodile, *C. moreletti* (Wu et al., 2000a), the American crocodile, *Crocodylus acutus* (Hall et al., 1979; Wu et al., 2000b), and the Nile crocodile, *C. niloticus* (Skaare et al., 1991). Indeed, alligator populations inhabiting Lake Apopka, where an OCP spill occurred in the 1980s, and other central Florida lakes contaminated with OCPs (through historic OCP use) produce eggs that contain concentrations of total OCPs that are over 100 times higher than concentrations found in eggs from reference lakes (Gross, unpublished data). In addition, the alligator populations inhabiting the OCP-contaminated lakes experience increased (and highly variable) rates of embryonic mortality, leading to reduced clutch success, and juvenile alligators appeared to have abnormal sex hormone concentrations as compared to those of reference sites (Masson, 1995; Rice, 1996; Woodward et al., 1993). However, a clear dose-response relationship has not been established with respect to individual or total OCP concentrations in egg yolks and reduced clutch success (Heinz et al., 1991). The lack of a clear dose-response suggests other factors (e.g., diet, population dynamics, and specific OCP mixtures) might be involved and/or that developmental effects result from altered maternal physiology resulting from OCP exposure, as opposed to direct embryotoxicity.

With respect to altered maternal physiology, alterations in steroid hormone levels have also been shown in alligators inhabiting OCP-contaminated sites (Guillette et al., 1994). Furthermore, maternal exposure suggests that OCPs may be maternally transferred from the adult female alligator to her offspring, as has been reported in other oviparous vertebrates (Russell et al., 1999). Assuming OCPs are maternally transferred, the possibility exists that yolks could be used as predictors of maternal exposure. A noninvasive method such as this would aid ecological risk assessments in understanding exposure levels for rare/endangered crocodilian species without having to capture and/or remove adults from the breeding population. Therefore, the objectives of the present study were to examine maternal transfer as a potential route for embryonic OCP exposure, and to evaluate the use of yolk burdens for predicting OCP burdens in maternal tissues in alligators. Our hypothesis was that OCP burdens in maternal tissues and yolks would be strongly correlated, which would allow yolk burdens to be used to predict maternal body burdens and suggest maternal transfer of OCPs as the major route for embryonic OCP exposure.

Materials and Methods

Site descriptions

Lakes Apopka (N 28° 35', W 81° 39'), Griffin (N 28° 53', W 81° 49'), and Lochloosa (N 29° 30', W 82° 09') in Florida were selected as collection sites because prior studies by our laboratory indicate vastly different levels of OCP exposure across these sites. All three lakes are part of the Ocklawaha Basin. Lake Lochloosa (which is connected to Orange Lake) was selected as a low exposure (reference) site. Four years (1999-2002) of data indicate mean total OCP concentrations in egg yolks from the reference sites (Lakes Orange and Lochloosa) were 231 ± 30 ppb (mean ± standard

deviation [SD], n = 56 clutches) with a concurrent mean clutch viability rate (number of live hatchlings/total number of eggs in a nest) of 71 ± 21% (Gross, unpublished data). Lake Griffin was selected as an intermediate exposure site since yolk concentrations averaged 4,414 ± 617 ppb (n = 47 clutches) and Lake Apopka was selected as a high exposure site since yolk concentrations averaged 15,911 ± 1,786 ppb (n = 42) for the same time period (Gross, unpublished data). Furthermore, mean clutch viability rates during this time period for Lakes Apopka (51 ± 31%, n = 42) and Griffin (44 ± 33%, n = 47) have been below rates observed for the reference site.

Animal Collections

Adult female alligators and their corresponding clutches of eggs were collected from Lakes Apopka (n = 4), Griffin (n = 8), and Lochloosa (n = 3) over the course of two nesting seasons (June 2001 and June 2002). Nests were located by aerial survey (helicopter) and/or from the ground (airboat). Once nests were located, all eggs were collected, and the nest cavity was covered. A snare-trap was set perpendicular to the tail-drag in order to capture the female as she crossed over the nest. After the traps were set, one member of the trapping crew subsequently transported the eggs to the Florida Fish and Wildlife Conservation Commission's Wildlife Research Unit (FWC; Gainesville, FL, USA) and placed the eggs in a temperature-controlled incubator. Snare-traps were checked later in the evening and early the next morning.

Trapped females were secured and transported from each lake to the United States Geological Survey's Florida Integrated Science Center (USGS; Gainesville, FL, USA). Upon arrival, the animals were weighed, measured, and blood samples were collected from the post-occipital sinus. Adult alligators were then euthanized by cervical dislocation followed by double pithing. A full necropsy was performed on each female.

Bile, liver, adipose (composite of abdominal fat and the abdominal fat pad), and tail muscle samples were collected for later determination of OCP burdens. Liver, adipose tissue, and muscle were wrapped in aluminum foil, while bile and blood were placed in scintillation vials. All samples were grouped according to nest identification number (ID), placed in plastic bags labeled with the appropriate ID, and stored in a –80 ^{o}C freezer. Each female's corresponding clutch of eggs was then transferred from FWC to USGS where yolk samples were collected (two eggs/clutch) and stored with the corresponding maternal tissues. The remaining eggs were set for incubation in a temperature/humidity-controlled incubator (31-33 ^{o}C, 88-92% relative humidity) located at USGS.

Analysis of OCPs in Maternal Tissues and Yolk

Analytical grade standards for the following compounds were purchased from the sources indicated: aldrin, alpha-benzene hexachloride (α-BHC), β-BHC, lindane, δ-BHC, *p,p'*-dichlorodiphenyldichloroethane (*p,p'*-DDD), *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), dichlorodiphenyltrichloroethane (*p,p'*-DDT), dieldrin, endosulfan, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor epoxide, hexachlorobenzene, kepone, methoxychlor, mirex, *cis*-nonachlor, and *trans*-nonachlor from Ultra Scientific (Kingstown, RI, USA); *cis*-chlordane, *trans*-chlordane, and the 525, 525.1 polychlorinated biphenyl (PCB) Mix from Supelco (Bellefonte, PA, USA); oxychlordane from Chem Service (West Chester, PA); *o,p'*-DDD, *o,p'*-DDE, *o,p'*-DDT from Accustandard (New Haven, CT, USA); and toxaphene from Restek (Bellefonte, PA, USA). All reagents were analytical grade unless otherwise indicated. Water was doubly distilled and deionized.

Adipose, liver, bile, and yolk samples were analyzed for OCP content using methods modified from Holstege et al. (1994 and Schenck et al. (1994). For extraction, a 2 g tissue sample was homogenized with ~1 g of sodium sulfate and 8 mL of ethyl acetate. The supernatant was decanted and filtered though a Büchner funnel lined with Whatman #4 filter paper (Fisher Scientific, Hampton, NH, USA) and filled to a depth of 1.25 cm with sodium sulfate. The homogenate was extracted twice with the filtrates collected together. The combined filtrate was concentrated to ~2 mL by rotary evaporation, and then further concentrated until solvent-free under a stream of dry nitrogen. The residue was reconstituted in 2 mL of acetonitrile. After vortexing (30 s), the supernatant was applied to a C18 solid phase extraction (SPE) cartridge (pre-conditioned with 3 mL of acetonitrile; Agilent Technologies, Wilmington, DE, USA) and was allowed to pass under gravity. This procedure was repeated twice with the combined eluent collected in a culture tube. After the last addition, the cartridge was rinsed with 1 mL of acetonitrile which was also collected. The eluent was then applied to a 0.5 g NH2 SPE cartridge (Varian, Harbor City, CA, USA), was allowed to pass under gravity, and collected in a graduated conical tube. The cartridge was rinsed with an additional 1 mL portion of acetonitrile which was also collected. The combined eluents were concentrated under a stream of dry nitrogen, to a volume of 300 μL, and transferred to a gas chromotography (GC) vial for analysis.

Whole blood was analyzed for OCP content using methods modified from Guillette et al. (1999). A 10 mL aliquot was transferred from the homogenized bulk sample and extracted in 15 mL of acetone by vortex mixer. The mixture was centrifuged for 5 min at 3000 rpm, after which the supernatant was transferred to a clean culture tube.

This process was repeated with the supernatants collected and concentrated under a stream of dry nitrogen until solvent-free. The residue was re-extracted in 11.5 mL of 1:1 methylene chloride-petroleum ether. After mixing, the sample was allowed to settle and the upper layer was transferred to a clean culture tube. This extraction was performed twice with the extracts collected together. The combined extracts were then applied to a prepared florisil cartridge (5 mL Fisher PrepSep, Fisher Scientific, Hampton, NH, USA). The cartridge had been prepared by filling the reservoir to a depth of 1.25 cm with anhydrous sodium sulfate and by prewashing the modified cartridge with 10 mL of 2:1:1 acetone: methylene chloride: petroleum ether. After the sample passed under gravity with the eluent collected in a 15-mL graduated conical tube, the cartridge was eluted with 4 mL of the 2:1:1 solvent mixture which was also collected. The combined eluents were concentrated under a stream of dry nitrogen, to a volume of 300 μL, and transferred to a GC vial for analysis.

GC/MS Analysis

Analysis of all samples was performed using a Hewlett Packard HP-6890 gas chromatograph (Wilmington, DE, USA) with a split/splitless inlet operated in splitless mode. The analytes were introduced in a 1 μL injection and separated across the HP-5MS column (30 m x 0.25 mm; 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA) under a temperature program that began at 60º C, increased at 10º C/min to 270º C, was held for 5 min, then increased at 25º C/min to 300º C and was held for 5 min. Detection utilized an HP 5973 mass spectrometer in electron impact mode. Identification for all analytes and quantitation for toxaphene was conducted in full scan mode, where all ions are monitored. To improve sensitivity, selected ion monitoring was used for the

quantitation for all other analytes, except kepone. The above program was used as a screening tool for kepone which does not optimally extract with most organochlorines. Samples found to contain kepone would be reextracted and analyzed specifically for this compound.

For quantitation, a five-point standard curve was prepared for each analyte ($r^2 \geq 0.995$). Fresh curves were analyzed with each set of twenty samples. Each standard and sample was fortified to contain a deuterated internal standard, 5 µL of US-108 (120 µg/mL; Ultra Scientific), added just prior to analysis. All samples also contained a surrogate, 2 µg/mL of tetrachloroxylene (Ultra Scientific) added after homogenization. Duplicate quality control samples were prepared and analyzed with every twenty samples (typically at a level of 1.00 or 2.50 µg/mL of γ-BHC, heptachlor, aldrin, dieldrin, endrin, and *p,p'*-DDT) with an acceptable recovery ranging from 70 – 130%. Limit of detection ranged from 0.1-1.5 ng/g for all OCP analytes, except toxaphene (120-236 ng/g), and limit of quantitation was 1.5 ng/g for all analytes, except toxaphene (1500 ng/g). Repeated analyses were conducted as allowed by matrix interferences and sample availability.

Data Analysis

OCP concentrations in maternal tissues and egg yolks were lipid-adjusted (wet weight concentration / proportion of lipid in tissue), and lipid-adjusted tissue-to-egg yolk ratios (maternal tissue OCP concentrations /egg OCP concentrations) were examined. Predictive models were determined by linear regression analysis of OCP concentrations in yolk against those of maternal tissues (log-transformed wet weight concentrations). Each model's ability to fit the data was evaluated by examining the p-value ($\alpha = 0.05$), the r^2 value , and the residual plots (SAS Institute Inc., 2002). ANOVA was used for

inter-site comparisons of adult female and clutch characteristics, and the Tukey test was used for multiple comparisons among sites. The relationship between maternal mass (kg) and concentrations of OCPs in eggs and maternal tissues (log-transformed wet weight concentrations) were evaluated using linear regression to assess whether increasing mass was associated with increasing concentrations of OCPs in eggs and maternal tissues, which may suggest adult females continue to bioaccumulate OCPs as they grow throughout their life. Adult females were grouped by site since the extreme differences in OCP exposure among sites would likely confound results. Unless otherwise noted, values are reported as mean ± standard deviation.

Results

Female Morphological and Reproductive Characteristics

For all females, mass and snout-vent length (SVL) averaged 74 ± 20 kg (range: 44-114) and 135 ± 11 cm (119-156), respectively. Clutch mass (mass of all eggs from a single nest) and fecundity (number of eggs collected from a single nest) of these individuals were 3.65 ± 0.86 kg (1.84-4.82) and 43 ± 10 eggs/nest (19-56), respectively. No significant differences were detected across sites with respect to female mass ($p = 0.14$), total length ($p = 0.90$), SVL ($p = 0.25$), tail girth ($p = 0.98$), head length ($p = 0.55$), clutch mass ($p = 0.23$), or fecundity ($p = 0.40$, Table 1).

With respect to lipid concentrations in egg yolk and muscle, no significant differences were detected across sites ($p > 0.05$). However, lipid concentration in liver of Lochloosa females was significantly higher ($p < 0.05$) than that of Apopka and Griffin females (which were not significantly different from one another). Furthermore, lipid concentration in abdominal adipose tissue of Apopka females was significantly less ($p < 0.05$) than that of Lochloosa and Griffin females (Table 1).

OCP concentrations in Yolk

Egg yolks from Lake Apopka females contained the highest total OCP concentration (15,108 ± 13,704) and greatest number of individual OCPs detected above the limit of quantitation (n = 18) with p,p'-DDE (66%) and toxaphene (32%) being main constituents. Lake Griffin females produced eggs with the next highest total OCP burdens (393 ± 300 ng/g; n = 13) being mainly composed of p,p'-DDE (69%), trans-nonachlor (10%), and dieldrin (7%). Lake Lochloosa females produced egg yolks with the smallest total OCP burden (124 ± 53 ng/g, n = 9), with main constituents being p,p'-DDE (73%), trans-nonachlor (10%), and cis-nonachlor (4%; Table 3-2). The OCP analytes with the highest average egg yolk concentrations were toxaphene (4,862 ± 4,177 ng/g), which was detected above the limit of quantitation in 3 of 15 clutches, followed by p, p'-DDE (2,828 ± 5,968 ng/g), dieldrin (191 ± 474 ng/g), and trans-nonachlor (126 ± 209 ng/g), which were above quantitation limit in all 15 clutches.

OCP concentrations in maternal tissues

Adipose tissue (a composite of abdominal fat and fat pad) contained the highest concentration of total OCPs (12,805 ± 31,678 ng/g wet weight) of all tissues. p,p'-DDE (67%) composed the majority of the total burden, followed by dieldrin (5%), and trans-nonachlor (3%). Although toxaphene was only detected in 3 individuals from Lake Apopka, its average burden in adipose tissue was 13,463 ± 1,267 ng/g (Table 3-2). In liver, OCP analytes were detected above the quantitation limit in 9 of 15 individuals, and total OCP concentrations averaged 1,008 ± 1,245 ng/g. Liver burdens were primarily composed of p,p'-DDE (76%) and dieldrin (6%). Total OCP concentrations in muscle averaged 716 ± 1,053 ng/g and were above quantitation limits in 10 of 15 individuals

with most of the burden being composed of p,p'-DDE (83%), dieldrin (6%) and trans-nonachlor (6%). Total OCP burdens in bile (412 ± 483 ng/g) were above quantitation limits in five individuals with p,p'-DDE (86%) and dieldrin (6%) comprising the majority of the burden. Total OCP concentrations in blood (43 ± 21 ng/g) were above quantitation limits in 4 individuals with p,p'-DDE (64%) and dieldrin (14%) comprising most of the burden. Overall, Lake Apopka alligators exhibited the highest OCP concentrations in maternal tissues and egg yolks, followed by Lakes Griffin and Lochloosa, respectively (Table 3-2).

Relationships between Maternal Tissue and Yolk Burdens

Examination of lipid-adjusted maternal tissue-to-egg yolk burdens showed differences among tissues. With respect to total OCPs, the adipose burden-yolk burden ratio was close to 1 (95% confidence interval (CI), $0.76 \leq \mu \leq 1.11$). In contrast, the liver-yolk ratio was significantly greater than 1 (95% CI, $1.49 \leq \mu \leq 9.19$), and muscle ratios showed considerable variation (95% CI, $-1.17 \leq \mu \leq 37.35$). As would be expected, most individual OCPs followed the above trend. However, cis-chlordane was an exception as liver ratios (95% CI, $2.85 \leq \mu \leq 6.75$) and muscle ratios (95% CI, $1.78 \leq \mu \leq 15.1$) were greater than 1, while adipose ratios (95% CI, $0.59 \leq \mu \leq 0.84$) were less than 1. With respect to total OCP concentrations, significant linear relationships (predictive models) were found for adipose, liver, muscle, and bile ($p \leq 0.05$, Fig. 1). With respect to individual OCP analytes, predictive models were derived for 12 of 14 (78%) of the OCPs co-detected in adipose tissue and egg yolk, followed by liver (9/12, 75%), bile (8/11, 73%), and muscle (2/12, 17%; Table 3-3). Although nine OCP analytes were concurrently detected in blood of the females and their respective egg yolks, no significant linear correlations were detected ($p > 0.05$).

As for individual OCP analytes, p,p'-DDE concentrations in yolk was significantly correlated with those of liver, muscle, bile, and adipose tissue. Blood p,p'-DDE concentrations did not exhibit a significant linear relationship ($p > 0.05$) with yolk p,p'-DDE concentrations. Heptachlor epoxide, trans-chlordane, cis-chlordane, trans-nonachlor, cis-nonachlor, mirex, and dieldrin concentrations in yolk were significantly correlated to their respective concentrations in adipose, liver, and bile. With respect to oxychlordane, significant correlations were only derived for liver and adipose tissue, and significant correlations for p,p'-DDD concentrations were found only for adipose and bile. Toxaphene and o,p'-DDT concentrations in adipose tissue were significantly correlated with respective egg yolk concentrations (Table 3-3).

Relationships between Maternal Mass and OCP concentrations in Eggs and Tissues

For females collected from Lakes Apopka (n = 4) and Lochloosa (n = 3), no significant correlations ($p > 0.05$) were found when maternal mass (kg) was compared against either individual or total OCP concentrations (log-transformed wet weight) in maternal tissues and eggs. However, significant correlations might have been difficult to detect because of the small sample size. In contrast, a larger number of Lake Griffin females (n = 8) were collected, and analyses indicated significant correlations between maternal mass and OCP concentrations in tissues and eggs indicating that larger females have higher concentrations of OCPs in their tissues and eggs, which may suggest females continue to bioaccumulate OCPs as they grow (increase in mass). For Lake Griffin females, OCP burdens in eggs had the greatest number of significant correlations ($p \leq 0.05$) with body mass (kg), which consisted of cis-nonachlor ($r^2 = 0.87$), cis-chlordane ($r^2 = 0.75$), trans-nonachlor ($r^2 = 0.73$), dieldrin ($r^2 = 0.69$), p,p'-DDE ($r^2 = 0.66$), o,p'-DDT ($r^2 = 0.61$), heptachlor epoxide ($r^2 = 0.59$), oxychlordane ($r^2 = 0.58$), trans-chlordane ($r^2 =$

0.57), and total OCPs ($r^2 = 0.71$). Following egg concentrations, abdominal fat OCP burdens-to-body mass correlations consisted of cis-nonachlor ($r^2 = 0.67$), cis-chlordane ($r^2 = 0.81$), trans-nonachlor ($r^2 = 0.63$), dieldrin ($r^2 = 0.62$), p,p'-DDE ($r^2 = 0.58$), , heptachlor epoxide ($r^2 = 0.53$), oxychlordane ($r^2 = 0.51$), and total OCPs ($r^2= 0.64$). Although egg burdens of o,p'-DDT and trans-chlordane were correlated with body mass, abdominal fat burdens were not. Lastly, liver OCP burdens-to-body mass correlations included only trans-nonachlor ($r^2= 0.99$) and p,p'-DDT ($r^2= 0.99$). No significant correlations were found for cis-chlordane, trans-chlordane, oxychlordane, dieldrin, heptachlor epoxide, o,p'-DDT, and cis-nonachlor.

Discussion

The presence of OCPs in the eggs and tissues of alligators is not novel; however, the value of our study was that OCP concentrations in maternal tissues and yolks appeared to be strongly correlated with one another, allowing yolk burdens to be used as predictors of OCP burdens in tissues of adult reproductive alligators, which may be a useful noninvasive technique that would aid risk assessments involving endangered crocodilians. Furthermore, our results are consistent with other studies that suggest OCPs are maternally transferred in wild alligators (Rauschenberger et al., 2004).

Several OCP analytes were detected in both maternal tissues and yolk (Table 3-3) suggesting that mixture composition may be an important consideration in risk assessment. One reason for this is that different xenobiotic compounds may induce or inhibit certain biotransformation enzymes. Specifically, alligators from Louisiana express several different xenobiotic biotransformation enzymes (e.g., liver cytochrome P-450 enzymes [CYP] such as CYP1A, CYP2B) in response to xenobiotic exposure (Ertl et al., 1999). Furthermore, genetic partitioning has been reported in spatially separated

alligator populations (Ryberg et al., 2002). Therefore, the possibility exists that certain individuals or populations may lack the genetic or epigenetic ability to produce a particular biotransformation enzyme, which may lead to increased risk of xenobiotic-induced toxicity. For example, certain populations of black-banded rainbowfish (*Melanotaenia nigrans*) were able to tolerate copper exposures (96-hr EC_{50}) that were 8.3 fold greater than the tolerance limits of other, spatially-separated populations of the same species. Genetic analyses suggested that allozyme frequencies of tolerant and susceptible populations were significantly different at AAT-1 and GPI-1 loci, suggesting differences in allozymes of exposed fish may have assisted in the increased copper tolerance (Woosley, 1996).

Examination of maternal tissue-to-egg concentration ratios (lipid-adjusted) showed differences among tissues. The adipose-to-yolk concentration ratio was close to 1, suggesting that OCPs reach equilibrium within abdominal adipose tissue, and that lipids and OCPs are mobilized and subsequently incorporated into the developing yolks. In contrast, liver-to-yolk concentration ratios were significantly greater than 1, and muscle-to-yolk concentration ratios showed considerable variation. One suggested explanation for the high liver-to-yolk ratios relates to one major function of the liver cells (hepatocytes), which is to accumulate and convert hydrophobic xenobiotics into hydrophilic metabolites to facilitate detoxication, excretion, and elimination. In addition, the low lipid content of liver (relative to the lipid content of adipose tissue and yolk, Table 3-1) may have contributed to the marked differences. With respect to the muscle-to-yolk ratios, the reasons for the large degree of variability are not as clear. One possible explanation is that muscle lipids are not mobilized during yolk formation and, as

a result, OCP burdens may continually accumulate in muscle lipids. Another potential explanation relates to the low lipid content of muscle when compared to yolk (Table 3-1). Lastly, cis-chlordane's exceptional liver, muscle, and adipose ratios underscore the fact that different OCP analytes may not always exhibit identical pharmacokinetics.

When compared to other vertebrates, adipose tissue-to-egg ratios in alligators are similar to those reported in the freshwater catfish, *Clarias batrachus*, in that adipose-to-egg ratios are approximately equal to 1. Furthermore, *C. batrachus* mobilizes lipids from its abdominal adipose tissue during vitellogenesis (Lal & Singh, 1987), similar to what this study suggests occurs in the American alligator. In contrast to adipose tissue OCP concentrations, muscle-to-egg OCP ratios in alligators appear to be quite different from fish. Alligator muscle-to-egg ratios were highly variable and, for the most part, greater than 1, while fish ratios appear to be consistently close to 1. With respect to more closely related species, muscle-to-egg OCP ratios are similar to those reported for the common snapping turtle (*Chelydra serpentina*) and several bird species with ratios exhibiting a great deal of variability and being greater than 1 (Russell et al., 1999). These differences suggest that fish differ from terrestrial vertebrates in regards to lipid content of muscle and/or lipid mobilization strategy (during vitellogenesis), which could lead to differences in embryonal exposure given equivalent maternal exposure.

Evaluation of Predictive Models

Although significant linear models were found for most tissues with respect to total OCP concentrations, caution should be used in the application of these "total OCP" models since it is probable that the concentrations and ratios of individual OCP analytes may vary across different locations. The greatest number of predictive linear models was derived for adipose tissue. This was not surprising considering that (for most analytes)

adipose-yolk lipid normalized ratios were close to 1. Next, with respect to the number of significant linear models, were liver and bile. The similarities between liver and bile should be expected since the liver produces bile, which transports OCP analytes to the intestinal lumen, leading to their eventual elimination from the body. However, OCP analytes may be reabsorbed from the intestine and redirected back to the liver via the portal vein through a process known as enterohepatic circulation, which may delay elimination of lipophilic xenobiotics, increase hepatic exposure and bioaccumulation (Stenner et al., 1997). For OCP concentrations in muscle, regression analysis indicated that only two out of 12 mutually detected analytes could be predicted using OCP concentrations in eggs. Lastly, nine OCP analytes were concurrently detected in blood and egg yolk with none exhibiting significant relationships. Possible explanations for the few significant linear relationships include the low lipid content of these tissues and thus the relatively low concentrations of OCP analytes in these tissues, as well as the possibility that each of these tissue burdens exhibit a nonlinear relationship with yolk burdens. In addition, blood samples were collected after the female had oviposited. Since blood was collected after eggs were excreted from the body, it is likely that the overall maternal body burden decreased, which would in turn lower the steady-state OCP concentrations in blood.

As for individual OCP analytes, predictive models for p,p'-DDE were derived for four of the five maternal tissues. One likely reason for this is that p,p'-DDE was detected in considerable concentrations in all eggs and in almost all tissues for all 15 females. Similarly, predictive models were derived for commonly detected analytes such as heptachlor epoxide, trans-chlordane, cis-chlordane, trans-nonachlor, cis-nonachlor,

mirex, and dieldrin for most tissues. Somewhat surprisingly, oxychlordane (a metabolite of cis- and trans-chlordane) and p,p'-DDD (an intermediate metabolite of p,p'-DDT) showed significant linear models only with respect to liver and adipose tissue. The fact that linear models for toxaphene and o,p'-DDT were derived only for adipose tissue was likely related to their low concentrations and infrequent detections in other tissues (Table 3-3).

Relationships between Maternal Mass and OCP concentrations in Eggs and Tissues

Although a portion of a female alligator's OCP body burden may be eliminated through egg deposition, adult female alligators from Lake Griffin had increased OCP concentrations in their tissues and eggs as they increased in mass, similar to size-related OCP bioaccumulation in smallmouth bass inhabiting contaminated sites in Michigan (Henry et al., 1998). Corresponding increases in OCP burdens and mass indicate that larger and possibly older females accumulate OCPs faster than they can excrete them. In addition, the relationship between OCP burdens in eggs and body mass was very similar to the relationship between abdominal fat burdens and body mass.

The correlation between OCP burdens in liver and body mass was significant for trans-nonachlor and p,p'-DDT; however the major metabolites of these compounds (oxychlordane and p,p'-DDE, respectively) were not significantly correlated with body mass. These results contrast those of egg and abdominal fat burdens and suggest that that alligator liver may not sequester OCP metabolites to the same extent as abdominal fat or egg.

Maternal body burdens: Toxicological Implications

Although our study's objective was to evaluate maternal transfer and prediction of the maternal OCP body burdens carried by the American alligator, we would be remiss if

we did not discuss whether these reported body burdens were capable of eliciting harmful effects. Although several studies report body and egg burdens in crocodilians, relatively few studies directly relate body and egg burdens to acute toxicological effects (Campbell, 2003), so we will briefly discuss how p,p'-DDE burdens in maternal alligator liver compare to reported p,p'-DDE burdens in liver of birds (birds were not from the present study areas) that have been associated with mortality (Blus, 1996).

In previous studies, mean DDE liver residues in birds which died due to DDT exposure ranged from 19,000–55,000 ng/g. When birds were exposed to DDE alone, liver residues of dead birds averaged 3,883,000 ng/g (range 460,000–11,725,000 ng/g) (Blus, 1996). When compared to the liver residues of the most contaminated alligators (Lake Apopka, upper 95% CI < 7,000 ng/g), it appears that death due to DDT/DDE exposure might be unlikely assuming bird and alligator susceptibilities are similar. However, since p,p'-DDE liver concentrations in alligators are almost half of lethal liver concentrations in birds, there is reason for some concern. In addition, the assumption that bird and alligator susceptibilities are similar might be argued as unfounded considering the variability in toxic responses between individuals of the same species, different species, and different vertebrate classes (James et al., 2000). To account for these uncertainties the risk assessment process identifies the different sources of uncertainty and incorporates the uncertainty in attempting to determine a "safe" tissue concentration based on levels associated with no adverse effects (NOAEL) or lowest observed adverse effect levels (LOAEL). Typically, interspecies extrapolation is assigned an uncertainty factor of 10, as are inter-individual uncertainty, uncertainty related to comparing different study designs (e.g., acute doses related to experimental bird studies, in contrast to chronic

exposure studies in wild alligators), and uncertainty related to database quality since DDE (p,p'-DDE + o,p-DDE) liver residues were reported, instead of p,p'-DDE. These four uncertainty factors constitute an overall uncertainty factor of 10,000, which is an order of magnitude greater than commonly used uncertainty factors (range: 300-1000) (James et al,. 2000). Considering the high degree of uncertainty, we suggest that more information is required before a "safe" level of p,p'-DDE exposure is determined for the American alligator based upon actual or predicted liver concentrations.

Sublethal effects are another possible consequence of OCP exposure. For example, exposure of the freshwater catfish, *Clarias batrachus*, to an OCP analyte (γ-BHC) at sublethal levels (2,000–8,000 ng/g) during vitellogenesis significantly decreased the biosynthesis and mobilization of phospholipids from liver to the developing follicles (Lal & Singh, 1987). Interestingly, alterations in fatty acid profiles of alligator eggs have been associated with reduced clutch success. Specifically, fatty acid profiles from wild, alligator eggs (normal hatch rates) showed considerable differences when compared to those of eggs from captive alligators (reduced hatch rates). One suggested explanation for this association between altered fatty acid profiles and reduced clutch success in captive alligators was that certain fatty acids are critical for reproductive success and that captive diets were deficient in essential fatty acids (Noble et al., 1993). Thus, the possibility exists that exposure to OCPs may alter the liver's ability to synthesize necessary fatty acids, leading to altered egg quality and decreased clutch success in wild alligators that inhabit OCP-contaminated sites. Chronic exposure to low doses of OCPs prior to and during vitellogenesis has been suggested as a cause for significant increases in OCP concentrations in egg yolk, as well as significantly decreased hatch rates in

captive adult female alligators. Importantly, the doses did not appear to induce acute toxicity in the adult females (Rauschenberger et al., 2004). Presently, we are using a captive breeding population of adult alligators, as well as data from field studies, to further evaluate the relationships between OCP exposure, altered fatty acid biosynthesis, nutritional content of eggs, and embryonic mortality.

In summary, the significant levels of OCP analytes observed across such a wide range of crocodilian species and geography suggests the need for a greater understanding of xenobiotic metabolism and toxicological responses in crocodilians. Such understanding would aid in the conservation of this ancient group by determining what risks are posed by contaminants with respect to species survival and how contaminant-related risks compare to other risks, such as habitat destruction. The results of the present study provide some evidence suggesting that maternal transfer of OCP analytes is the major route for embryonic exposure. In addition, it provides several models for the prediction of OCP concentrations in maternal tissues of American alligators, which may be extrapolated to other crocodilians. Hopefully, the present study will encourage new investigations into the pharmacokinetics and pharmacodynamics of contaminants in other crocodilian species.

Table 3-1. Morphological and reproductive characteristics of adult female alligators collected during June 2001 and 2002 from Lakes Apopka, Griffin, and Lochloosa in central Florida.

Parameter [a,b]	Apopka	Griffin	Lochloosa
Number of females collected	4	8	3
Total Length (cm)	252 ± 38	258 ± 17	258 ± 7
Snout-Vent Length (cm)	142 ± 15	134 ± 9	129 ± 5
Mass (kg)	94 ± 30	70 ± 17	63 ± 4
Clutch Mass (kg)	3.78 ± 0.98	3.33 ± 0.82	4.31 ± 0.45
Fecundity (# eggs/clutch)	43 ± 10	40 ± 10	49 ± 6
Lipid % Adipose	47.0 ± 32.5 B	78.1 ± 8.0 A	81.4 ± 4.0 A
Lipid % Liver	1.3 ± 1.0 A	0.8 ± 0.2 A	5.0 ± 2.3 B
Lipid % Muscle	0.8 ± 0.9	1.3 ± 0.9	0.2 ± 0.02
Lipid % Yolk	19.9 ± 1.1	18.1 ± 1.7	18.2 ± 1.6

[a] Values represent mean ± standard deviation. [b] Different letters indicate significant differences ($p < 0.05$).

Table 3-2. Pesticide concentrations (ng/g wet wt.) in tissues and yolks of adult female alligators collected during June 2001 and 2002 from Lakes Apopka, Griffin, and Lochloosa in central Florida.

Lake [a]	Chemical [b, c]	Bile	Blood	Adipose	Liver	Muscle	Yolk
Apopka	Aldrin	X	X	X	X	X	1
(4)	α-BHC	X	X	X	X	X	X
	β-BHC	X	X	7.5 ± 6.7	X	X	2 ± 1.4
	cis-Nonachlor	10 ± 3.2	2 ± 0.4	521 ± 602.7	31 ± 6.7	23 ± 18.3	123 ± 81.9
	cis-Chlordane	4 ± 1.9	1 ± 0.4	190 ± 241.2	11 ± 9.1	14.1 ± 11.7	62 ± 59.2
	δ-BHC	X	X	X	X	X	X
	Dieldrin	38 ± 10.2	5 ± 0.4	2,376 ± 3,770.9	105 ± 80.2	68 ± 48.3	663 ± 803.0
	Endosulfan I	X	X	X	X	X	X
	Endosulfan II	X	X	X	21	X	X
	Endosulfan Sulfate	X	X	X	X	X	X
	Endrin	X	X	X	X	X	X
	Endrin Aldehyde	3 ± 0.4	X	X	X	X	X
	Endrin Ketone	X	X	X	X	X	X
	γ-BHC	X	X	X	X	X	X
	Heptachlor	X	X	X	X	8 ± 11.7	1 ± 0.04
	Heptachlor Epoxide	3 ± 2.1	0.3 ± 0	67 ± 81.5	6 ± 2.2	4 ± 3.6	26 ± 15.0
	Hexachlorobenzene	1 ± 0	1 ± 0	1 ± 0	1 ± 0.0	1	1 ± 0.0
	Kepone	X	X	X	X	X	X
	Methoxychlor	X	X	X	5	X	X
	Mirex	2 ± 2.6	X	19 ± 13.1	7 ± 9.0	1 ± 0.4	7 ± 7.1
	o,p'-DDD	X	X	3 ± 3.4	X	X	X
	o,p'-DDE	X	X	52 ± 55.8	X	X	45 ± 17.7
	o,p'-DDT	2 ± 0.2		27 ± 26.4	4 ± 1.8	4 ± 2.2	17 ± 7.8
	Oxychlordane	7 ± 1.3	1 ± 0.2	247 ± 336.4	17 ± 9.9	12 ± 10.7	75 ± 68.2
	p,p'-DDD	2 ± 0.2	1 ± 0.2	43 ± 67.5	11 ± 10.3	17 ± 8.0	52 ± 61.4
a	*p,p'*-DDE	806 ± 341	42 ± 5.7	29,840 ± 34,366	1,846 ± 918.1	1,392 ± 1,0782	9,994 ± 8,529
	Toxaphene	X	X	13,436 ± 12,670.2	X	X	4,862 ± 4,177
	trans-Nonachlor	21 ± 7.8	3 ± 0.4	1,153 ± 1,378.7	65 ± 22.7	68 ± 57.0	387 ± 277.7
	Total OCP	900 ± 369.7	55 ± 7	44,650 ± 53,230	2,140 ± 1,024	1,610 ± 1,226	15,108 ± 13,704

Table 3-2. (Continued)

Lake	Chemical	Bile	Blood	Adipose	Liver	Muscle	Yolk
Griffin	Aldrin	X	X	X	X	X	X
(8)	α-BHC	X	X	X	X	X	X
	β-BHC	X	X	2.1 ± 1.1	X	X	X
	cis-Nonachlor	4 ± 2.4	1 ± 0.4	75 ± 74.9	8 ± 4.4	9 ± 10.6	14 ± 7.6
	cis-Chlordane	2 ± 0.5	1 ± 0	30 ± 10.8	2 ± 0.7	3 ± 3.4	11 ± 3.7
	δ-BHC	X	X	X	X	X	X
	Dieldrin	13 ± 4.9	7	109 ± 133.4	17 ± 8.0	22 ± 20.8	26 ± 25.7
	Endosulfan I	X	X	X	X	X	X
	Endosulfan II	X	X	X	X	X	X
	Endosulfan Sulfate	X	X	X	X	X	X
	Endrin	X	5	X	X	X	X
	Endrin Aldehyde	X	X	X	X	X	X
	Endrin Ketone	X	2	X	X	X	X
	γ-BHC	X	X	2	X	X	X
	Heptachlor	X	X	X	X	2± 1.4	X
	Heptachlor Epoxide	3 ± 3.3	1	34 ± 45.7	5 ± 3.6	10 12.0	8± 8.8
	Hexachlorobenzene	1± 0	1	1± 0	1± 0	1	1± 0.0
	Kepone	X	X	X	X	X	X
	Methoxychlor	X	X	X	X	X	2
	Mirex	0.3 ± 0	1	5± 3.6	1	1 ± 0.5	1 ± 0.2
	o,p'-DDD	X	X	X	X	X	X
	o,p'-DDE	X	X	X	X	X	3
	o,p'-DDT	1 ± 0.2	1± 0.0	10 6.9	X	2 ± 0.1	3 ± 1.8
	Oxychlordane	7 ± 4.9	X	56 ± 84	8 ± 6.2	16 ± 17.8	12 ± 14.9
	p,p'-DDE	54 ± 25.7	13 ± 9.1	1,030 ± 931.3	75 ± 46.6	131 ± 132.4	273 ± 204.0
	p,p'-DDT	1	13	3 ± 1.5	29 ± 0.9	X	3
	Toxaphene	X	X	X	X	X	X
	trans-Chlordane	1 ± 0.3	1 ± 0	3 ± 1.7	1 ± 0.3	1	2 ± 1.0
	trans-Nonachlor	9 ± 5.9	1 ± 0.09	171 ± 213.5	18 ± 13.2	28 ± 36.0	40 ± 38.3
	Total OCP	87 ± 46.4	31 ± 28.4	1,533 ± 1,439	153 ± 78.1	208 ± 227	393 ± 299

Table 3-2. Continued.

Lake	Chemical	Bile	Blood	Adipose	Liver	Muscle	Yolk
Lochloosa	Aldrin	X	X	X	X	X	X
(3)	α-BHC	NA	X	X	X	X	X
	β-BHC	NA	X	1	X	X	X
	cis-Nonachlor	NA	X	17 ± 1.8	1	X	5 ± 1.9
	cis-Chlordane	NA	X	8 ± 1.4	X	X	3 ± 0.1
	δ-BHC	NA	X	X	X	X	X
	Dieldrin	NA	X	14 ± 4.7	2.6	1.4	4 ± 2.8
	Endosulfan I	NA	X	X	X	15.6	X
	Endosulfan II	NA	X	X	X	X	X
	Endosulfan Sulfate	NA	X	X	X	X	X
	Endrin	NA	X	X	X	X	X
	Endrin Aldehyde	NA	X	X	X	X	X
	Endrin Ketone	NA	X	X	X	X	X
	γ-BHC	NA	X	X	X	X	X
	Heptachlor	NA	X	X	X	18 ± 9.1	X
	Heptachlor Epoxide	NA	X	11 ± 9.6	X	X	3± 2.9
	Hexachlorobenzene	NA	X	X	X	X	X
	Kepone	NA	X	X	X	X	X
	Methoxychlor	NA	X	X	X	X	X
	Mirex	NA	X	2.6	X	X	X
	o,p'-DDD	NA	X	X	X	X	X
	o,p'-DDE	NA	X	X	X	X	X
	o,p'-DDT	NA	X	3 ± 0.2	X	7.1	1 ± 0.0
	Oxychlordane	NA	X	17 ± 11.0	1	X	5 ± 4.3
	p,p'-DDD	NA	X	1 ± 0.1	X	1	2± 0.9
	p,p'-DDE	NA	X	297 ± 90.1	20 ± 20.9	11 ± 6.7	91 ± 32.5
	p,p'-DDT	NA	X	1.4 ± 0.1	X	1.4	X
	Toxaphene	NA	X	X	X	X	X
	trans-Chlordane	NA	X	1 ± 0.1	X	1.4	X
	trans-Nonachlor	NA	X	38 ± 24.6	2.6	1.4	12 ± 8.8
	Total OCP	NA	X	407 ± 143.6	28 ± 32.6	33 ± 33.9	124 ± 53.3

[a] Number of females and clutches collected noted in parentheses beneath name of lake. [b] Values represent mean ± standard deviation

[SD], values without SD indicate a single measurement. X indicates values which were below limit of detection (LOD) or below limit of quantitation (LOQ) and NA indicates not analyzed. LOD ranged from 0.1-1.5 ng/g for most OCP analytes (toxaphene LOD ranged from 120-236 ng/g), and LOQ ranged was 1.5 ng/g for all analytes except for toxaphene (1500 ng/g). Percent recovery ranged from 70-130%. The following chemicals were neither detected in females nor their eggs: α-BHC, δ-BHC, endosulfan sulfate, and kepone.
[c] BHC = Benzene hexachloride; DDD = Dichlorodiphenyldichloroethane; DDE = Dichlorodiphenyldichloroethylene; DDT = Dichlorodiphenyltrichloroethane; Total OCP = ∑ organochlorine pesticide concentrations for all analytes.

Table 3-3. Regression equations for predicting organochlorine pesticide (OCP) concentrations in maternal tissues, where LOG [Tissue-OCP] = bo + b1 LOG [Yolk-OCP].

Tissue	Chemical[a]	b_o	b_1	n	r^2	p
Adipose	Dieldrin	0.6624	0.8785	15	0.87	< 0.0001
	cis-Nonachlor	0.6737	0.9136	15	0.75	< 0.0001
	cis-Chlordane	0.4037	0.9633	15	0.69	0.0001
	Heptachlor Epoxide	0.6294	0.8134	14	0.62	0.0008
	Mirex	0.8217	0.6030	6	0.89	0.0028
	o,p -DDT	0.5840	0.6040	14	0.41	0.0141
	Oxychlordane	0.6694	0.8544	15	0.80	<.0001
	p,p'-DDD	0.2375	0.7 597	14	0.50	0.0046
	p,p'-DDE	0.6968	0.9216	15	0.93	<.0001
	Toxaphene	0.0880	1.0928	3	0.99	0.0486
	trans-Chlordane	0.1733	0.9397	12	0.58	0.0041
	trans-Nonachlor	0.6430	0.8960	15	0.84	< 0.0001
Bile	Dieldrin	-0.6196	0.9559	4	0.90	0.0494
	cis-Nonachlor	-0.3863	0.7646	5	0.97	0.0017
	cis-Chlordane	-0.4308	0.6314	5	0.83	0.0301
	Heptachlor Epoxide	-0.3207	0.6959	5	0.79	0.0435
	p,p'-DDD	-1.1407	1.0748	4	0.95	0.0246
	p,p'-DDE	-0.6385	0.9472	5	0.94	0.0057
	trans-Nonachlor	-0.2919	0.6867	5	0.96	0.0039
	trans-Chlordane	-0.2245	-0.4531	5	0.87	0.0220
Blood	NS[b]					
Liver	Dieldrin	0.0248	0.7162	7	0.98	<0.0001
	cis-Nonachlor	-0.2471	0.8448	8	0.92	0.0002
	cis-Chlordane	-0.5557	0.8876	7	0.97	<0.0001
	Heptachlor Epoxide	-0.3878	0.8323	6	0.85	0.0084
	Mirex	-0.0547	0.9557	5	0.89	0.0155
	Oxychlordane	-0.2855	0.8123	7	0.92	0.0005
	p,p'-DDE	-0.7696	1.0156	10	0.93	<.0001
	trans-Chlordane	-0.0722	0.3300	7	0.94	0.0003
	trans-Nonachlor	-0.2854	0.8263	8	0.98	<.0001
Muscle	*p,p'*-DDE	-0.3733	0.8153	10	0.54	0.0160
	Mirex	0.1816	-0.2797		0.96	0.0040

[a] BHC = Benzene hexachloride; DDD = Dichlorodiphenyldichloroethane; DDE = Dichlorodiphenyldichloroethylene; DDT = Dichlorodiphenyltrichloroethane. [b] NS = no significant linear regressions were determined for the 9 chemicals which were detected both in blood and in yolk.

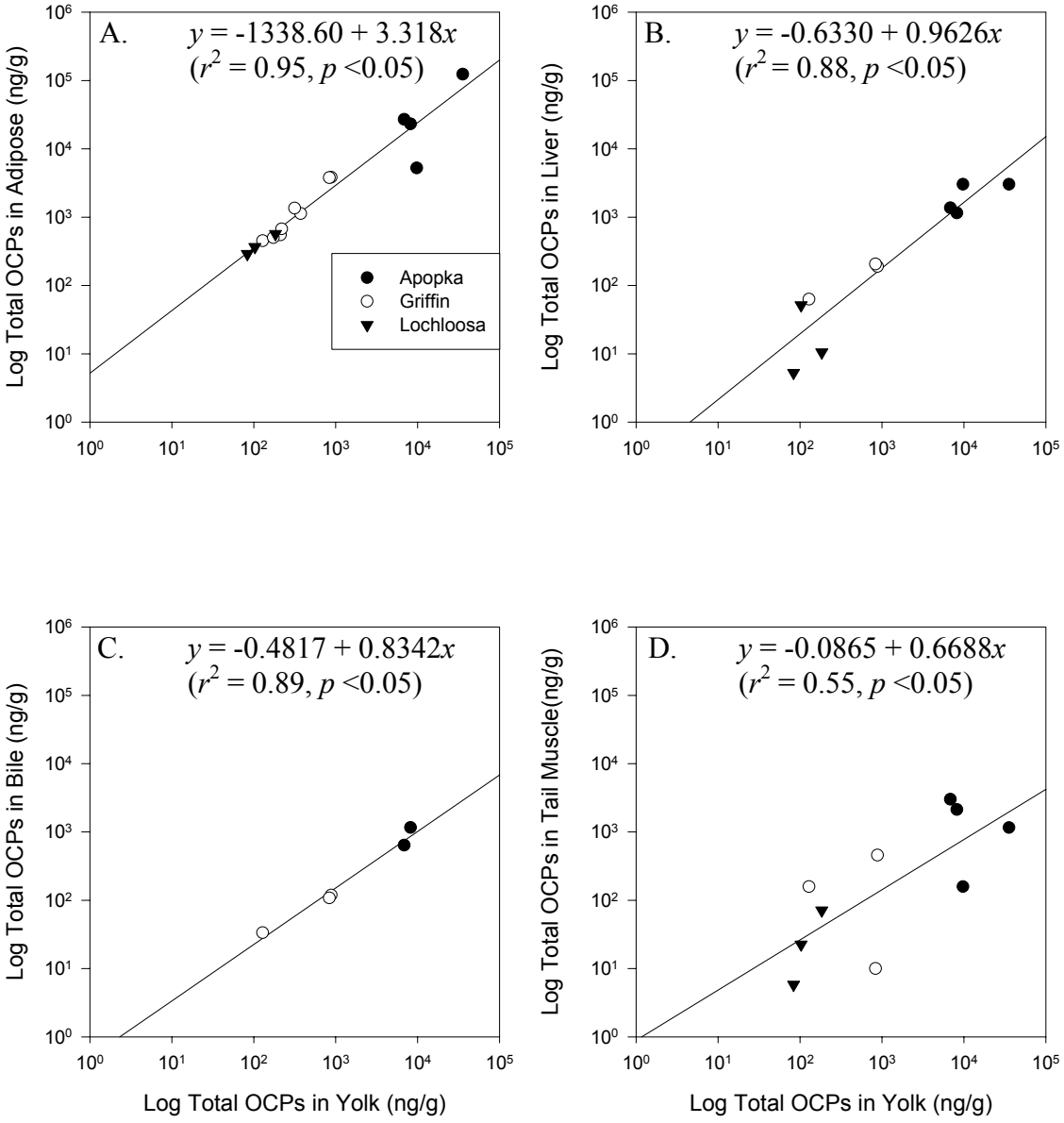

Figure 3-1. Linear regressions of total organochlorine pesticide (OCP) concentrations in maternal tissues against total OCP concentrations in egg yolks. A. Adipose tissue. B. Liver. C. Bile. D. Muscle.

CHAPTER 4
MATERNAL FACTORS ASSOCIATED WITH DEVELOPMENTAL MORTALITY IN THE AMERICAN ALLIGATOR

Recent data suggested maternal organochlorine pesticide (OCP) body burdens and OCP egg yolk concentrations are significantly correlated, and that significant relationships between maternal size and maternal body burdens exist. Maternal age and size has also been shown to have a strong relationship with clutch viability (number of live hatchings / total number of eggs) and clutch size characteristics (i.e., fecundity, clutch mass). Specifically, females between 15 and 30 years old (~ 2.3–2.8 m in total length) produce larger clutches (35-40 eggs / clutch) with increased clutch viability compared to younger females, which themselves produce smaller clutches (15–25 eggs) with smaller eggs and have decreased clutch viability. Females older than 30 years tend to produce clutches similar to 15-30 year old females, with the only exception being smaller clutches (15–25 eggs) (Ferguson, 1985). Therefore, female size or age may be a confounding factor when examining the relationship between OCP burdens in yolk and reproductive performance. In addition, age (or size) and maternal OCP exposure could cause interactive effects. For example, females of optimum reproductive age may be more resistant to effects of OCPs; while, younger (or older) females may show increased susceptibility. Therefore, the objective of the present study was to test the hypotheses that reproductive efficiency, clutch viability, and mortality rates are significantly correlated with maternal OCP body burdens, maternal size, or both; and (2) that clutch size characteristics are significantly correlated with maternal OCP body burdens, maternal size, or both.

Materials and Methods

The greatest difficulty in examining the relationship between maternal age and OCP exposure and effects is that determining the age of an alligator requires either long term monitoring or counting the rings that form in the femur as a result of annual calcium deposition (Ferguson, 1985). However, this technique is not valid for reproductive females since femoral bone resorption provides calcium necessary for eggshell formation and egg yolk nutrition, and subsequently causes the removal of "bone rings" and underestimation of age (Elsey & Wink, 1985; Wink & Elsey, 1986). In addition, removing an alligator's limb simply to age it is ethically unacceptable. Given these difficulties with assigning a chronological age, female size will be used lieu of age. One potential limitation in using female size as an indicator of age class is that female growth rates between lakes may differ since dietary composition has been suggested to differ among OCP-contaminated sites and reference sites (Rice, 2004). Therefore, the possibility exists that a female from a reference site may be smaller than one from a contaminated site, even though both are of the same age. This is important since age, in addition to size, has been shown to be an important determinant of sexual maturity in alligators. Indeed, alligator ranchers are able to accelerate growth so that a female may reach six feet in length in 3-4 years, however, these females do not seem to be able to reproduce until they reach 8-10 years of age (Ferguson, 1985). To control for potential confounding due to differential growth rates, relationships between female size and OCP burdens and clutch viability will be evaluated using site and year as covariates. If the effects of covariates are determined statistically negligible, female data will be grouped together.

Site Descriptions

Lakes Apopka (N 28° 35', W 81° 39'), Griffin (N 28° 53', W 81° 49'), and Lochloosa (N 29° 30', W 82° 09') in Florida were selected as collection sites because prior studies by our laboratory indicate vastly different levels of OCP exposure across these sites. All three lakes are part of the Ocklawaha Basin. Lake Lochloosa (which is connected to Orange Lake) was selected as a low exposure (reference) site. Three years (2000-2002) of data indicate mean total OCP concentrations in egg yolks from the reference sites (Lakes Orange and Lochloosa) were 102 ± 16 ppb (mean ± standard deviation [SD], n = 19 clutches) with a concurrent mean clutch viability rate (number of live hatchlings/total number of eggs in a nest) of 70 ± 4% (Gross, unpublished data). Lake Griffin was selected as an intermediate exposure site since yolk concentrations averaged 1169 ± 423 ppb (n = 42 clutches) and Lake Apopka was selected as a high exposure site since yolk concentrations averaged 7,582 ± 2,008 ppb (n = 23) for the same time period (Chapter 2). Furthermore, mean clutch viability rates during this time period for Lakes Apopka (52 ± 6%, n = 23) and Griffin (43 ± 5%, n = 42) have been below rates observed for the reference site.

Animal Collections

Adult female alligators and their corresponding clutches of eggs were collected from Lakes Apopka (*n* = 19), Griffin (*n* = 18), and Lochloosa (*n* = 3) over the course of four nesting seasons (June 1999 to June 2002). Nests were located by aerial survey (helicopter) and/or from the ground (airboat). Once nests were located, all eggs were collected, and the nest cavity was covered. A snare-trap was set perpendicular to the tail-drag in order to capture the female as she crossed over the nest. After the traps were set, one member of the trapping crew subsequently transported the eggs to the Florida Fish

and Wildlife Conservation Commission's Wildlife Research Unit (FWC; Gainesville, FL, USA) and placed the eggs in a temperature-controlled incubator. Snare-traps were checked later in the evening and early the next morning.

In 1999 and 2000, trapped females were secured and measurements (total length, snout-vent length, head length and tail girth) were collected along with a blood sample and a scute for OCP analysis. These females were then immediately released. In 2001 and 2002, females were captured and transported from each lake to the United States Geological Survey's Florida Integrated Science Center (USGS; Gainesville, FL, USA). Upon arrival, the animals were weighed, measured, and blood samples were collected from the post-occipital sinus. Adult alligators were then euthanized by cervical dislocation followed by double pithing. A full necropsy was performed on each female. Bile, liver, adipose (composite of abdominal fat and the abdominal fat pad), and tail muscle samples were collected for later determination of OCP burdens. Liver, adipose tissue, and muscle were wrapped in aluminum foil, while bile and blood were placed in scintillation vials. All samples were grouped according to nest identification number (ID), placed in plastic bags labeled with the appropriate ID, and stored in a –80 ^{o}C freezer. Each female's corresponding clutch of eggs was then transferred from FWC to USGS where yolk samples were collected (two eggs/clutch) and stored with the corresponding maternal tissues. The remaining eggs were set for incubation in a temperature/humidity-controlled incubator (31-33 ^{o}C, 88-92% relative humidity) located at USGS.

Analysis of OCPs in Maternal Tissues and Yolk

Analytical grade standards for the following compounds were purchased from the sources indicated: aldrin, alpha-benzene hexachloride (α-BHC), β-BHC, lindane, δ-BHC,

p,p'-dichlorodiphenyldichloroethane (*p,p'*-DDD), *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), dichlorodiphenyltrichloroethane (*p,p'*-DDT), dieldrin, endosulfan, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor epoxide, hexachlorobenzene, kepone, methoxychlor, mirex, *cis*-nonachlor, and *trans*-nonachlor from Ultra Scientific (Kingstown, RI, USA); *cis*-chlordane, *trans*-chlordane, and the 525, 525.1 polychlorinated biphenyl (PCB) Mix from Supelco (Bellefonte, PA, USA); oxychlordane from Chem Service (West Chester, PA); *o,p'*-DDD, *o,p'*-DDE, *o,p'*-DDT from Accustandard (New Haven, CT, USA); and toxaphene from Restek (Bellefonte, PA, USA). All reagents were analytical grade unless otherwise indicated. Water was doubly distilled and deionized.

Adipose, liver, bile, and yolk samples were analyzed for OCP content using methods modified from Holstege et al. (1994) and Schenck et al. (1994). For extraction, a 2 g tissue sample was homogenized with ~1 g of sodium sulfate and 8 mL of ethyl acetate. The supernatant was decanted and filtered though a Büchner funnel lined with Whatman #4 filter paper (Fisher Scientific, Hampton, NH, USA) and filled to a depth of 1.25 cm with sodium sulfate. The homogenate was extracted twice with the filtrates collected together. The combined filtrate was concentrated to ~2 mL by rotary evaporation, and then further concentrated until solvent-free under a stream of dry nitrogen. The residue was reconstituted in 2 mL of acetonitrile. After vortexing (30 s), the supernatant was applied to a C18 solid phase extraction (SPE) cartridge (pre-conditioned with 3 mL of acetonitrile; Agilent Technologies, Wilmington, DE, USA) and was allowed to pass under gravity. This procedure was repeated twice with the combined eluent collected in a culture tube. After the last addition, the cartridge was rinsed with 1

mL of acetonitrile which was also collected. The eluent was then applied to a 0.5 g NH2 SPE cartridge (Varian, Harbor City, CA, USA), was allowed to pass under gravity, and collected in a graduated conical tube. The cartridge was rinsed with an additional 1 mL portion of acetonitrile which was also collected. The combined eluents were concentrated under a stream of dry nitrogen, to a volume of 300 μL, and transferred to a gas chromotography (GC) vial for analysis.

Whole blood was analyzed for OCP content using methods modified from Guillette et al. (1999). A 10 mL aliquot was transferred from the homogenized bulk sample and extracted in 15 mL of acetone by vortex mixer. The mixture was centrifuged for 5 min at 3000 rpm, after which the supernatant was transferred to a clean culture tube. This process was repeated with the supernatants collected and concentrated under a stream of dry nitrogen until solvent-free. The residue was re-extracted in 11.5 mL of 1:1 methylene chloride-petroleum ether. After mixing, the sample was allowed to settle and the upper layer was transferred to a clean culture tube. This extraction was performed twice with the extracts collected together. The combined extracts were then applied to a prepared florisil cartridge (5 mL Fisher PrepSep, Fisher Scientific, Hampton, NH, USA). The cartridge had been prepared by filling the reservoir to a depth of 1.25 cm with anhydrous sodium sulfate and by prewashing the modified cartridge with 10 mL of 2:1:1 acetone: methylene chloride: petroleum ether. After the sample passed under gravity with the eluent collected in a 15-mL graduated conical tube, the cartridge was eluted with 4 mL of the 2:1:1 solvent mixture which was also collected. The combined eluents were concentrated under a stream of dry nitrogen, to a volume of 300 μL, and transferred to a GC vial for analysis.

GC/MS Analysis

Analysis of all samples was performed using a Hewlett Packard HP-6890 gas chromatograph (Wilmington, DE, USA) with a split/splitless inlet operated in splitless mode. The analytes were introduced in a 1 µL injection and separated across the HP-5MS column (30 m x 0.25 mm; 0.25 µm film thickness; J & W Scientific, Folsom, CA, USA) under a temperature program that began at 60º C, increased at 10º C/min to 270º C, was held for 5 min, then increased at 25º C/min to 300º C and was held for 5 min. Detection utilized an HP 5973 mass spectrometer in electron impact mode. Identification for all analytes and quantitation for toxaphene was conducted in full scan mode, where all ions are monitored. To improve sensitivity, selected ion monitoring was used for the quantitation for all other analytes, except kepone. The above program was used as a screening tool for kepone which does not optimally extract with most organochlorines. Samples found to contain kepone would be reextracted and analyzed specifically for this compound.

For quantitation, a five-point standard curve was prepared for each analyte ($r^2 \geq$ 0.995). Fresh curves were analyzed with each set of twenty samples. Each standard and sample was fortified to contain a deuterated internal standard, 5 µL of US-108 (120 µg/mL; Ultra Scientific), added just prior to analysis. All samples also contained a surrogate, 2 µg/mL of tetrachloroxylene (Ultra Scientific) added after homogenization. Duplicate quality control samples were prepared and analyzed with every twenty samples (typically at a level of 1.00 or 2.50 µg/mL of γ-BHC, heptachlor, aldrin, dieldrin, endrin, and *p,p'*-DDT) with an acceptable recovery ranging from 70 – 130%. Limit of detection (LOD) ranged from 0.1-1.5 ng/g for all OCP analytes, except toxaphene (120-236 ng/g), and limit of quantitation (LOQ) was 1.5 ng/g for all analytes, except toxaphene (1500

ng/g). Repeated analyses were conducted as allowed by matrix interferences and sample availability.

Data Analysis

Specific OCP analytes were removed from analysis if measurable concentrations were not found in at least 5% of the clutches. Numerical data were log-transformed [ln(x)], while proportional data were arcsine square root transformed to conform to statistical assumptions. Maternal OCP burdens for females collected during 1999 and 2000 were estimated using the females' respective yolk burdens and predictive models described in Chapter 3.

ANOVA (PROC GLM; SAS Institute Inc., 2002) was used for inter-site comparisons of adult female and clutch characteristics, and the Tukey test was used for multiple comparisons among sites ($\alpha = 0.05$ since no interactions were tested). Because relationships between response variables and explanatory variables (Table 4-1) in ecological studies are often complex with interactions occurring, an indirect gradient multivariate analysis method, Detrended Correspondence Analysis (DCA) (ter Braak, 1986) was used to initially evaluate data structure. Two matrices were constructed for DCA, with the first representing the response variables (female-clutch pair number x clutch parameters) and the second representing the explanatory variables (female-clutch pair number x maternal size and OCP burdens) (Table 2). DCA results indicated that a direct gradient, multivariate linear analysis, Redundancy Analysis (RDA) (Rao, 1964), was appropriate since the lengths of the DCA ordination axes were equal to or less than 2 standard deviations (ter Braak, 1995). For the RDA, similar matrices were constructed with the exception that response variables measured as a percentage (i.e., clutch viability) and response variables measured as a number (i.e., clutch mass) were split into separate

matrices because percentage data were ln(x+1) transformed and not standardized, while continuous data were ln(x) transformed and standardized (ter Braak & Smilauer, 2002). Automatic forward selection of the best four explanatory variables was conducted for both sets of RDA analyses and tested for significance by Monte Carlo permutation test DCA and RDA were conducted using the program CANOCO (ter Braak & Smilauer, 2002). Biplots of environmental variables and response variables were then constructed to facilitate interpretation.

Results

A total of 40 female alligators and their respective clutches (female-clutch pairs) were collected during the summers of 1999-2002 from Lake Apopka (n = 19), Lake Griffin (n = 18), and Lake Lochloosa (n = 3). No significant differences between lakes were determined with respect to fecundity, average egg mass, clutch viability, percentage of unbanded eggs, percentage of early embryonic mortality, percentage of late embryonic mortality, female head length, female snout-vent length, female tail girth, female total length, and female body condition index (Table 4-3). Significant differences were detected with respect to total OCP concentration in female adipose tissue with Lake Apopka female burdens (22,737 ± 5,767.6 ng/g) being greater than those of Lake Griffin (1,821 ± 702.7 ng/g), and Lake Lochloosa female burdens (375 ± 63.1 ng/g). No significant differences were determined between adipose burdens of Lake Griffin and Lake Lochloosa females (Table 4-3).

Results of the forward selection RDA evaluating reproductive efficiency (clutch viability, percentage unbanded eggs, early embryo mortality and late embryo mortality) indicated that the four explanatory variables that best accounted for the variance of clutch survival parameters were interaction variable: % *trans*-chlordane (TC%) (lambda A =

12%), percentage *p,p'*-DDE (DDE%) (lambda A = 6%), heptachlor epoxide ([HE]) (lambda A = 4%), and percentage oxychlordane (λ_A = 4%). These factors accounted for 28% of the total variation of reproductive efficiency. Two of the four variables, TC% and PDDE% were determined significant and together accounted for 18% of total variation (Table 4-4).

Biplots of the extracted factors and reproductive variables (Fig. 4-1) suggested that clutch viability had strong negative correlations with TC% (*r* = -0.5451) and %OX-[OX] (*r* = -0.2885), but was weakly correlated with PDDE% (*r* = 0.0548). Unbanded egg percentages, however, were positively strongly correlated with DDE% (*r* = 0.4012), weakly correlated with %OX-[OX] (*r* = -0.1298) and TC%-[TC] (*r* = 0.0228). Early embryonic mortality percentages were weakly correlated with TC%-[TC] (*r* = 0.1860), %OX-[OX] (*r* = 0.1556), and PDDE% (*r* = 0.133). Late embryo mortality percentages were positively correlated with TC%-[TC] (*r* = 0.3361) and %OX-[OX] (*r* = 0.3144), but showed negative correlations with DDE% (*r* = -0.3230).

For clutch size characteristics (fecundity, clutch mass, and average egg mass), RDA results indicated that the four explanatory variables that best explained clutch size variance were concentration of cis-chlordane ([CC]) (LambdaA = 6%), (LambdaA = 6%), percentage dieldrin (DL%) (LambdaA = 6%), concentration of *p,p*'-DDD ([PDDD]) (LambdaA = 4%), and concentration of toxaphene ([TOX]) (LambdaA = 4%). None of these variables were determined to be significantly associated with clutch size parameters (Table 4-5).

Discussion

With respect to the first hypothesis, results of the present study suggest that certain OCPs in maternal adipose tissue were significantly associated with decreased clutch

survival parameters (clutch viability, percentage, unbanded eggs, early and late embryo mortality), but that clutch survival parameters were neither significantly correlated with maternal morphometrics. Although maternal burdens of certain OCPs were correlated with clutch survival parameters, extracted variables only explained 18% of the variation. However, it is important to note that compositional percentage of an OCP analyte appears to be an important factor with respect to clutch survival parameters. Indeed both OCP variables found to be significantly associated with clutch survival parameters were compositional variables (TC% and pDE%) (Table 4-4). This suggests that the composition of the OCP mixture may be more important in altering clutch viability than the total OCP burden or total number of OCPs detected in maternal tissues. Furthermore, biplots (Fig. 4-1) suggest that the rates of unbanded eggs, early embryo mortality, and late embryo mortality, which all contribute to reproductive efficiency, have different relationships with the each of the extracted OCP variables, suggesting that certain mixtures differentially affect certain aspects of reproductive function. Two reasons for the importance of mixture composition are that the effects and the toxicity (potency) of OCPs vary considerably from analyte to analyte. For example, Japanese quail orally dosed with technical grade DDE (300 ppm) had decreased rates of fertility and increased rates of mortality; however, similar exposures to technical grade DDT (300 ppm) did not cause adverse effects (Robson et al., 1976). Decreased fertility in quail exposed to DDE is consistent with the results of the present study in that as DDE% increased in female alligator adipose tissue, the percentage of unbanded eggs also increased.

Although total OCP burdens in maternal adipose tissue were significantly higher in females from Lake Apopka, no significant differences were detected between lakes with

respect to clutch viability, percentages of unbanded eggs, early embryo mortality, or late embryo mortality,. The significant differences in total OCP burdens among sites and lack thereof for clutch parameters, again suggests that total OCP burdens may be less important than mixture composition.

With respect to the second hypothesis, OCP burdens and maternal morphometrics were not found to be associated with clutch size characteristics. This may be due to similar sized females and clutches being collected with in and among sites (Table 4-3).

The correlations between certain OCPs and clutch survival parameters suggest decreased reproductive efficiency may be related to increased maternal OCP burdens, however, correlations alone do not establish causal relationships. Indeed, several viewpoints must be considered before causality is concluded, however, the only viewpoint, or "criterion", that can rule out a cause-effect relationship is temporality (i.e., exposure must precede effect) (Hill, 1965). The major criteria used in the current practice of causal inference is temporality, biological plausibility, consistency of the association, strength of the association, and biological gradient, (Weed et al., 2002; Gadbury & Schreuder, 2003).

Since females were exposed to OCPs prior to vitellogenesis and oviposition, temporality is satisfied. The second criterion, biological plausibility, is also satisfied since studies in other oviparous vertebrates have shown that OCP exposure can cause adverse reproductive effects through a variety of mechanisms (Fry, 1995)

The third criterion is consistency of association, which means that similar results have been found among other studies examining the same problem. Few studies have examined reproductive effects of maternal OCP exposure in alligators with one of these

studies reporting no significant correlations between DDE concentrations in maternal tissues and clutch anomalies for Lake Apopka alligators (Giroux, 1998). The earlier study's focus was on a single analyte while this study looked a several analytes concurrently, so the two studies differ somewhat. Given relatively small number of studies and the ambiguous interpretations, no clear conclusion can be reached as to whether the consistency criterion has been satisfied.

The fourth and fifth criteria, strength of association and biological gradient, are somewhat similar in nature. Strength of association refers to how strongly correlated the causal factor is to the response variable, and the biological gradient refers to whether the response variable increases as the dose increases. With respect to strength of association, the present study's results indicate weak-moderate associations between maternal OCP burdens and clutch survival parameters (18% of variance explained). With respect to dose-response, biplots indicated that biological gradients existed between certain maternal factors and reproductive responses in that as percentage p,p'-DDE and percentage trans-chlordane increased, incidence of unbanded eggs increased and clutch viability decreased, respectively (Fig. 4-1).

In summary, rarely does a single observational study establish clear causal relationships, and the present study is no exception. However, the present study does satisfy some of the criteria used for establishing causality. Importantly, results suggest that a moderate part of the variation associated with reproductive function in the American alligator can be attributed to maternal OCP body burdens. Hopefully, the results of the present study will stimulate future efforts aimed at increasing our understanding of the effects of environmental contaminants of crocodilians.

Table 4-1. Reproductive, morphometric, and contaminant parameters measured on adult female alligators collected during June 1999, 2000, 2001, and 2002.

Female Parameter	Definition	Measured as
Response variables		
Fecundity	Total No. of eggs in one clutch	*n*
Clutch mass	Total mass of eggs in one clutch	kg
Ave. Egg Weight	Clutch mass / Fecundity	g
% Unbanded eggs[a]	No. of unbanded eggs / fecundity x 100	Percentage
% Early embryo mortality	No. of deaths < dev. Day 35 / fecundity x 100	Percentage
% Late embryo mortality	No. of deaths ≥ dev. Day 35 / fecundity x 100	Percentage
Clutch Viability	No. eggs yielding live hatchling / fecundity x 100	Percentage
Explanatory variables		
Head Length	Tip of snout to posterior base of skull (dorsal)	cm
Snout-Vent Length	Tip of snout to posterior base of vent (dorsal)	cm
Tail Girth (cm)	Circumference of tail at vent	cm
Total Length (cm)	Tip of snout to tip of tail (dorsal)	cm
Body condition index	Snout-vent length / Tail girth x 100	Percentage
[OCP analyte] in adipose tissue[b]	ng OCP analyte / g adipose tissue wet weight	ppb
% OCP analyte	[OCP analyte] / ∑ [OCP] x 100	Percentage

[a]An egg with no evidence of embryonic attachment

[b]See text for list of measured OCP analytes. For 1999 and 2000 females, adipose OCP concentrations were estimated using predictive equations (see Chapter 3).

Table 4-2. Explanatory variables included in RDA with forward selection of four best variables ($\alpha = 0.05$).

Variable	Code
Head Length	HL
Snout-vent length	SVL
Total Length	TL
Tail Girth	TG
Body Index	BI
Age	Age
No. OCPs quantitated	NOC
∑ [OCP]	TOC
% *cis*-Chlordane	CC%
[*cis*-Chlordane]	[CC]
% *cis*-Nonachlor	CN%
[*cis*-Nonachlor]	[CN]
% Dieldrin	DL%
[Dieldrin]	[DL]
% Heptochlor epoxide	HE%
[Heptachlor epoxide]	[HE]
% Mirex	MX%
[Mirex]	[MX]
% *o,p*-DDT	ODDT%
[*o,p*-DDT]	[ODDT]
% Oxychlordane	OX%
[Oxychlordane]	[OX]
% *p,p*'-DDE	PDDE%
[*p,p*'-DDE]	PDDE]
% *p,p*'-DDD	PDDD%
[*p,p*'-DDD]	[PDDD]
% *p,p*'-DDT	PDDT%
[*p,p*'-DDT]	[PDDT]
% *trans*-Chlordane	TC%
trans-Chlordane	[TC]
% *trans*-Nonachlor	TN%
[*trans*-Nonachlor]	[TN]
% Toxaphene	TX%
[Toxaphene]	[TX]

Table 4-3. Reproductive, morphometric, and contaminant summary statistics[a] of adult female alligators collected during June of 1999-2002.

Parameter	Apopka	Griffin	Lochloosa	Summary
Female-clutch pairs (n)	19	18	3	40
Fecundity (n)	45 ± 2 (22–54)	44 ± 1.9 (19–56)	49 ± 3.5 (45–56)	44 ± 1.3 (19–56)
Clutch mass (kg)	4.2 ± 0.14 (2.4–5.1)	3.6 ± 0.18 (1.8–5.2)	4.3 ± 0.26 (4–4.8)	3.9 ± 0.11 (1.8–5.2)
Egg Mass (g)	89 ± 1.2 (77.6–100)	84 ± 2.8 (70.8–112.6)	88 ± 1.1 (86.1–90)	87 ± 1.4 (70.8–112.6)
Clutch 'viability (%)[b]	61 ± 6.8 (0–98)	42 ± 8.5 (0–92)	60 ± 8.3 (48–76)	52 ± 5.2 (0–98)
Unbanded eggs (%)[c]	19 ± 6.9 (0–100)	21 ± 5.3 (0–70)	9 ± 5.5 (3–20)	19 ± 4 (0–100)
Early embryo mortality (%)[d]	12 ± 3 (0–45)	14 ± 3.4 (0–52)	25 ± 3.2 (20–31)	14 ± 2.1 (0–52)
Late embryo mortality (%)[e]	7 ± 2 (0–25)	22 ± 6.8 (0–89)	6 ± 3 (0–10)	14 ± 3.4 (0–89)
Head Length (cm)	37 ± 1.3 (22–52)	36 ± 0.7 (28–41)	35 ± 0.4 (35–36)	36 ± 0.7 (22–52)
Snout-Vent Length (cm)	140 ± 3.6 (83–156)	135 ± 1.9 (120–148)	129 ± 2.8 (125–134)	137 ± 2 (83–156)
Tail Girth (cm)	68 ± 2.8 (36–92)	66 ± 1.7 (52–77)	62 ± 2 (59–66)	67 ± 1.5 (36–92)
Total Length (cm)	263 ± 7.9 (161–304)	260 ± 4.5 (220–298)	258 ± 4.2 (253–266)	262 ± 4.2 (161–304)
Body index[f]	2.09 ± 0.059 (1.46–2.7)	2.05 ± 0.04 (1.79–2.38)	2.07 ± 0.023 (2.03–2.11)	2.07 ± 0.033 (1.46–2.7)
TotalOCPs (ng/g)[g]	22,734 ± 5767.6 A (5,224.7–123,081.5)	1,821 ± 702.7 B (355.6–12,938.7)	375 ± 63.1 B (289.8–498.1)	11,648 ± 3,200.9 (289.8–123,081.5)

[a]Values represent mean ± standard error with range in parentheses. Significant differences ($\alpha = 0.05$) between sites indicated by letters (A-B) beside mean. Same letters = not significant
[b] % of eggs in a clutch that yield a live hatchling.
[c] % of eggs with no evidence of embryonic attachment
[d] % of embryos in a clutch that perish during first half (35 days) of development.
[e] % of embryos in a clutch that perish during last half (35 days) of development.
[f] snout-vent length / tail girth
[g] ∑[ng OCP analyte / g adipose tissue (wet weight)]

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Table 4-4. Results of redundancy analysis with automatic selection of four best maternal factors associated with variation in reproductive efficiency.

Variable	LambdaA[a]	*P*	*F*
TC%*	0.12	0.004	5.18
pDE%*	0.06	0.04	2.78
[HE]	0.04	0.202	1.65
Lochloosa	0.04	0.146	1.80

[a]Proportion of total variance explained by each variable (total variance = 1.0). *$P < 0.05$

Table 4-5. Results of redundancy analysis with automatic selection of four best maternal factors associated with variation in clutch size characteristics.

Variable	LambdaA	*P*	*F*
[CC]	0.06	0.174	2.21
[pDD]	0.04	0.190	1.94
[TOX]	0.04	0.162	1.49
[DL]	0.06	0.134	2.54

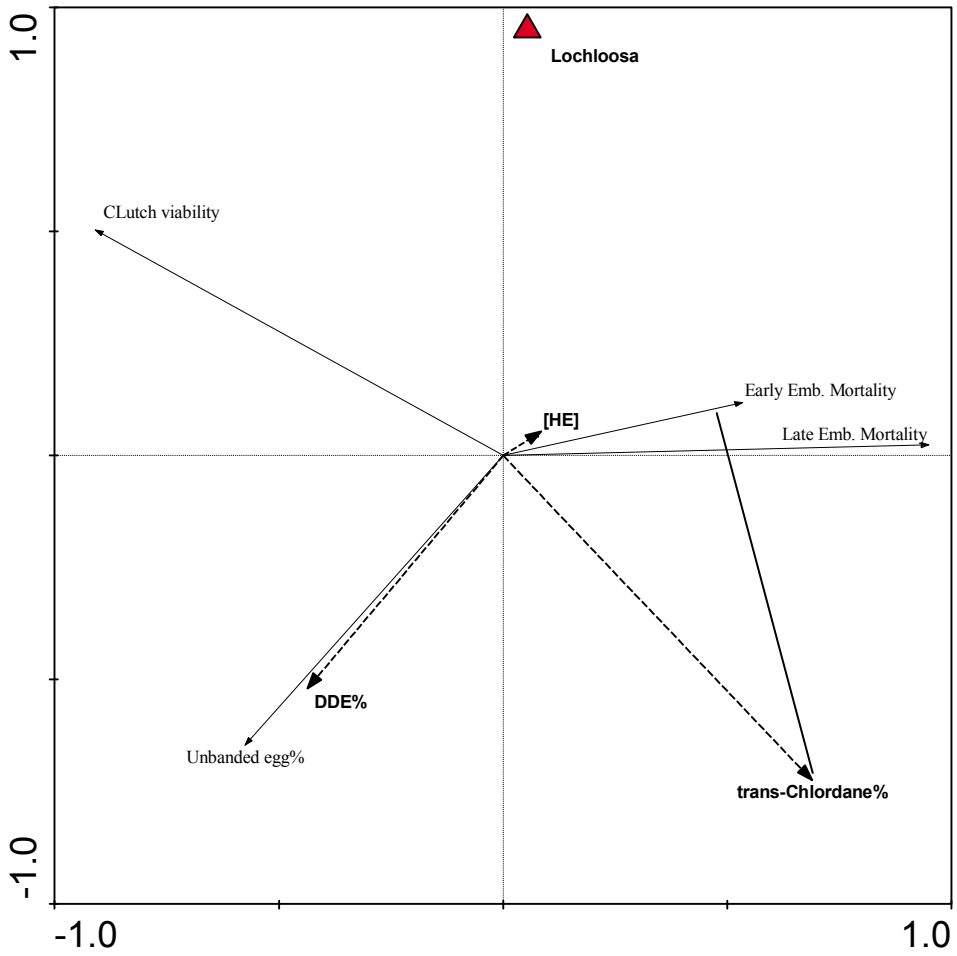

Figure 4-1. Biplot of maternal factors (dashed lines) and clutch survival parameters (solid lines) of American alligators collected during June 1999-2002. Arrows pointing in the same direction indicate a positive correlation (e.g., unbanded egg% and DDE%), arrows that are approximately perpendicular indicate near-zero correlation (e.g., clutch viability and DDE%), and arrows pointing in opposite directions indicate negative correlations (clutch viability and trans-chlordane%]. Arrow lengths indicate rank order of correlations. For example, extending a perpendicular line from the early emb. mort. axis to tip of trans-chlordane% arrow indicates that early emb. mort. and trans-chlordane have a stronger positive correlation compared to early emb. mort. correlation and [HE]. The cosine of the angle formed at the origin between individual clutch variables and individual OCP variables is the correlation coefficient (r). For example, arrows pointing in exactly opposite directions have an angle of 180°, and since cos(180) = -1.0, the arrows are perfectly negatively correlated (r) (ter Braak, 1995).

CHAPTER 5
MORPHOLOGY AND HISTOPATHOLOGY OF AMERICAN ALLIGATOR (*ALLIGATOR MISSISSIPPIENSIS*) EMBRYOS FROM REFERENCE AND OCP-CONTAMINATED HABITATS

In central Florida, American alligators living in habitats contaminated with organochlorine pesticides (OCPs) have poor reproductive success in comparison to populations inhabiting reference sites (Woodward et al., 1993) (Wiebe et al., 2001). Decreased reproductive efficiency has been largely attributed to increased rates of early embryo mortality (mortality occurring first 35 days of development) and, to a lesser extent, late embryo mortality (mortality after day 35), as well as increased incidence of unbanded eggs, with unbanded eggs likely being a product of infertility, or pre-ovipositional embryo mortality, or a combination of both (Masson, 1995; Wiebe et al., 2001; Rotstein et al., 2002).

A clear dose-response relationship between embryo mortality and total OCP burdens in eggs has not been established (Heinz et al., 1991), and recent studies, on Lake Apopka, suggested poor egg viability was more closely associated with muck farm reclamation (wetland restoration) sites than with tissue and egg concentrations of the predominant pesticide residue (DDE) (Giroux, 1998). Muck farming typically refers to a farming practice where a dike is built around a marshy area adjacent to a lake, then the water is pumped out of the marsh, and the fertile peat (i.e., "muck") is then used for crop production. In addition, altered endocrine function and decreased egg viability were documented among alligators at another site, Lake Griffin, where tissue and egg concentrations of OCP residues such as DDE are intermediate in comparison with Lake

Apopka, but Lake Griffin is also highly eutrophic and has adjacent muck farms and muck farm reclamation areas.

Although a clear dose-response relationship has not been established with respect to embryo mortality and total organochlorine pesticide burdens, great differences exist, nonetheless, between sites with respect to OCP egg burdens and OCP constituent composition, suggesting mixture composition may play a role or even be more important than simple cumulative OCP burdens.

OCPs are of concern because they are prevalent and persistent environmental contaminants that are lipid soluble, resistant to metabolic degradation, bioaccumulate in animal tissues, and may cause altered function of the immune system, as well as neural toxicity (Blus, 1996). Furthermore, *in vitro* and *in vivo* experiments using laboratory organisms, as well as epidemiology studies involving OCP-exposed human and wildlife populations, suggest a variety of OCPs and OCP metabolites, such as dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyltrichloroethylene (DDE), methoxychlor, dicofol, chlordane, dieldrin, and toxaphene, may be associated with disrupted endocrine function and altered embryo development (Colborn et al., 1993; Gray et al.; 1997; Fairbrother et al., 1999; Longnecker et al., 2002; Rattner & Heath, 2003).

Although increased early embryonic mortality and late embryo mortality have been documented, few histopathology studies have examined live, moribund, or dead alligator embryos to determine whether alterations in morphology and/or specific pathogenicities are associated with increased mortality rates or specific OCPs and OCP burdens in eggs. Such histopathology studies are arduous due to rapidity of tissue autolysis, confounded by the difficulty in determining whether an embryo is alive or dead. Determining

whether an embryo is alive or dead, when it is still inside the egg, is difficult because bright light candling is currently the only practical method for determining embryo viability in large studies involving thousands of eggs. Using bright light candling, live embryos are differentiated from dead embryos based on the color of the illuminated egg, with bright red indicating a live embryo (or very recently dead) and orange-pink indicating a dead embryo (color changes may be related to breakdown of red bloods cells and general autolysis of egg and embryo membranes).

Although difficult, examining morphological development and histopathology of live and dead embryos would aid in understanding the causes and mechanisms associated with the embryo mortality. For example, histopathology may indicate occurrence of acute chemical toxicity since it is known that many types of pesticides, including OCP compounds, induce toxicopathic lesions in vital organs, with liver being the predominant target organ (Metcalfe, 1998). Furthermore, evaluating how changes in morphology and histopathology relate to clutch mortality rates and OCP egg burdens may provide insight as to whether OCPs play a role in the increased incidence of embryo mortality observed in OCP-contaminated lakes.

Therefore, the objective of the present study was to evaluate embryo morphology as a function of embryo condition (live/dead), lake of origination, clutch quality, and OCP egg burden, and to evaluate the histopathology of embryos from clutches with diverse OCP egg burdens and mortality rates. To accomplish this objective the following hypotheses will be tested. First, morphological development of live alligator embryos is different from dead embryos of the same chronological age. Second, morphological development of live embryos of the same chronological age is different among reference

and OCP-contaminated sites. Third, morphology of live embryos from clutches with low mortality rates is different from those of clutches with high mortality rates, and fourth, variation in morphological development of live embryos is associated with composition and/or concentration of OCPs in eggs. Lastly, histopathology of live embryos from clutches with low mortality rates and low OCP egg burdens is different from those of clutches with high mortality rates and high OCP egg burdens.

Materials and Methods

Site Descriptions

Lakes Apopka (N 28° 35', W 81° 39'), Griffin (N 28° 53', W 81° 46'), Emeralda Marsh Conservation Area ((N 28° 55', W 81° 47'), and Lochloosa (N 29° 30', W 82° 09') in Florida were selected as collection sites because prior studies by our laboratory indicate vastly different levels of OCP exposure across these sites. All three lakes are part of the Ocklawaha Basin. Lake Lochloosa (which is connected to Orange Lake) was selected as a low exposure (reference) site. Four years (2000-2002) of data indicate mean total OCP concentrations in egg yolks from the reference sites (Lake Lochloosa) were 102 ± 15 ppb (mean ± standard error [SE], n = 19 clutches) with a concurrent mean clutch viability rate (number of live hatchlings/total number of eggs in a nest) of 70 ± 4% . Lake Griffin was selected as an intermediate exposure site since yolk concentrations averaged 1,169 ± 423 ppb (n = 42 clutches) and Lake Apopka was selected as a high exposure site since yolk concentrations averaged 7,582 ± 2,008 ppb (n = 23) for the same time period (Gross, unpublished data). Furthermore, mean clutch viability rates during this time period for Lakes Apopka (51 ± 6%, n = 23) and Griffin (44 ± 5%, n = 42) have been below rates observed for the reference site.

Egg Collections

In the field, clutches were located via aerial surveys (helicopter) and ground surveys (airboat). Each clutch was provided with a unique identification number, and immediately transported in plastic pans (43 cm x 33 cm x 18 cm) containing the original nest substrate material to the US Geological Survey's Center for Aquatic Resources Studies, Gainesville, Florida (CARS). Upon arrival, complete clutches were evaluated for embryonic viability using a bright light candling procedure. Viable eggs (i.e. having a visible band) were nested in pans containing moist sphagnum moss and incubated at 30.5ºC and ~98% humidity, in an incubation building (7.3 m x 3.7 m). This intermediate incubation temperature will normally result in a 1:1 male/female sex ratio. One or two eggs were sacrificed from each clutch to identify the embryonic stage of development at the time of collection, and to evaluate the concentrations of OCPs in yolk. From each clutch, information on the following parameters was collected: total number of eggs found per nest (fecundity); number of unbanded eggs, number of damaged eggs, number of dead banded eggs, number of live banded eggs, total clutch mass and average egg mass of clutch. Then, each clutch was evenly divided between two pans, with half of the clutch left relatively undisturbed (except for weekly monitoring of embryo viability) to determine clutch viability (the number of live hatchlings / fecundity), and the other half of the clutch used to study embryo development and morphometry.

Embryo Sampling and Measurement

After initial determination of morphological ages (MA) for all clutches (Ferguson, 1985), 2-4 live embryos were collected from each clutch at each of four selected chronological ages. Morphological age (MA) refers to the age of the embryo as determined by level of morphological development and chronological age (CA) refers to

the calendar age when an embryo was sampled. For example, two clutches are initially examined and it is determined that embryos of clutch A are of MA Day 12 and those of clutch B are of MA Day 10. With respect to the initial age determination, it is assumed that MA = CA. Furthermore, if embryos of both clutches are to be sampled at CA age-14, then clutch A would be sampled two days after initial examination and Clutch B would be sampled 4 days after initial examination. MA can then be determined (based on morphological features), and can be compared to CA to see if actual morphological development (MA) differs from what would be expected, given the particular CA. The four chronological ages sampled were Day 14, Day 25, Day 33, Day 43. These ages were selected because each are clearly distinguishable from other ages, and provide a good representation of progression of organogenesis and growth (Ferguson, 1985). These ages also correspond to periods of increased embryo mortality, as determined by previous studies (Masson, 1995).

The following parameters were measured on fresh embryos (live and dead): egg mass; embryo condition (live or dead), embryo mass, embryo morphological age, eye length, head length, and total length of embryo. Dead embryos were differentiated from live embryos based on the lack of a visible cardiac contractions and signs of autolysis, such as atypical coloration and loss of tissue integrity. Other parameters were derived in an attempt to determine more subtle differences in development. These derived parameters included the following ratios: eye length / head length (Eye L.: Head L.); head length / total length (Head L.: Total L.); and total length / embryo mass (Total L.: Emb. M.).

Morphological age was determined using Ferguson's (1985) crocodilian developmental staging scheme. Fresh embryos were photographed with either a Olympus model DPII digital camera mounted to a Zeiss model Stemi SV 6 dissecting scope (for embryos of age Day 9 or less) or with a Canon EOS D30 digital camera mounted to a photographer's stand (for embryos of age Day 10 or greater) (Fig. 5-1). Embryos were measured from digitized photographs using an image analysis software program, SigmaScan Pro (Systat Inc., 1999). After being photographed, embryos were fixed in formalin and stored in labeled containers for histopathology.

Histopathology

Subsamples of live embryos from "best case" clutches (clutch viability > 71%, which is equal to overall mean clutch viability + 1 standard deviation) and low total OCP egg burdens (≤ 350 ng/g) and live embryos from "worst case" clutches (< 47%) and high total OCP egg burdens (i.e., ≥ 3,700) were selected and processed for histopathology. Comparing best case to worst case provided the best opportunity for determining if differences existed with respect to frequency of lesions and identifying target organs and tissues. If large differences were found between embryos of best case and worst case clutches, subsequent examinations could be conducted on embryos of intermediate quality clutches. Conversely, if no differences were found, it would be unlikely to detect differences in intermediate quality clutches; therefore, subsequent examinations would not be warranted.

Embryos were cross-sectioned, and then four equidistance step-sections were taken from each of the following regions: the head, the thorax, and the abdomen. For each of the 7 μm sections, distances between step-sections ranged from ~42-300 μm, depending on the age of the embryo, with inter-sectional distances increasing with embryo size.

Sections were mounted to slides and stained with hematoxylin and eosin. Slides were screened for lesions, the type of lesion present, and the organ or tissue involved.

Expected morphological changes due to chronic OCP exposure include hepatocellular hypertrophy and focal necrosis. Hypertrophy is due to enlargement of the smooth endoplasmic reticulum (SER) and formation of a lipid droplet in the center of the SER (caused by OCP-induced expression of microsomal enzymes within the SER) (Smith, 1991). Other morphological changes found in the liver include foci of vacuolated hepatocytes and spongiosis hepatic (lesions of hepatic parenchyma). Renal lesions induced by chronic OCP exposure include dilation of tubular lumina, and vacuolization (degeneration) and necrosis of tubular epithelium (Metcalfe, 1998).

Other than hepatic and renal toxicopathic lesions, OCPs may cause death by disrupting neural transmission to the point of cardiovascular failure. Neural morphology is rarely changed by OCP exposure, which causes difficulty in determining whether cardiovascular failure was caused by OCP exposure or some other factor.

Analysis of OCPs in Yolk

Analytical grade standards for the following compounds were purchased from the sources indicated: aldrin, alpha-benzene hexachloride (α-BHC), β-BHC, lindane, δ-BHC, *p,p'*-dichlorodiphenyldichloroethane (*p,p'*-DDD), *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), dichlorodiphenyltrichloroethane (*p,p'*-DDT), dieldrin, endosulfan, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor epoxide, hexachlorobenzene, kepone, methoxychlor, mirex, *cis*-nonachlor, and *trans*-nonachlor from Ultra Scientific (Kingstown, RI, USA); *cis*-chlordane, *trans*-chlordane, and the 525, 525.1 polychlorinated biphenyl (PCB) Mix from Supelco (Bellefonte, PA, USA); oxychlordane from Chem Service (West Chester, PA); *o,p'*-

DDD, *o,p'*-DDE, *o,p'*-DDT from Accustandard (New Haven, CT, USA); and toxaphene from Restek (Bellefonte, PA, USA). All reagents were analytical grade unless otherwise indicated. Water was doubly distilled and deionized.

Egg yolk samples were analyzed for OCP content using methods modified from Holstege et al. (1994) and Schenck et al. (1994). For extraction, a 2 g tissue sample was homogenized with ~1 g of sodium sulfate and 8 mL of ethyl acetate. The supernatant was decanted and filtered though a Büchner funnel lined with Whatman #4 filter paper (Fisher Scientific, Hampton, NH, USA) and filled to a depth of 1.25 cm with sodium sulfate. The homogenate was extracted twice with the filtrates collected together. The combined filtrate was concentrated to ~2 mL by rotary evaporation, and then further concentrated until solvent-free under a stream of dry nitrogen. The residue was reconstituted in 2 mL of acetonitrile. After vortexing (30 s), the supernatant was applied to a C18 solid phase extraction (SPE) cartridge (pre-conditioned with 3 mL of acetonitrile; Agilent Technologies, Wilmington, DE, USA) and was allowed to pass under gravity. This procedure was repeated twice with the combined eluent collected in a culture tube. After the last addition, the cartridge was rinsed with 1 mL of acetonitrile which was also collected. The eluent was then applied to a 0.5 g NH2 SPE cartridge (Varian, Harbor City, CA, USA), was allowed to pass under gravity, and collected in a graduated conical tube. The cartridge was rinsed with an additional 1 mL portion of acetonitrile which was also collected. The combined eluents were concentrated under a stream of dry nitrogen, to a volume of 300 μL, and transferred to a gas chromatography (GC) vial for analysis.

GC/MS Analysis

Analysis of all samples was performed using a Hewlett Packard HP-6890 gas chromatograph (Wilmington, DE, USA) with a split/splitless inlet operated in splitless mode. The analytes were introduced in a 1 µL injection and separated across the HP-5MS column (30 m x 0.25 mm; 0.25 µm film thickness; J & W Scientific, Folsom, CA, USA) under a temperature program that began at 60º C, increased at 10º C/min to 270º C, was held for 5 min, then increased at 25º C/min to 300º C and was held for 5 min. Detection utilized an HP 5973 mass spectrometer in electron impact mode. Identification for all analytes and quantitation for toxaphene was conducted in full scan mode, where all ions are monitored. To improve sensitivity, selected ion monitoring was used for the quantitation for all other analytes, except kepone. The above program was used as a screening tool for kepone which does not optimally extract with most organochlorines. Samples found to contain kepone would be reextracted and analyzed specifically for this compound.

For quantitation, a five-point standard curve was prepared for each analyte ($r^2 \geq$ 0.995). Fresh curves were analyzed with each set of twenty samples. Each standard and sample was fortified to contain a deuterated internal standard, 5 µL of US-108 (120 µg/mL; Ultra Scientific), added just prior to analysis. All samples also contained a surrogate, 2 µg/mL of tetrachloroxylene (Ultra Scientific) added after homogenization. Duplicate quality control samples were prepared and analyzed with every twenty samples (typically at a level of 1.00 or 2.50 µg/mL of γ-BHC, heptachlor, aldrin, dieldrin, endrin, and *p,p'*-DDT) with an acceptable recovery ranging from 70 – 130%. Limit of detection ranged from 0.1-1.5 ng/g for all OCP analytes, except toxaphene (120-236 ng/g), and limit of quantitation was 1.5 ng/g for all analytes, except toxaphene (1500 ng/g).

Repeated analyses were conducted as allowed by matrix interferences and sample availability.

Results

Inter-Site Clutch Comparisons

A total of 58 clutches were collected during June 2001 and 2002 from Lakes Apopka, Griffin, Lochloosa and Emeralda Marsh Conservation Area. No differences ($\alpha = 0.05$) were determined between sites with respect to the following clutch parameters: fecundity (overall mean ± standard error: 43 ± 1), clutch viability (56 ± 3.9%), damaged eggs (4 ± 1.7%), unbanded eggs (12 ± 1.9%), early embryo mortality (i.e., mortality prior to Day 36; 14 ± 2.7%), and late embryo mortality (i.e., on or after Day 36; 14 ± 2.5%). However, the average egg mass of clutches from Emeralda Marsh was greater than that of Lake Griffin (Table 5-1). Significant differences were noted between sites in recent studies (Chapter 2) which a had a larger total sample size (n = 168). The lack of significant differences was likely due to the large variance noted in Emeralda clutches (Table 5-3).

Many differences were detected between sites with respect to egg yolk OCP concentrations. Alligator eggs from Emeralda Marsh and Lake Apopka were found to have a greater number of OCP analytes (n = 12 and 11, respectively) as compared to Lake Griffin (n = 10), which was greater than Lochloosa (n = 8.5) (Table 1). Eggs from Emeralda Marsh yielded the highest total OCP concentrations (29,838 ± 4,844 ng/g), which were over three-fold greater than those of Lake Apopka, 32-fold greater than those of Lake Griffin, and 290-fold greater than those of Lake Lochloosa. Furthermore, 45% of individual OCP analytes were at greater concentrations in Emeralda eggs as compared to those of Lake Apopka, with major differences in total OCP egg yolk concentrations

related to amounts of toxaphene (three-fold greater in Emeralda) and *p,p'*-DDE (two-fold greater in Emeralda). Other OCP analyte concentrations were similar between Emeralda and Lake Apopka, except for a few DDT and chlordane analytes. When individual OCP egg yolk concentrations from Apopka and Emeralda were compared against Lakes Griffin and Lochloosa, 82% of the individual OCP analyte concentrations were greater in eggs from Emeralda Marsh and Lake Apopka as compared to the other lakes (Table 5-1).

Intra-Site Live Embryo/Dead Embryo Morphological Comparisons

Comparisons between live and dead embryos sampled at chronological age (CA) Day 14 yielded the following results. For Lakes Lochloosa, Apopka, and Griffin clutches, live embryos sampled at CA Day 14 had an overall morphological age (MA) of 15 ± 0.3 (mean ± standard error), which was greater than the MA (11 ± 1) of dead embryos, and live embryos were of greater mass compared to dead embryos, suggesting that dead embryos may have been developmentally retarded. One other notable difference between live and dead embryos sampled at CA Day 14 was that eggs of dead embryos were greater in mass compared to live cohorts for Lakes Apopka and Griffin; however, for Lake Lochloosa, eggs of live embryos were greater in mass compared to dead cohorts (Table 5-2). Comparisons between eye length, head length, and total length were not made because dead embryos could not be uniformLy positioned for photographs due to their size and fragility of tissues resulting from the early stage of development and limited autolysis.

For Lake Lochloosa, live and dead embryos of CA Day 25 differed with respect to egg mass, embryo mass, and MA (for all endpoints: live > dead), and Total L.: Emb. M., with live Total L.: Emb. M. ratios being less than those of dead embryos. For Lake Apopka, live and dead embryos differed with respect to embryo mass, Eye L.: Head L.,

morphological day (for all endpoints: live > dead), and Head L.: Total L. (live < dead). No significant differences were detected between live and dead embryos from Emeralda clutches for the measured endpoints. For Lake Griffin, differences between live and dead embryos were determined for embryo mass, eye length, head length, and MA (for all endpoints: live > dead) (Table 5-2).

For Lake Lochloosa, live and dead embryos of CA Day 33 differed with respect to embryo mass and MA (for both endpoints: live > dead). For Lake Apopka, live embryos had greater mass, eye length, and MA than dead embryos, but dead embryos had greater Total L.: Emb. M.. Live embryos from Emeralda Marsh clutches were of greater mass and Total L.: Emb. M. than dead cohorts. Live embryos from Lake Griffin clutches were also of greater mass and were of greater MA in comparison to dead cohorts (Table 5-2). For Lake Lochloosa, live and dead embryos sampled at CA Day 43 differed with respect to embryo mass, eye length, head length, Head L.:Total L., and MA (for all endpoints: live > dead).

For Lake Apopka, live embryos were of greater mass, head length, total length, and morphological age compared to dead cohorts. Live embryos of Emeralda Marsh were of greater morphological age than dead cohorts, however, power of detection was low since only one dead embryo was sampled. Lastly, live embryos of Lake Griffin were of greater mass and morphological age than their dead cohorts (Table 5-2).

Inter-Site Comparisons of Morphology of Live Embryos

Egg mass of live embryos of CA Day 14 differed between sites, with respect to egg mass and MA. Eggs of CA Day 14 embryos from Lake Lochloosa clutches were of greater mass compared to those of Emeralda and Griffin, and MA of Lake Griffin embryos was greater than that of Emeralda Marsh (Table 3). For embryos sampled at CA

Day 25, egg mass, embryo mass, eye length, head length, total length, and TL: EM differed among sites. Eggs from Lake Lochloosa were of greater mass than all other sites, but embryo mass for Lochloosa clutches was less than that of Griffin. Embryos from Emeralda and Lake Griffin had the greater eye lengths in comparison to Lake Apopka with no significant differences detected for Lake Lochloosa embryos. With respect to head lengths and total lengths, embryos from Lake Apopka clutches were less than those of all other sites and embryos from Lake Griffin were greater than those of all other sites except for Emeralda. Lastly, Lochloosa embryos had higher Total L.: Emb. M. than all other sites except for Lake Apopka (Table 5-3).

For CA Day 33 embryos, only egg mass and MA differed between sites. Similar to earlier sampling periods, Lake Lochloosa eggs were of greater mass than all other sites except for Apopka. Lake Griffin embryos were of greater MA than all other sites, and Lake Apopka embryos were of lesser mass than all other sites except for those of Emeralda.

For CA Day 43 embryos, Lochloosa eggs were of greater mass than all other sites, and Lochloosa embryos were of lesser mass than all other sites except for Lake Apopka. Embryos of Lake Griffin were of greater mass than all other sites. Embryos of Lake Apopka and Lake Griffin also had greater eye lengths than those of Emeralda. For TL: EM ratios, Lake Griffin embryos had smaller ratios compared to all other sites except for Emeralda. In addition, the MA of embryos of Lochloosa was less than all other sites except Emeralda (Table 5-3).

Live Embryo Morphology and Embryo Survival Relationships

Redundancy analysis with forward selection (Monte Carlo permutation tests for significance) was used to examine whether embryo morphometric parameters (eye

length, head length, total length, and embryo mass) were strongly associated with embryo survival parameters (clutch viability, early embryo mortality, and late embryo mortality percentages) for each of the chronological ages (CA) sampled (Day 14, Day 25, Day 33, Day 43). For CA Day 14 embryos, results of the RDA indicated only late embryo mortality percentage was significantly associated with the observed variation embryo morphology, but accounted for only 7% of the morphological variation. For CA Day 25 and Day 33 embryos, no significant associations were found between morphological and embryo survival parameters. For CA Day 43, clutch viability was determined significant but accounted for only 5% of the variation in morphology.

Live Embryo Morphology and Egg Yolk OCP Burdens

In contrast to embryo survival parameters, OCP concentrations in egg yolks were significantly associated with variation in embryo morphology. For CA Day 14 embryos, partial-redundancy analysis using site (i.e., Emeralda, Apopka, Griffin, Lochloosa) as the covariate (since OCP burdens differed among sites), with forward selection of the best five OCP variables (Table 4), indicated that four of five selected variables were determined to be significant via Monte Carlo permutation tests. The four OCP variables were oxychlordane concentration ([OX]), heptachlor epoxide percentage of total OCP burden (HE%), toxaphene percentage of total OCP burden (TX%), and *trans*-nonachlor percentage of total OCP burden (TN%). These OCP variables accounted for 47% of the observed variation in embryo morphometric parameters. Individually, [OX] explained 20% of the variation in the morphometric parameters, followed by HE% (11%), TX% (10%), and TN% (6%) (Table 5). Embryo head length was negatively correlated with [OX] and HE%. Embryo eye length was negatively correlated with TN% and HE%.

Embryo mass was positively correlated with [OX] and HE%, and embryo total length was negatively correlated with [OX] but positively correlated with TN%.(Fig. 5-2).

For CA Day 25 embryos, four of five selected variables were determined to be significant, with HE%, cis-Nonachlor percentage of total OCP burden (CN%), dieldrin percentage of total OCP burden (DL%), and dieldrin concentration ([DL]) accounting for 24% of variation of embryo morphological parameters. HE% accounted for 11% of embryo morphological variation, followed by CN% (6%), DL% (5%) and [DL] (2%) (Table 5). Embryo head length was positively correlated with DL%, CN%, HE%, and [DL]. Total embryo length was also positively correlated with HE%, DL%, and [DL], but showed little correlation with CN%. In contrast to head length and total length, embryo mass and eye length were negatively correlated with HE%, DL%, and [DL], but showed little correlation with CN% (Fig. 5-3).

For CA Day 33 embryos, three of five selected variables were determined to be significant and consisted of CN%, DL%, cis-chlordane percentage of total OCP burden (CC%) and accounted for 24% of morphological variation (Table 5). Embryo head length and total length were positively correlated with DL% and negatively correlated with CC% and CN%. In contrast, embryo mass and eye length were positively correlated with CC% and CN%, but showed little correlation with DL% (Fig. 5-4).

For CA Day 43 embryos, three of five selected variables were determined to be significant and together accounted for 20% of the morphological variation. These variables consisted of *p,p'*-DDT concentration ([pDDT]), *cis*-chlordane concentration ([CC]), and total number of individual OCP analytes detected in yolk (NOC) (Table 5). Embryo mass was positively correlated with NOC and [pDDT], but showed little

correlation with [CC]. Embryo eye length was positively correlated with NOC, negatively correlated with [CC], and showed no correlation with [pDDT]. Embryo head length was positively correlated with NOC and negatively correlated with [CC] and [pDDT]. Total embryo length was negatively correlated with NOC, not correlated with [pDDT], and positively correlated with [CC] (Fig. 5-4).

Embryo Morphological Age, Derived Morphometric Variables and Egg Yolk OCP Burdens

For embryos sampled at CA Day 14, four of five RDA-selected OCP variables were determined to be significant and accounted for 44% of the variation associated with morphological age (MA) and derived morphometric variables (DMV), which consisted of Eye L.: Head L., Head L.: Total L., and Total L.: Emb. M.. The four extracted OCP variables were [OX], CN%, OX%, and HE%, and each respectively accounted for 21%, 12%, 7%, and 4% of variance associated with MA and DMV (Table 5-6). With the exception of Total L.: Emb. M., all DMV and MA were positively correlated with were [OX], OX%, and HE%. Total L.: Emb. M. was positively correlated with CN%, and CN% was negatively correlated with MA and the other DMV (Fig. 5-6).

For CA Day 25 embryos, three of five selected OCP variables were determined significant and accounted for 22% of MA and DMV variance. The three OCP variables consisted of HE%, NOC, and [pDD] and each respectively accounted for 12%, 7%, and 3% of the variation in MA and DMV (Table 6). HE% was positively correlated with Total L.: Emb. M. and negatively correlated with MA, Head L.: Total L., and Eye L.: Head L. NOC was negatively correlated with Total L.: Emb. M. and positively correlated with MA, Head L.: Total L., and Eye L. [pDD] was positively correlated with Head L.:

Total L. and Eye L.: Head L., but showed little correlation with MA and Total L.: Emb. M (Fig. 5-7).

For Day 33 embryos, three OCP variables (DL%, PDE%, and CN%) evenly accounted for 15% of variation in MA and DMV (Table 6). DL% was negatively correlated with Head L.: Total L. and Eye L.: Head L., but showed little correlation with MA and was positively correlated with Total L.: Emb. Mass. PDE%, and CN% were positively correlated with MA, Head L.: Total L. and Eye L.: Head L., but showed a negative correlation with Total L.: Emb. M. (Fig. 5-8).

Lastly, for CA Day 43 embryos, four of five OCP variables selected via partial RDA were determined to be significant and accounted for 24% of variation in MA and DMV. These four OCP variables consisted of, with respect to amount of variation accounted for, PDT% (8%), [CC] (5%), NOC (5%), and [PDT] (4%) (Table 6). PDT%, NOC, and [PDT] were positively correlated with Head L.: Total L., Eye L.: Head L., and MA, but were negatively correlated with Total L.: Emb. M. [CC] was negatively correlated with Head L.: Total L., Eye L.: Head L., and MA, but was positively correlated with Total L.: Emb. M. (Fig. 5-9).

Histopathology of Live and Dead Embryos

Results of histopathology of live embryos (n = 34) from five reference clutches (clutch viability > 71% and OCP yolk burdens < 350 ng/g) and live embryos (n = 26) from four OCP-contaminated clutches (clutch viability < 47% and OCP yolk burdens > 3,700 ng/g) indicated that 16% of all embryos exhibited at least one type of hepatic lesion, followed by lesions of the skeletal muscle (5%), and kidney (3%). Hepatic lesions included necrosis (characterized by pyknotic nuclei and vacuolated hepatocytes) and cholestasis. Lesions detected in skeletal muscle included necrosis characterized by

pyknotic nuclei and segmented sarcoplasm. Kidney lesions included necrosis of tubules characterized by vacuolization and pyknotic nuclei. No significant differences were determined between reference embryos and OCP-contaminated embryos with respect to incidence of hepatic lesions ($\chi^2 = 0.87$, $p = 0.49$), renal lesions ($\chi^2 = 1.58$, $p = 0.50$), or muscular lesions ($\chi^2 = 2.41$, $p = 0.25$).

Histopathology results of dead embryos ($n = 20$) from OCP-contaminated sites indicated that generalized autolysis was the predominant finding, with fungi hyphae present in 2 embryos, and a single case of menigoencephalitis, that would be consistent with a bacterial infection. Advance autolysis, in some cases, likely impeded detection of cytoxic lesions.

Discussion

With respect to the first hypothesis, results suggest that certain morphological parameters of live alligator embryos differ from those of dead embryos of the same chronological age. Intra-site comparisons suggested that among all sites and all sampled ages (CA) embryo MA and mass were greater for live embryos as compared to dead embryos. Importantly, the concurrent decreases in MA and mass of dead embryos suggests that embryos may have been developing normally up to a point at which development stalled and the embryo eventually perished, or embryos could have developed at a much slower overall rate until the point at which they perished. Either way it appears that the mass of dead embryos was appropriate for their MA. For example, live embryos of Lake Griffin sampled at CA Day 14 had an average MA of ~ Day 16 and an average mass of 0.41 g, which was similar to the MA (~ Day 15) and mass (0.41 g) of dead embryos sampled at CA Day 25 (Table 2). Other measured parameters and derived parameters showed variation in patterns among sites and ages, but one

consistency was that when differences were detected, measured parameters were almost always greater in live embryos as compared to dead embryos.

With the exception of Lake Apopka, few significant differences were found between live and dead embryos with respect to derived morphometric parameters (i.e., Eye L.: Head L.; Head L.: Total L.; and Total L.: Emb. M.). Differences were found between derived morphometric parameters of live and dead embryos sampled at older ages (CA) from Lake Apopka, and suggest that morphology of dead embryos of Lake Apopka is disproportionate compared to live cohorts. The differences between the patterns of morphometric relationships of live and dead embryos from Lake Apopka as compared to other sites, may indicate the causes or mechanisms associated with mortality of Lake Apopka embryos differ from other sites, since it has been shown that the type of teratogenic effect may depend on the specific teratogenic agent or cause (Schmidt & Johnson, 1997).

With respect to the second hypothesis, results suggested that morphology of live embryos was not consistently different among sites, except for live embryos of CA Day 25. For Day 25 live embryos, embryos of Lake Griffin and Emeralda Marsh were consistently larger, with respect to measurement parameters, than those of Lakes Apopka and Lochloosa. The only differences found, with respect to derived parameters, was for Total L.: Emb. M., with Lochloosa embryos appearing to be leaner embryos compared to those of Emeralda and Griffin. Since Day 25 is during the middle of organogenesis, this stage of development may be more sensitive to OCP exposure or variation in yolk nutrient content since it has been shown in other species the period of organogenesis is

most susceptible to alterations caused by teratogen exposure or nutrient excess or deficiency (Schmidt & Johnson, 1997).

With respect to the third hypothesis, redundancy analysis results indicated that variation in morphometry of live embryos is not significantly related to variation in clutch mortality rates, suggesting that live embryos from clutches with high mortality rates develop similarly to those of low mortality clutches. This finding may suggest a threshold-type response in which embryos exposed to stressors below a certain threshold have the ability to overcome stressors through various cellular homeostatic mechanisms, but above a certain threshold, developmental retardation and lethality occur. Such threshold dose-response patterns have been accepted as a major dose-response pattern in mammalian developmental toxicology (Rogers & Kavlock, 2001).

With respect to the fourth hypothesis, variation in morphological development of live embryos was significantly associated with variation in the composition and concentration of OCPs in eggs. However, the strength of the relationships appeared to decrease with the age sampled (CA), with youngest embryos sampled (CA Day 14) showing the strongest relationships between OCP egg burden and morphometric parameters, followed by each subsequent CA, respectively (Table 5-5). Interestingly, the percentage of the total OCP burden (concentration) composed by an OCP analyte (i.e., HE%), appeared to be more important than OCP analyte concentrations alone. With respect to all sampled ages, except the eldest (CA Day 43), OCP percentage variables accounted from a minimum of 47% to a maximum of 100% of the total variation attributed to all OCP variables found to be significantly associated with variation in measured morphometric parameters (Table 5-5). For derived morphometric parameters

and morphological age (MA), similar patterns were observed in that embryos sampled at younger CA showed stronger relationships with OCP burdens than older cohorts (Table 5-6).

With respect to individual OCP analytes, the cyclodienes appear to be more important than the dichlorodiphenylethanes, in that cyclodienes accounted for an average of 70% of the morphometric variation that could be attributed to OCP variables for all sampled ages. This is surprising considering that dichlorodiphenylethanes (*p,p'*-DDT + *p,p'*-DDD + *p,p'*-DDE) make up an average of 66% of the total OCP burden among all sites (Table 5-1).

Another important observation was that different cyclodienes appeared to be associated with morphological variation of embryos of different ages (CA). Most important were the components of technical grade chlordane and its metabolites, which include *cis* and *trans*-chlordane, *cis* and *trans*-nonachlor, oxychlordane, and heptachlor epoxide. One or more of these components were found to be significantly associated with variation in embryo morphology for each CA sampled. These data suggest that the chlordane group may merit further study in relation to developmental effects in reptiles, especially considering other studies have suggested that sexual differentiation in turtles may be altered by low dose *in ovo* exposures of these compounds (Willingham, 2004).

With respect to the final hypothesis, no significant differences were found between the histopathology of live and dead embryos from best-case clutches (low mortality rates and low OCP egg burdens) compared to those of worst-case clutches (high mortality rates and high OCP egg burdens). Few signs of bacterial or fungal infections were found. These results may suggest that lesions were not a causal factor in death, and may not be

associated with variation in OCP exposure or increased mortality rates. However, the death and autolysis of delicate embryonic tissues may have obscured lesions associated with death and/or OCP exposure. In addition, OCPs may cause dysfunction in neural transmission, leading to cardiovascular failure and mortality. Future studies may consider *in ovo* monitoring of neural transmission and cardiovascular function to determine if increased OCP exposure is associated with altered neural transmission and cardiovascular failure in alligator embryos.

In conclusion, the present study found that embryo mortality occurring in alligator populations inhabiting reference and OCP-contaminated sites was characterized by developmental retardation without gross deformities or overt presence of lesions to vital organs. However, variation in embryo morphology appeared to be associated with variation in OCP burdens of eggs and the percentage composition composed by an OCP analyte was equally as important as concentration, suggesting the importance of mixture composition. Younger embryos appeared more susceptible to OCP influence but OCP influence may not necessarily be the result of direct embryo effects. Similar types of embryo mortality has been documented in quail, with embryo mortality determined to be maternally mediated, where maternal liver function was altered, resulting in nutrient deficiencies in eggs that were severe enough to induce embryo mortality (Donaldson & Fites, 1970). In summary, subsequent studies should evaluate embryo mortality in alligators as a function of OCP exposure and egg nutrient content.

Table 5-1. Summary statistics for parameters measured on American alligator clutches collected during June 2001 and 2002.

Parameter	Apopka	Emeralda	Griffin	Lochloosa	Summary
N$^{o.}$ Clutches (n)	15	7	18	18	58
Fecundity (*n*)	46 ± 2	50 ± 3	43 ± 2	40 ± 2	43 ± 1
	(28–56)	(42–64)	(19–58)	(26–56)	(19–64)
Egg mass (g)	86 ± 3.3 AB	107 ± 18.1 A	79 ± 3.2 B	89 ± 3.2 AB	87 ± 2.8
	(67–120)	(65–180)	(46–113)	(71–139)	(46–180)
Clutch viability (%)	53 ± 8.6	63 ± 13.8	46 ± 6.7	64 ± 5.7	56 ± 3.9
	(0–92)	(0–96)	(0–87)	(0–95)	(0–96)
Damaged eggs (%)	1 ± 0.4	1 ± 0.7	7 ± 4.2	4 ± 3.3	4 ± 1.7
	(0–4)	(0–4)	(0–63)	(0–60)	(0–63)
Unbanded eggs (%)	13 ± 3.4	10 ± 3.9	15 ± 4.8	11 ± 2.1	12 ± 1.9
	(0–40)	(0–30)	(0–65)	(0–33)	(0–65)
Early Emb. Mort. (%)	14 ± 6.2	24 ± 12.7	13 ± 4.4	13 ± 3.1	14 ± 2.7
	(0–90)	(0–95)	(0–73)	(0–36)	(0–95)
Late Emb. Mort. (%)	19 ± 6.3	3 ± 2.1	19 ± 4.8	9 ± 2.6	14 ± 2.5
	(0–77)	(0–15)	(0–58)	(0–34)	(0–77)
Dieldrin (ng/g)	405.7 ± 121.32 A	227.3 ± 40.09 A	24.8 ± 5.18 B	3.6 ± 0.5 C	146.1 ± 39.53
	(23.5–1,859)	(88–386.7)	(4.4–76.9)	(1.3–8.2)	(1.3–1,859)
Hep. Epoxide (ng/g)	14.4 ± 3.01 A	5.3 ± 1.38 AB	7.1 ± 2.05 B	2.8 ± 0.76 B	7.8 ± 1.24
	(1.2–46.8)	(1.4–11.6)	(1.1–29.6)	(1.2–9.7)	(1.1–46.8)
cis-Chlordane (ng/g)	48.3 ± 13.74 B	150.4 ± 26.21 A	10.7 ± 1.01 C	1.9 ± 0.21 D	34.3 ± 7.71
	(6.6–179.2)	(62.4–281)	(4.3–16.9)	(1.2–4.1)	(1.2–281)
cis-Nonachlor (ng/g)	70.4 ± 16.41 A	89.2 ± 15.7 A	17.1 ± 2.7 B	4.6 ± 0.63 C	35.7 ± 6.29
	(10.5–238.4)	(55–166)	(4.4–54.2)	(2.4–12.5)	(2.4–238.4)
Oxychlordane (ng/g)	45.7 ± 10.94 A	29.3 ± 4.1 A	12.6 ± 3.17 B	3.8 ± 1.06 C	20.4 ± 3.71
	(3.9–176)	(17.9–46.1)	(1.1–45.9)	(1.2–17.8)	(1.1–176)

Table 5-1. Continued.

Parameter	Apopka	Emeralda	Griffin	Lochloosa	Summary
Toxaphene (ng/g)	3,308 ± 658.5 B	8,269 ± 1,077.2 A	2,677 ± 376.5 B	nd C	4872 ± 722.8
	(1,896.1–9,678.3)	(4,512.8–11,485.4)	(1,927.9–3,110.8)	(0–0)	(1,896–11,485.4)
p, p′-DDD (ng/g)	45.8 ± 13.49 B	1,986 ± 333.9 A	5.7 ± 1.1 C	1.9 ± 0.21 D	277.6 ± 101.28
	(10.6–192.8)	(617.3–2962.8)	(1.5–18.5)	(1.2–2.9)	(1.2–2962.8)
p, p′-DDE (ng/g)	5,792 ± 1,490.4 B	18,056.3 ± 3,113.7 A	337 ± 55.2 C	74.8 ± 12.38 D	3,805 ± 924
	(18.3–22,421.9)	(6,811.7–33,554.8)	(94.6–979.1)	(28–231)	(18.3–33,554.8)
p, p′-DDT (ng/g)	9.8 ± 3.81 A	17.7 ± 3.24 A	2.7 ± 0.25 AB	1.3 ± 0.02 B	9.4 ± 2.17
	(1.2–45.6)	(5.8–25.3)	(2.5–3)	(1.2–1.3)	(1.2–45.6)
trans-Chlor. (ng/g)	7.4 ± 2.38 B	44 ± 4.61 A	1.6 ± 0.19 C	2.6 ± 0.73 BC	11.3 ± 2.81
	(1.3–27.4)	(23.2–58.2)	(0.8–3.4)	(1.2–3.7)	(0.8–58.2)
trans-Nonachl. (ng/g)	202.5 ± 54.77 A	278.2 ± 56.93 A	42.4 ± 9.31 B	8.4 ± 1.67 C	101.7 ± 20.46
	(10.5–787.6)	(148–554.9)	(8.9–155.2)	(2.5–24.6)	(2.5–787.6)
∑[OCP] (ng/g)	9,177 ± 2,391.2 B	29,838 ± 4,844.3 A	911 ± 302.4 C	103 ± 16.3 D	6,238.6 ± 1,508.35
	(555.2–35,587.8)	(13,183.5–53,559.7)	(128.7–4,487.7)	(42.7–289.4)	(42.7–53,559.7)
$N^{o.}$ OCPs (*n*)	11 ± 0.28 A	12 ± 0.22 A	10 ± 0.18 B	8.6 ± 0.28 C	10.1 ± 0.2
	(9–12)	(11–13)	(9–12)	(6–10)	(6–13)

[a]Values represent mean ± standard error with ranges in parentheses. Letters beside values (A-D) indicate differences between sites (α = 0.05). Clutch viability % = number of live hatchlings / fecundity x 100, damaged eggs % = number of damaged egg / fecundity x 100, unbanded eggs % = number of unbanded eggs / fecundity x 100, early embryo mortality % = number of embryos that died at ages ≤Day 35 / fecundity x 100, late embryo mortality % = number of embryos that died at ages >Day 35 / fecundity x 100, Hep. Epoxide = heptachlor epoxide, *trans*-Chlor. = *trans*-chlordane, *trans*-Nonachl. = *trans*-nonachlor, and No. OCPs = number of OCPs detected at measurable levels.

Table 5-2. Comparisons of egg and embryo morphometrics of live and dead embryos collected during June-August of 2001 and 2002.

Age[a]	Parameter[b]	Apopka		Emeralda		Griffin	
		Live	Dead	Live	Dead	Live	Dead
14	*n*	7	9	12		23	8
	Egg mass (g)	86.3 ± 1.23	92.5 ± 1.35*	78.6 ± 1.35		81.6 ± 1.92	86.7 ± 2.6
	Embryo mass (g)	0.38 ± 0.12*	0.14 ± 0.089	0.35 ± 0.019		0.41 ± 0.032	0.2 ± 0
	Eye L. (mm)	2.9 ± 0	0 ± 0	0 ± 0		3.5 ± 0.06	0 ± 0
	Head L. (mm)	8 ± 0	0 ± 0	0 ± 0		9.1 ± 0.28	0 ± 0
	Total L. (mm)	51.4 ± 0	0 ± 0	0 ± 0		57.5 ± 0.57	0 ± 0
	Eye L.: Head L.	0.36 ± 0	0 ± 0	0 ± 0		0.39 ± 0.012	0 ± 0
	Head L.: Total L.	0.16 ± 0	0 ± 0	0 ± 0		0.16 ± 0.006	0 ± 0
	Total L.: Emb. M.	171.33 ± 0	0 ± 0	0 ± 0		111.5 ± 15.353	0 ± 0
	Morph. Day	16 ± 1.195	12.6 ± 1.661	14 ± 0		15.96 ± 0.4*	12 ± 3
25	*n*	20	13	24	6	30	37
	Egg mass (g)	81.1 ± 2.01	87.1 ± 2.1	80 ± 0.98	75.7 ± 2.55	80.7 ± 1.49	79.7 ± 1.05
	Embryo mass (g)	1.15 ± 0.11*	0.56 ± 0.131	1.45 ± 0.116	1.57 ± 0.27	1.51 ± 0.088*	0.41 ± 0.188
	Eye L. (mm)	5 ± 0.18	4.2 ± 1.17	5.5 ± 0.12	5.9 ± 0	5.9 ± 0.17*	2.5 ± 0.98
	Head L. (mm)	11.5 ± 0.55	11.2 ± 2.66	13.5 ± 0.41	16.7 ± 0	14.9 ± 0.65*	7.7 ± 3.54
	Total L. (mm)	73.7 ± 3.38	65.6 ± 14.12	87.6 ± 2.44	98.7 ± 0	89.1 ± 2.32	72.3 ± 0
	Eye L.: Head L.	0.44 ± 0.01*	0.36 ± 0.024	0.41 ± 0.007	0.35 ± 0	0.4 ± 0.011	0.33 ± 0.029
	Head L.: Total L.	0.16 ± 0.012	0.17 ± 0.015	0.15 ± 0.002	0.17 ± 0	0.16 ± 0.003	0.2 ± 0*
	Total L.: Emb. M.	68.25 ± 4.72	146.51 ± 41.27*	58.89 ± 2.47	47.02 ± 0	52.24 ± 3.526	0 ± 0
	Morph. Day	24.2 ± 0.99*	17.63 ± 1.475	26.42 ± 0.58	28 ± 0	26.13 ± 0.619*	14.63 ± 1.75
33	*n*	15	13	29	3	29	17
	Egg mass (g)	82.4 ± 2.08	81.5 ± 3.4	80.8 ± 1.15	75 ± 3.09	77.7 ± 1.42	81 ± 1.4

Table 5-2. Continued.

Age[a]	Parameter[b]	Apopka		Emeralda		Griffin	
		Live	Dead	Live	Dead	Live	Dead
33	Embryo mass (g)	3.14 ± 0.09*	1.2 ± 0.423	3.8 ± 0.196*	1.2 ± 0	4.04 ± 0.203*	0.75 ± 0.552
	Eye L. (mm)	6.2 ± 0.1*	3.3 ± 0.52	6.4 ± 0.12	6 ± 0	6.5 ± 0.1	6.6 ± 0.27
	Head L. (mm)	19.1 ± 0.31*	10.7 ± 3.21	20.1 ± 0.49	19.1 ± 0	20.6 ± 0.35	19.9 ± 0.34
	Total L. (mm)	108.2 ± 1.1*	67.8 ± 13.25	114.4 ± 2.54	108.8 ± 0	117.8 ± 2.19	111.9 ± 5.02
	Eye L.: Head L.	0.32 ± 0.008	0.32 ± 0.049	0.32 ± 0.007	0.31 ± 0	0.32 ± 0.006	0.33 ± 0.008
	Head L.: Total L.	0.18 ± 0.00*	0.15 ± 0.017	0.18 ± 0.002	0.18 ± 0	0.18 ± 0.001	0.18 ± 0.005
	Total L.: Emb. M.	34.77 ± 0.81	291.4 ± 254.57*	31.36 ± 1.56	90.69 ± 0*	31.41 ± 1.467	30.53 ± 0
	Morph. Day	33.3 ± 0.33*	19.83 ± 3.323	35.69 ± 0.57	33 ± 0	39.28 ± 0.854*	19 ± 3.167
43	*n*	44	10	24	1	41	24
	Egg mass (g)	81.2 ± 1.45	85.4 ± 2.56	79.8 ± 1.19	73.5 ± 0	78 ± 1.32	80.6 ± 1.34
	Embryo mass (g)	9.91 ± 0.27*	3.11 ± 1.463	10.69 ± 0.30	0 ± 0	13.01 ± 0.514*	6.77 ± 2.706
	Eye L. (mm)	7.3 ± 0.14	7 ± 0.4	6.5 ± 0.11	0 ± 0	7.5 ± 0.2	6.8 ± 0.75
	Head L. (mm)	27.5 ± 0.53*	21.6 ± 5.57	26.1 ± 0.41	0 ± 0	27.4 ± 0.9	24.6 ± 4.08
	Total L. (mm)	172.1 ± 5.2*	130.1 ± 36.33	170.1 ± 2.14	0 ± 0	184.9 ± 7.05	167.8 ± 29.4
	Eye L.: Head L.	0.27 ± 0.004	0.34 ± 0.069*	0.25 ± 0.006	0 ± 0	0.35 ± 0.08	0.29 ± 0.041
	Head L.: Total L.	0.16 ± 0.003	0.17 ± 0.004	0.15 ± 0.002	0 ± 0	0.19 ± 0.042	0.15 ± 0.003
	Total L.: Emb. M.	15.89 ± 1.00	238 ± 188.667*	16.59 ± 0.35	0 ± 0	14.26 ± 0.741	21.2 ± 7.317
	Morph. Day	47.8 ± 0.68*	30.5 ± 4.119	47.6 ± 0.42*	38 ± 0	48.5 ± 0.482*	36 ± 5.04

Table 5-2. Continued.

Age[a]	Parameter[b]	Lochloosa	
		Live	Dead
14	*n*	13	8
	Egg mass (g)	90.4 ± 1.64*	79.5 ± 0.98
	Embryo mass (g)	0.32 ± 0.032	0 ± 0
	Eye L. (mm)	4.3 ± 1.22	0 ± 0
	Head L. (mm)	12.2 ± 3.18	0 ± 0
	Total L. (mm)	56.6 ± 12.21	0 ± 0
	Eye L.: Head L.	0.35 ± 0.025	0 ± 0
	Head L.: Total L.	0.18 ± 0.03	0 ± 0
	Total L.: Emb. M.	151.48 ± 52.437	0 ± 0
	Morph. Day	14.46 ± 0.666*	8.33 ± 1.202
25	*n*	32	22
	Egg mass (g)	87.5 ± 1.25*	81.7 ± 1.36
	Embryo mass (g)	1.03 ± 0.051*	0.52 ± 0.175
	Eye L. (mm)	5.5 ± 0.16	5 ± 0.37
	Head L. (mm)	13.4 ± 0.51	13 ± 1.89
	Total L. (mm)	78.8 ± 2.18	80.9 ± 9.26
	Eye L.: Head L.	0.42 ± 0.007	0.39 ± 0.025
	Head L.: Total L.	0.16 ± 0.003	0.16 ± 0.005
	Total L.: Emb. M.	74.64 ± 12.461	186.08 ± 78.758*
	Morph. Day	24.28 ± 0.49*	14.5 ± 2.045
33	*n*	27	11
	Egg mass (g)	86.5 ± 1.28	82.9 ± 1.83
	Embryo mass (g)	3.41 ± 0.226*	3.13 ± 1.788
	Eye L. (mm)	6.4 ± 0.18	7.4 ± 0
	Head L. (mm)	19.8 ± 0.37	25.8 ± 0*
	Total L. (mm)	115.1 ± 2.63	144.8 ± 0*
	Eye L.: Head L.	0.33 ± 0.008	0.29 ± 0
	Head L.: Total L.	0.17 ± 0.001	0.18 ± 0
	Total L.: Egg M.	31.93 ± 1.262	0 ± 0
	Morph. Day	36.48 ± 0.676*	16.75 ± 4.304
43	*n*	40	14
	Egg mass (g)	85.7 ± 1	84.4 ± 1.11
	Embryo mass (g)	9.25 ± 0.346*	5.74 ± 3.25
	Eye L. (mm)	7 ± 0.14*	3 ± 0
	Head L. (mm)	26.2 ± 0.63*	18.3 ± 9.75
	Total L. (mm)	165 ± 4.15	212 ± 0
	Eye L.: Head L.	0.27 ± 0.006	0.35 ± 0
	Head L.: Total L.	0.16 ± 0.002*	0.13 ± 0

Table 5-2. Continued.

Age[a]	Parameter[b]	Lochloosa	
		Live	Dead
43	Total L.: Emb. M.	14.29 ± 1.212	139.47 ± 139.47*
	Morph. Day	45.69 ± 0.496*	20.74 ± 4.456

[a]Age = chronological (calendar) age of embryo (days).
[b]Values = mean ± standard error. L. = length, Eye L.: Head length = eye length / head length, Head L.: Total L. = head length / total length, Total L.: Emb. M. = total length / embryo mass, and Morph. Day = age as determined by morphological characteristics.
*indicate significant differences ($\alpha = 0.05$).

Table 5-3. Morphometric comparisons of live embryos collected during June-August 2001 and 2002.

Age[a]	Parameter[b]	Apopka	Emeralda	Griffin	Lochloosa	Summary
14	*n*	7	12	23	13	55
	Egg mass (g)	86.3 ± 1.23 AB (82.8–90.9)	78.6 ± 1.35 B (70.8–85.4)	81.6 ± 1.92 B (61.8–100.1)	90.4 ± 1.64 A (77–99.7)	83.6 ± 1.11 (61.8–100.1)
	Embryo mass (g)	0.38 ± 0.115 A (0–0.8)	0.35 ± 0.02 AB (0.2–0.4)	0.41 ± 0.032 AB (0.1–0.8)	0.32 ± 0.03 B 2 (0.2–0.5)	0.37 ± 0.022 (0–0.8)
	Eye length (mm)	2.9 ± 0 (2.9–2.9)	0 ± 0 (0–0)	3.5 ± 0.06 (3.4–3.7)	4.3 ± 1.22 (2–6.6)	3.8 ± 0.53 (2–6.6)
	Head length (mm)	8 ± 0 (8–8)	0 ± 0 (0–0)	9.1 ± 0.28 (8.7–9.9)	12.2 ± 3.18 (5.8–19.1)	10.4 ± 1.43 (5.8–19.1)
	Total length (mm)	51.4 ± 0 (51.4–51.4)	0 ± 0 (0–0)	57.5 ± 0.57 (56.3–58.9)	56.6 ± 12.21 (44–81)	56.4 ± 4.07 (44–81)
	Eye L.: Head L.	0.36 ± 0 (0.36–0.36)	0 ± 0 (0–0)	0.39 ± 0.012 (0.36–0.42)	0.35 ± 0.025 (0.29–0.41)	0.37 ± 0.013 (0.29–0.42)
	Head L.: Total L.	0.16 ± 0 (0.16–0.16)	0 ± 0 (0–0)	0.16 ± 0.006 (0.15–0.18)	0.18 ± 0.03 (0.13–0.24)	0.17 ± 0.011 (0.13–0.24)
	Total L.: Emb. M.	171.33 ± 0 (171.33–171.33)	0 ± 0 (0–0)	111.49 ± 15.353 (72.25–147.3)	151.48 ± 52.437 (0–223.86)	135.91 ± 23.698 (0–223.86)
	Morph. Day	16 ± 1.195 AB (10–19)	14 ± 0 B (14–14)	15.96 ± 0.4 A (12–21)	14.46 ± 0.67 AB (9–18)	15.18 ± 0.291 (9–21)
25	*n*	20	24	30	32	106
	Egg mass (g)	81.1 ± 2.01 B (62.4–96.4)	80 ± 0.98 B (71.6–90.5)	80.7 ± 1.49 B (59.4–100.2)	87.5 ± 1.25 A (73.3–99)	82.7 ± 0.77 (59.4–100.2)
	Embryo mass (g)	1.15 ± 0.111 AB (0.2–1.9)	1.45 ± 0.12 AB (0.2–2.7)	1.51 ± 0.088 A (0.7–2.4)	1.03 ± 0.051 B (0.3–1.6)	1.29 ± 0.049 (0.2–2.7)
	Eye length (mm)	5 ± 0.18 B (4.5–7.2)	5.5 ± 0.12 A (4.6–6.5)	5.9 ± 0.17 A (4.9–8.6)	5.5 ± 0.16 AB (4.5–7.6)	5.5 ± 0.08 (4.5–8.6)
	Head length (mm)	11.5 ± 0.55 B (9.1–17.4)	13.5 ± 0.41 A (10.4–17.7)	14.9 ± 0.65 A (10.8–24.3)	13.4 ± 0.51 A (10–18.5)	13.5 ± 0.29 (9.1–24.3)

Table 5-3. Continued.

Age[a]	Parameter[b]	Apopka	Emeralda	Griffin	Lochloosa	Summary
25	Total length (mm)	73.7 ± 3.38 C (38.4–102.9)	87.6 ± 2.44 AB (71–105.6)	89.1 ± 2.32 A (72.8–118.5)	78.8 ± 2.18 BC (63.4–102.5)	82.9 ± 1.43 (38.4–118.5)
	Eye L.: Head L.	0.44 ± 0.01 (0.38–0.51)	0.41 ± 0.007 (0.36–0.48)	0.4 ± 0.011 (0.24–0.48)	0.42 ± 0.007 (0.34–0.46)	0.42 ± 0.005 (0.24–0.51)
	Head L.: Total L.	0.16 ± 0.012 (0.13–0.32)	0.15 ± 0.002 (0.13–0.18)	0.16 ± 0.003 (0.14–0.21)	0.16 ± 0.003 (0.14–0.18)	0.16 ± 0.003 (0.13–0.32)
	Total L.: Emb. M.	68.25 ± 4.717 AB (46.28–102.39)	58.89 ± 2.473 B (39.12–75.47)	52.24 ± 3.526 B (0–79.12)	74.64 ± 12.461 A (0–317)	63.36 ± 4.046 (0–317)
	Morph. Day	24.17 ± 0.988 (12–33)	26.42 ± 0.583 (15–28)	26.13 ± 0.619 (17–30)	24.28 ± 0.49 (17–28)	25.29 ± 0.33 (12–33)
33	*n*	15	29	29	27	100
	Egg mass (g)	82.4 ± 2.08 AB (65.9–92.6)	80.8 ± 1.15 B (69.5–91.3)	77.7 ± 1.42 B (62.4–90.7)	86.5 ± 1.28 A (69.5–97.3)	81.7 ± 0.77 (62.4–97.3)
	Embryo mass (g)	3.14 ± 0.09 (2.4–3.7)	3.81 ± 0.196 (1.7–6.1)	4.04 ± 0.203 (1.4–5.8)	3.41 ± 0.226 (1.1–8)	3.67 ± 0.107 (1.1–8)
	Eye length (mm)	6.2 ± 0.1 (5.6–7)	6.4 ± 0.12 (4.9–7.7)	6.5 ± 0.1 (5.5–7.5)	6.4 ± 0.18 (4.3–7.5)	6.4 ± 0.06 (4.3–7.7)
	Head length (mm)	19.1 ± 0.31 (16.7–21.4)	20.1 ± 0.49 (11.8–23.4)	20.6 ± 0.35 (14.5–23.2)	19.8 ± 0.37 (15.7–23.4)	20 ± 0.21 (11.8–23.4)
	Total length (mm)	108.2 ± 1.11 (101–114.7)	114.4 ± 2.54 (76.6–135.9)	117.8 ± 2.19 (79.1–132.9)	115.1 ± 2.63 (91.2–153.6)	114.6 ± 1.22 (76.6–153.6)
	Eye L.: Head L.	0.32 ± 0.008 (0.28–0.41)	0.32 ± 0.007 (0.26–0.42)	0.32 ± 0.006 (0.24–0.38)	0.33 ± 0.008 (0.26–0.38)	0.32 ± 0.004 (0.24–0.42)
	Head L.: Total L.	0.18 ± 0.002 (0.16–0.19)	0.18 ± 0.002 (0.15–0.2)	0.18 ± 0.001 (0.17–0.19)	0.17 ± 0.001 (0.15–0.18)	0.17 ± 0.001 (0.15–0.2)
	Total L.: Emb. M.	34.77 ± 0.812 (28.6–42.06)	31.36 ± 1.562 (22.28–52.18)	31.41 ± 1.467 (22.58–56.5)	31.93 ± 1.262 (19.2–49.51)	32.07 ± 0.726 (19.2–56.5)
	Morph. Day	33.33 ± 0.333 C (33–38)	35.7 ± 0.57 BC (28–38)	39.28 ± 0.854 A (28–48)	36.48 ± 0.676 B (25–43)	36.62 ± 0.406 (25–48)

Table 5-3. Continued.

Age[a]	Parameter[b]	Apopka	Emeralda	Griffin	Lochloosa	Summary
43	*n*	44	24	41	40	
	Egg mass (g)	81.2 ± 1.45 B (63.7–96)	79.8 ± 1.19 B (67.5–91.5)	78 ± 1.32 B (60.3–91.3)	85.7 ± 1 A (69.6–97.9)	81.4 ± 0.68 (60.3–97.9)
	Embryo mass (g)	9.91 ± 0.274 BC (7.3–14.41)	10.69 ± 0.297 B (7.5–13.4)	13.01 ± 0.514 A (7.71–23.53)	9.25 ± 0.346 C (4.5–17.14)	10.69 ± 0.229 (4.5–23.53)
	Eye length (mm)	7.3 ± 0.14 A (5.8–9.5)	6.5 ± 0.11 B (5.5–7.1)	7.5 ± 0.2 A (5.9–10.9)	7 ± 0.14 AB (4.3–8.6)	7.2 ± 0.08 (4.3–10.9)
	Head length (mm)	27.5 ± 0.53 (15.8–36.9)	26.1 ± 0.41 (21.6–28.4)	27.4 ± 0.9 (3.2–35.8)	26.2 ± 0.63 (9.6–33.8)	26.9 ± 0.35 (3.2–36.9)
	Total length (mm)	172.1 ± 5.19 (92.6–250.4)	170.1 ± 2.14 (147.5–181.3)	184.9 ± 7.05 (19.9–278.8)	165 ± 4.15 (122.5–251.3)	173.6 ± 2.87 (19.9–278.8)
	Eye L.: Head L.	0.27 ± 0.004 (0.22–0.37)	0.25 ± 0.006 (0.21–0.3)	0.35 ± 0.08 (0.21–2.66)	0.27 ± 0.006 (0.23–0.45)	0.29 ± 0.02 (0.21–2.66)
	Head L.: Total L.	0.16 ± 0.003 (0.13–0.22)	0.15 ± 0.002 (0.13–0.16)	0.19 ± 0.042 (0.01–1.48)	0.16 ± 0.002 (0.13–0.18)	0.17 ± 0.012 (0.01–1.48)
	Total L.: Emb.M.	15.89 ± 1.004 A (0–23.01)	16.6 ± 0.35 AB (14.39–19.67)	14.26 ± 0.741 B (0–24.48)	14.29 ± 1.212 A (0–27.22)	15.06 ± 0.517 (0–27.22)
	Morph. Day	47.76 ± 0.676 A (41.45–55)	47.6 ± 0.42 AB (38–48)	48.54 ± 0.482 A (43–55)	45.69 ± 0.496 B (37–48)	47.35 ± 0.289 (37–55)

[a]Age = chronological (calendar) age of embryo (days).

[b]Values represent mean ± standard error with ranges in parentheses. Letters beside values (A-D) indicate differences between sites ($\alpha = 0.05$). L. = length, Eye L.: Head length = eye length / head length, Head L.: Total L. = head length / total length, Total L.: Emb. M. = total length / embryo mass, and Morph. Day = age as determined by morphological characteristics.

Table 5-4. Explanatory variables included in partial redundancy analysis evaluating relationship between organochlorine pesticide burdens in eggs and embryo morphometrics.

Variable[a]	Code	Measured as
cis-Chlordane	[CC]	ng/g yolk wet weight
cis-Nonachlor	[CN]	ng/g yolk wet weight
Dieldrin	[DL]	ng/g yolk wet weight
Hep. Epoxide	[HE]	ng/g yolk wet weight
o,p-DDD	[ODD]	ng/g yolk wet weight
Oxychlordane	[OX]	ng/g yolk wet weight
p,p'-DDD	[PDD]	ng/g yolk wet weight
p,p'-DDE	[PDE]	ng/g yolk wet weight
p,p'-DDT	[PDT]	ng/g yolk wet weight
Toxaphene	[TX]	ng/g yolk wet weight
trans-Chlordane	[TC]	ng/g yolk wet weight
trans-Nonachlor	[TN]	ng/g yolk wet weight
∑All OCP burdens	[TOC]	ng/g yolk wet weight
$N^{o.}$ OCPs measured	NOC	*n*
cis-Chlordane%	CC%	[CC] / [TOC] x 100
cis-Nonachlor%	CN%	[CN] / [TOC] x 100
Dieldrin%	DL%	[DL] / [TOC] x 100
Hep. Epoxide%	HE%	[HE] / [TOC] x 100
o,p-DDD%	ODD%	[ODD] / [TOC] x 100
o,p-DDT%	ODT%	[ODT] / [TOC] x 100
Oxychlordane%	OX%	[OX] / [TOC] x 100
p,p'-DDD%	PDD%	[PDD] / [TOC] x 100
p,p'-DDE%	PDE%	[PDE] / [TOC] x 100
p,p'-DDT%	PDT%	[PDT] / [TOC] x 100
Toxaphene%	TX%	[TX] / [TOC] x 100
trans-Chlordane%	TC%	[TC] / [TOC] x 100
trans-Nonachlor%	TN%	[TN] / [TOC] x 100

Table 5-5. Best five organochlorine pesticide (OCP) variables accounting for variation in embryo morphology, selected using redundancy analysis with forward selection and Monte Carlo permutation tests for significance.

Age[a]	OCP variable	LambdaA[b]	*P*	*F*
14	[OX]	0.20	0.012	12.31
	HE%	0.11	0.02	7.03
	TX%	0.1	0.028	8.48
	ODT%	0.05	0.178	3.87
	TN%	0.06	0.018	6.25
25	HE%	0.11	0.002	12.09
	CN%	0.06	0.01	7.79
	DL%	0.05	0.016	5.80
	[DL]	0.02	0.04	3.49
	PDD%	0.02	0.076	2.80
33	CC%	0.06	0.042	6.68
	DL%	0.08	0.012	9.85
	PDE%	0.03	0.082	4.00
	CN%	0.1	0.006	14.51
	PDD%	0.03	0.052	6.01
43	[CC]	0.07	0.002	11.11
	[PDT]	0.08	0.002	12.58
	NOC	0.05	0.002	9.60
	[DL]	0.02	0.108	2.14
	[HE]	0.01	0.064	2.84

[a]Age = chronological (calendar) age of embryo (days).

[b]LambdaA = amount of morphometric variance accounted for by explanatory variable.

Table 5-6. Best five organochlorine pesticide (OCP) variables that account for embryo morphological age and derived morphological parameters as determined by redundancy analysis with forward selection and Monte Carlo permutation tests for significance.

Age	Code	LambdaA	*P*	*F*
14	[OX]	0.21	0.004	14.66
	ODT%	0.08	0.064	5.41
	CN%	0.12	0.006	11.56
	OX%	0.07	0.026	6.67
	[HE]	0.04	0.012	4.58
25	HE%	0.12	0.002	14.46
	NOC	0.07	0.01	8.14
	[PDD]	0.03	0.044	3.63
	[CN]	0.01	0.154	2
	[OX]	0.03	0.056	4.14
33	CC%	0.04	0.054	4.94
	DL%	0.06	0.016	7
	PDD%	0.03	0.082	4.07
	PDE%	0.05	0.006	6.36
	CN%	0.06	0.004	8.39
43	PDT%	0.08	0.008	11.2
	[CC]	0.05	0.006	8.42
	[PDT]	0.04	0.018	6.7
	NOC	0.05	0.008	8.82
	[DL]	0.02	0.068	3.02

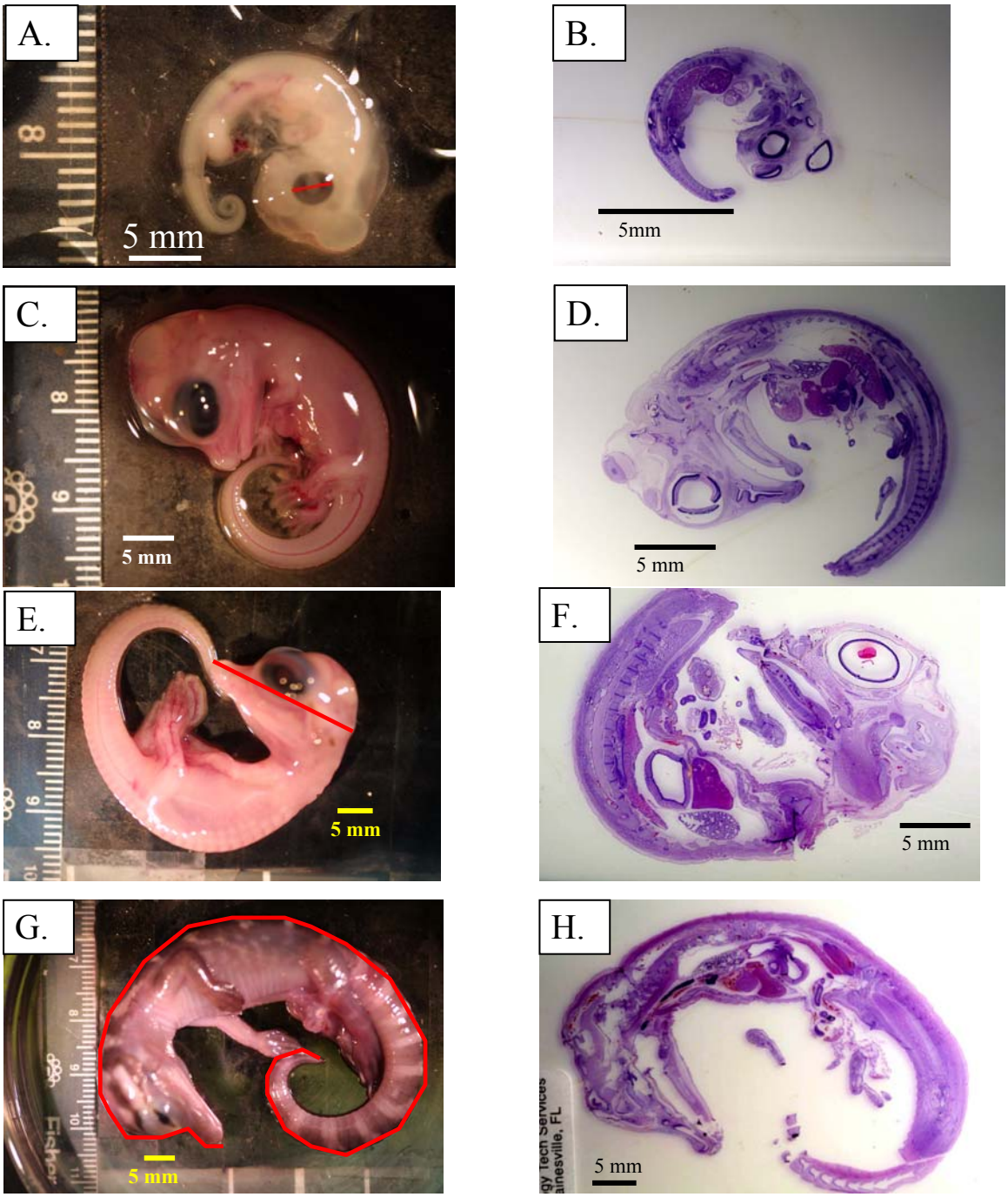

Figure 5-1. Representative developmental stages of embryos that were collected from Lakes Lochloosa (reference site), Apopka, and Griffin, and Emeralda Marsh during 2001-2002. A) Live embryo at Day 14 with red line indicating eye length. B) Saggital section of Day 14 embryo. C) Day 25 live embryo. D) Saggital section of Day 25 embryo. E) Day 33 live embryo with red line indicating head length. F) Saggital section of Day 33 embryo. G) Day 43 embryo with red line indicating total length. H) Saggital section of Day 43 embryo (organogenesis nearly complete).

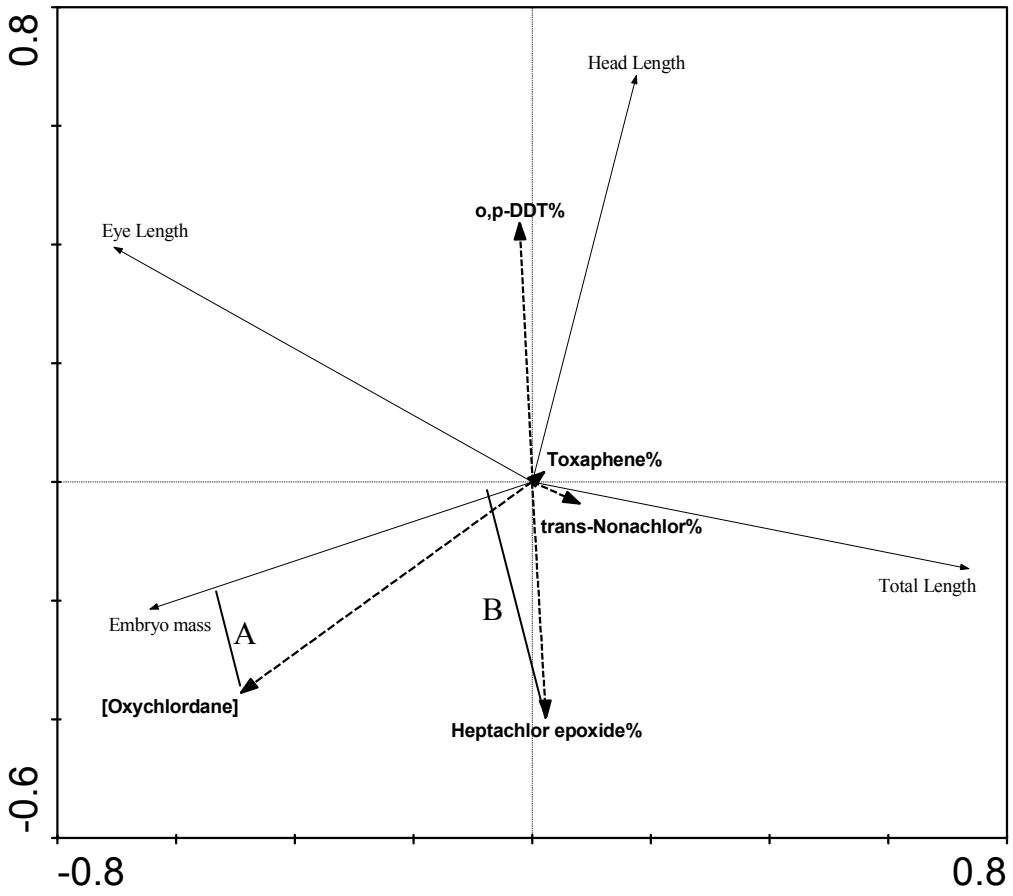

Figure 5-2. Ordination biplot of embryo morphometric parameters (solid lines) and organochlorine pesticide (OCP) variables (dashed lines) for embryos collected at chronological age Day 14. Arrows pointing in the same direction indicate a positive correlation (e.g., embryo mass and [oxychlordane), arrows that are approximately perpendicular indicate near-zero correlation, and arrows pointing in opposite directions indicate negative correlations (head length and [oxychlordane]. Arrow lengths indicate rank order of correlations. For example, extending a perpendicular line (A) from the embryo mass axis to tip of [oxychlordane] arrow indicates that [oxychlordane] has a stronger positive correlation with embryo mass than heptachlor epoxide% (B). The cosine of the angle formed at the origin between individual clutch variables and individual OCP variables is the correlation coefficient (r). For example, if arrows pointing in exactly opposite directions have an angle of 180°, and cos(180) = -1.0, then the arrows would be perfectly, negatively correlated (r) (ter Braak, 1995).

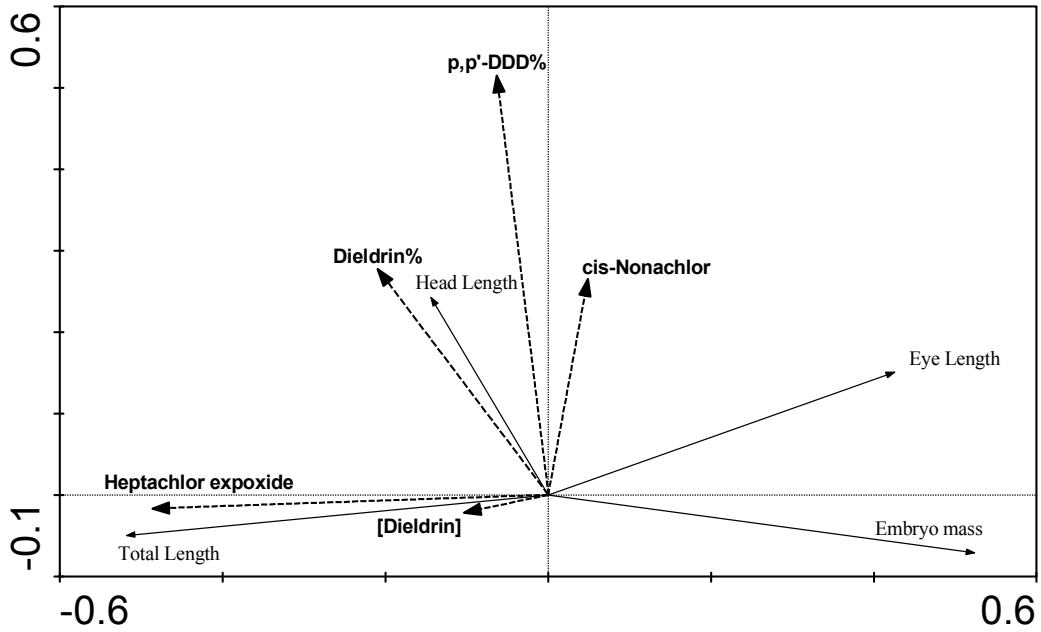

Figure 5-3. Ordination biplot of embryo morphometric parameters (solid lines) and organochlorine pesticide (OCP) variables (dashed lines) for embryos collected at chronological age Day 25 .

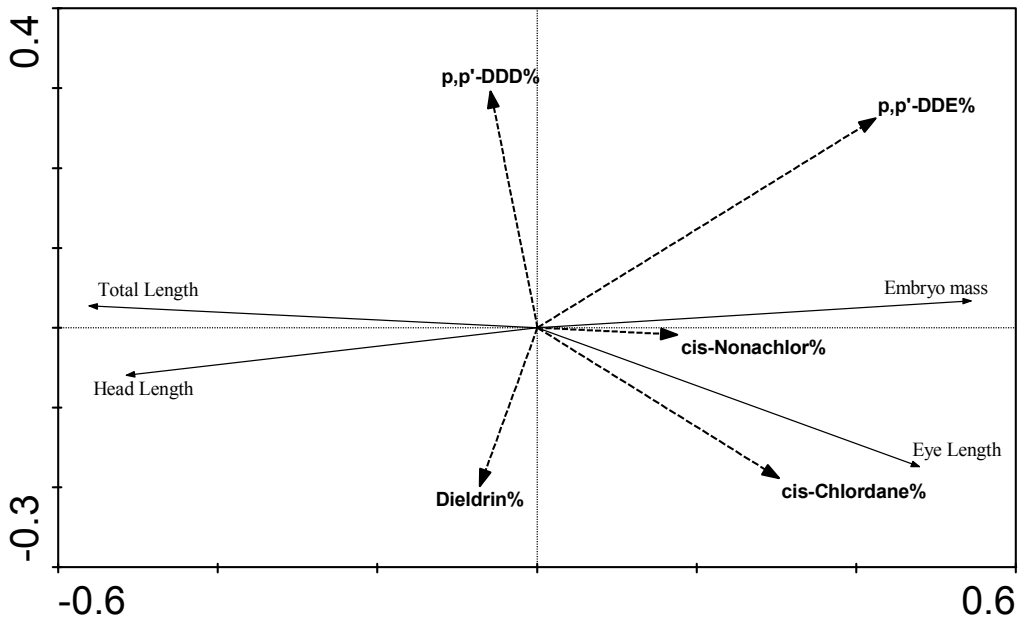

Figure 5-4. Ordination biplot of embryo morphometric parameters (solid lines) and organochlorine pesticide (OCP) variables (dashed lines) for embryos collected at chronological age Day 33.

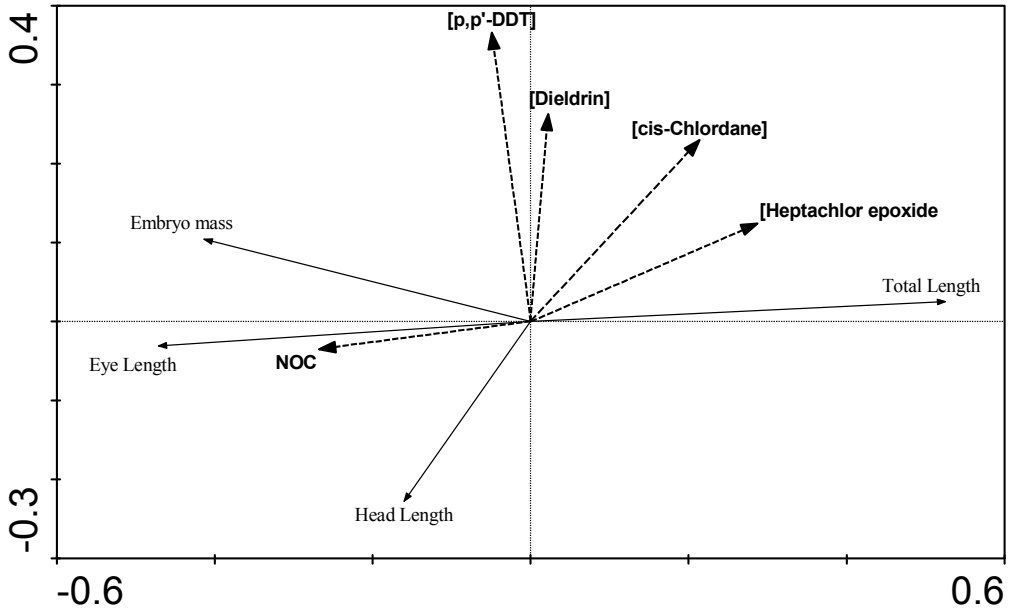

Figure 5-5. Ordination biplot of embryo morphometric parameters (solid lines) and organochlorine pesticide (OCP) variables (dashed lines) for embryos collected at chronological age Day 43.

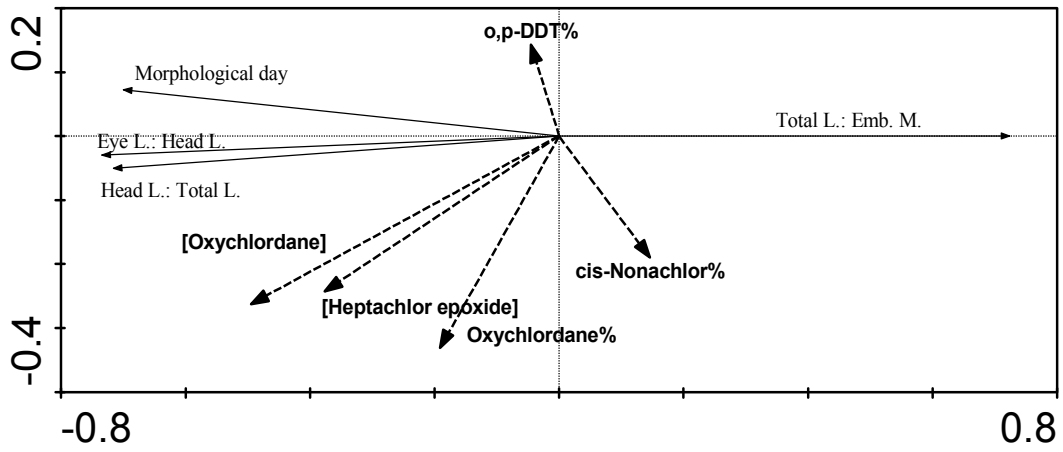

Figure 5-6. Ordination biplot of derived embryo morphometric parameters (solid lines) and organochlorine pesticide (OCP) variables (dashed lines) for embryos collected at chronological age Day 14.

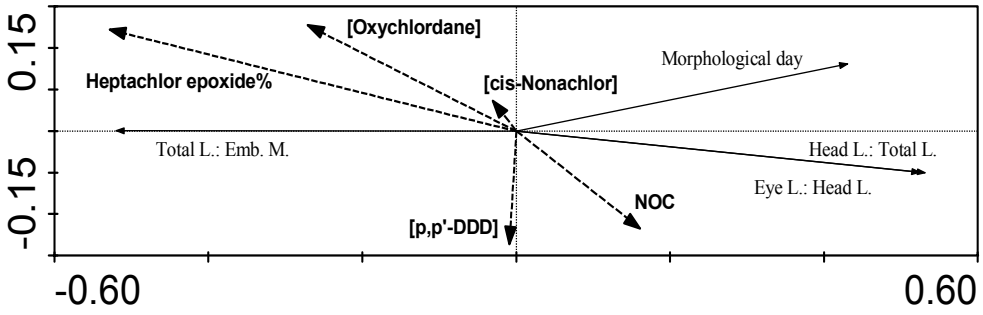

Figure 5-7. Ordination biplot of derived embryo morphometric parameters (solid lines) and organochlorine pesticide (OCP) variables (dashed lines) for embryos collected at chronological age Day 25.

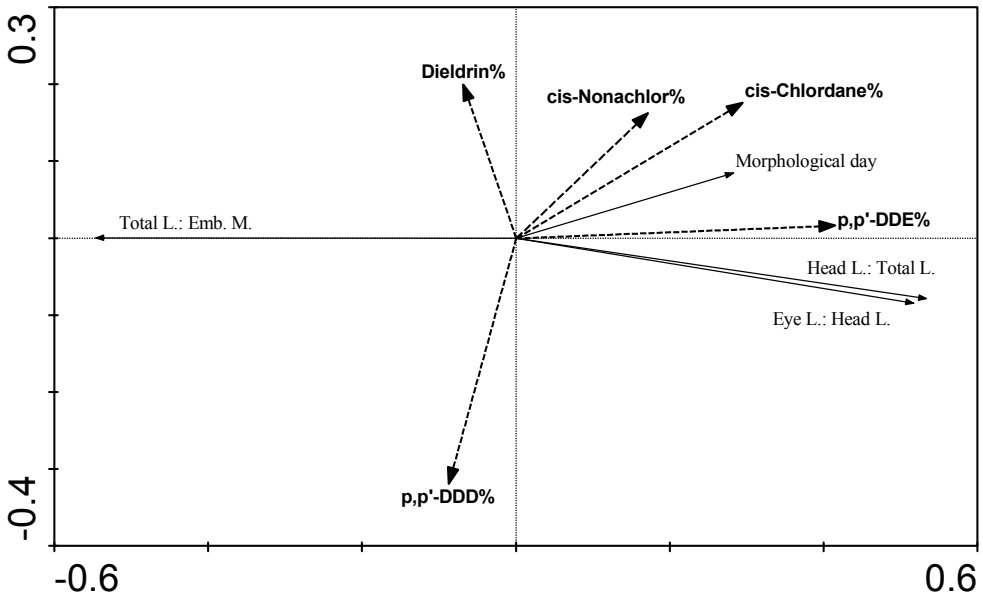

Figure 5-8. Ordination biplot of derived embryo morphometric parameters (solid lines) and organochlorine pesticide (OCP) variables (dashed lines) for embryos collected at chronological age Day 33.

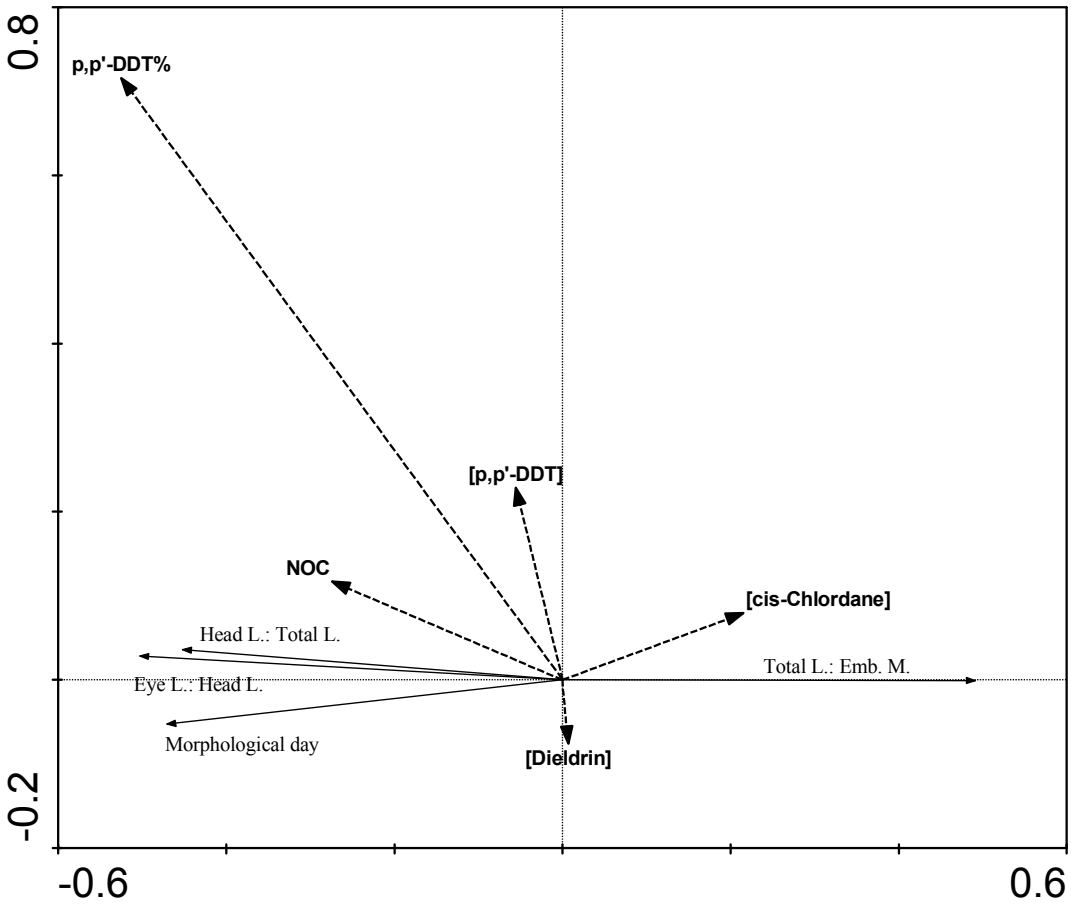

Figure 5-9. Ordination biplot of derived embryo morphometric parameters (solid lines) and organochlorine pesticide (OCP) variables (dashed lines) embryos collected at chronological age Day 43.

CHAPTER 6
NUTRIENT AND CHLORINATED HYDROCARBON CONCENTRATIONS IN AMERICAN ALLIGATOR EGGS AND ASSOCIATIONS WITH DECREASED CLUTCH VIABILITY

In central Florida, American alligator (*Alligator mississippiensis*) populations inhabiting lakes contaminated with organochlorine pesticides (OCPs) have poor reproductive success, primarily due to increased embryo mortality (Woodward et al., 1993; Woodward et al., 1989). During 2000-2002, clutch viability (percentage of eggs that yield a live hatchling) was monitored on 168 clutches from reference and OCP-contaminated sites, indicating that clutches from a reference site, Lake Lochloosa-Orange, had higher clutch viability (mean clutch viability = 70%) as compared than the OCP-contaminated sites, Lake Apopka (51%), Emeralda Marsh Restoration Area (48%), and Lake Griffin (44%). Furthermore, 115 of these clutches were analyzed for OCPs, and results indicated that alligators inhabiting Emeralda Restoration Marsh (total average egg OCPs = 15,480 ng/g), Lake Apopka (7,582 ng/g), and Lake Griffin (1,169 ng/g) contained significantly higher OCP burdens in eggs compared to those of Lake Lochloosa-Orange (102 ng/g) (Chapter 2). Although total embryo mortality was highest in eggs from sites with high OCPs, the amount of variation in embryo mortality rates explained by OCP egg burdens differs among OCP-contaminated sites (Chapter 2), suggesting the presence of additional factor(s).

With respect to vertebrates, examples of non-OCP factors that have been associated with increased embryo mortality include nutritional deficiencies and excesses (Wilson, 1997; McEvoy et al., 2001), exposure to polychlorinated biphenyls (PCBs) (Summer et

al., 1996), and exposure to polyaromatic hydrocarbons (PAHs) (Hoffman, 1990). For example, early-life stage (embryo) mortality has been associated with: thiamine deficiency in trout and salmon (Fitzsimons et al., 1999); PCB exposure in chickens (Summer et al., 1996); and PAH exposure in mallard eggs (Hoffman & Gay, 1981).

More recent data suggested that thiamine deficiency may be involved in the increased incidence of embryo mortality in American alligators inhabiting the aforementioned OCP-contaminated lakes in central Florida. Indeed, thiamine concentrations in egg yolks were positively correlated with clutch viability and accounted for 40% of variation in clutch viability among Lakes Lochloosa, Griffin, Apopka, and Emeralda Marsh (Sepúlveda et al., 2004). However, further investigation into thiamine's potential role in embryo mortality is warranted before any conclusions are drawn. Reasons for further study are that only five clutches were sampled per site, sampling occurred during a single nesting season (2000), and the potential role of other nutrients (i.e., vitamin E) and contaminants (i.e., PCBs) were not evaluated. With respect to other vitamins, vitamin E (tocopherol) has been suggested as having a potential role in the reduced clutch viability of captive alligators from Louisiana (Lance et al., 1983). Lastly, besides the embryotoxic effects of PCBs and PAHs, studies indicate that these contaminants may reduce thiamine storage in laboratory animals (Yagi et al., 1979), and that the presence of high contaminant burdens may affect thiamine's role in the production of metabolic energy (de Roode et al., 2002a). Together these data suggest the need for a detailed examination of contaminant burdens and nutrient content of eggs, and their association with clutch viability and embryo mortality in American alligators from OCP-contaminated sites in Florida.

The present study's specific aims were to conduct a case-control, cohort study to examine the relationship between multiple nutrients and contaminants, to further examine hypotheses derived from the case-cohort study via an expanded field study, and to test hypotheses derived from the expanded field study using laboratory experiments.

Materials and Methods

Egg Collections and Incubation

Alligator eggs were collected during 2001, 2002, and 2003 nesting seasons (June-July) from the following OCP-contaminated sites: Lakes Apopka (N 28° 35', W 81° 39'), Griffin (N 28° 53', W 81° 46'), and Emeralda Marsh Conservation Area ((N 28° 55', W 81° 47'), and from a reference site, Lake Lochloosa (N 29° 30', W 82° 09') in central Florida. Alligator nests were located via aerial (helicopter) and ground surveys (airboat), and clutches were subsequently collected by ground crews. The top of each egg was marked before eggs were removed from the nest to ensure proper orientation; thus, preventing embryo mortality due to inversion. Embryo mortality due to inversion occurs because, once an embryo has attached to the top of the egg, inverting the egg's orientation may either break embryonic attachment or cause the yolk mass to settle on top of the embryo, crushing it.

After marking each egg and placing about 5 cm of nest substrate in a uniquely numbered plastic pan (43 cm x 33 cm x 18 cm), all eggs found in each clutch were placed in the pan in five rows with six eggs per row. If a clutch contained more than 30 eggs, a second layer of nest substrate was added and the remaining eggs were collected. The top layer of eggs was covered with nest substrate so that there was no space left between the top of the pan and the top of the eggs (approximately 10 cm). Clutches were transported to the US Geological Survey's Center for Aquatic Resources Studies in Gainesville, FL.

Upon arrival, clutches were evaluated for embryonic viability using a bright light candling procedure. One or two eggs were opened from each clutch to identify the embryonic stage of development at the time of collection, and to collect yolk samples for later measurement of OCP, PAH, and PCB burdens and selected nutrient content with all yolk and albumin samples being stored at -80 °C. From each clutch, the following parameters were measured: total number of eggs per nest (fecundity); number of unbanded eggs, number of damaged eggs, number of dead, banded eggs, number of live banded eggs, total clutch mass, and average egg mass of clutch. Viable eggs (i.e. having a visible band) were nested in pans containing moist sphagnum moss and incubated at 30.5ºC and ~98% humidity in an incubation building (7.3 m x 3.7 m). This intermediate incubation temperature will normally result in a 1:1 male/female sex ratio, as alligators have temperature dependent sexual differentiation (Ferguson, 1985). On a daily basis, temperature and humidity were monitored at several locations throughout the incubator, clutches were rotated within the incubator, and air was circulated to mitigate any thermal gradients. Eggs were monitored for viability via bright-light candling every 10 days during incubation. Clutches collected during 2001 and 2002 were used for the field study and those collected during 2003 were used for the laboratory experiment. Experimental Design

Field studies

A case-control cohort study was conducted that involved the selection of clutches based upon their viability and their OCP egg burdens. Clutches were assigned to one of nine possible categories based on clutch viability and OCP egg burdens (Table 6-1). The purpose of the case-control cohort study was to determine if PAH, PCB, zinc, selenium, vitamins A, E, and B_1 concentrations differed or showed trends among clutch viability-

OCP categories. Selenium (Spallholz & Hoffman, 2002), zinc, vitamins A, E, (Ashworth & Antipatis, 2001) and B_1 (de Roode et al., 2002b)were examined because they are important for embryo development and survival, and their activity and/or levels may be affected by chlorinated hydrocarbons. This strategy aided in forming hypotheses related to the association between non-OCP factors and embryo mortality and the relationship between non-OCP factors and OCP exposure. For example, if increasing levels of a non-OCP factor showed a strong positive association with embryo viability, regardless of OCP burden, and levels did not differ between OCP exposure groups, then it could be hypothesized that the potential effects were likely related to the non-OCP factor(s) and unrelated to OCP exposure(s). Conversely, if increasing levels of the non-OCP factor(s) showed a strong positive association with embryo viability, but only with respect to low OCP exposure groups, then it could be hypothesized that the potential effects were likely due to a combination of OCP exposure and the level of the non-OCP factor(s).

Based on the case-control cohort study, hypotheses were derived that focused on the major non-OCP factors associated with embryo mortality and OCP exposure. Lastly, results of this expanded field study were used to design an egg treatment experiment to examine the hypotheses in a more controlled setting.

Laboratory experiments

In 2003, laboratory experiments were conducted using clutches collected from Lakes Dexter and Griffin, and from Emeralda Marsh. Based upon the case-control cohort study and the expanded field study (see results), the purpose of this experiment was to test the hypothesis that increasing thiamine levels in eggs would result in decreased embryo mortality. This experiment consisted of increasing thiamine concentrations in eggs that were known to have low thiamine concentrations, moderate to high yolk OCP

concentrations (Lakes Griffin and Emeralda Marsh), and high embryo mortality. Thiamine HCL was applied at high (60 mg thiamine/mL dimethyl sulfoxide, DMSO) and low concentrations (12 mg/mL DMSO) over the surface of each egg (application volume of 50 µl) using a micropipette. Controls received only vehicle treatment (DMSO). These doses were calculated to achieve yolk thiamine concentrations similar to those measured from the reference site (Lakes Orange-Lochloosa complex). Eggs from each site were labeled and randomLy distributed among each treatment group, so that all clutches were equally represented in the study. There were two replicates per treatment with a minimum of 26 eggs (maximum of 31) per replicate. After being dosed, eggs were placed in the incubator, and candled weekly to determine effects on embryo and hatchling survival. For Emeralda Marsh clutches, three eggs from each replicate were sampled 7 days after treatment to determine the amount of thiamine present in albumin and yolk. Embryo mortality rates were recorded for each treatment group as the percentage of eggs failing to hatch over the number of eggs treated.

The second experiment's purpose was to test the hypothesis that, in the absence of high OCP exposure, decreased thiamine bioactivity (functional deficiency) would result in increased embryo mortality rates. This experiment involved inducing decreases in thiamine bioactivity in eggs known to have relatively high thiamine concentrations, low OCP burdens, and low embryo mortality. Since clutches from Lake Lochloosa-Orange were assigned to another study during 2003, eggs were collected from another reference site (Lake Dexter, N 29º 98', W 81º 47'). To decrease thiamine bioactivity, oxythiamine-HCL, a thiamine antagonist (Akerman et al., 1998), was topically applied at concentrations of 12 or 60 mg/mL using DMSO as the carrier. Controls received only

DMSO. Eggs were labeled and randomLy distributed among each treatment group, so that all clutches were equally represented. There were two replicates per treatment with a minimum of 20 eggs (maximum of 21) per replicate. After being dosed, eggs were placed in the incubator, and candled weekly to determine effects on embryo and hatchling survival. Hatch rates for each replicate were determined as the percentage of eggs that produced a live hatchling.

Analysis of Chlorinated Hydrocarbons in Yolk

Analytical grade standards for the following compounds were purchased from the sources indicated: aldrin, alpha-benzene hexachloride (α-BHC), β-BHC, lindane, δ-BHC, *p,p'*-dichlorodiphenyldichloroethane (*p,p'*-DDD), *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), dichlorodiphenyltrichloroethane (*p,p'*-DDT), dieldrin, endosulfan, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor epoxide, hexachlorobenzene, kepone, methoxychlor, mirex, *cis*-nonachlor, and *trans*-nonachlor from Ultra Scientific (Kingstown, RI, USA); *cis*-chlordane, *trans*-chlordane, and the 525, 525.1 polychlorinated biphenyl (PCB) Mix from Supelco (Bellefonte, PA, USA); oxychlordane from Chem Service (West Chester, PA); *o,p'*-DDD, *o,p'*-DDE, *o,p'*-DDT from Accustandard (New Haven, CT, USA); and toxaphene from Restek (Bellefonte, PA, USA). All reagents were analytical grade unless otherwise indicated. Water was doubly distilled and deionized.

Egg yolk samples were analyzed for chlorinated hydrocarbon content using methods modified from Holstege et al. (1994) and Schenck et al. (1994). For extraction, a 2 g tissue sample was homogenized with ~1 g of sodium sulfate and 8 mL of ethyl acetate. The supernatant was decanted and filtered though a Büchner funnel lined with Whatman #4 filter paper (Fisher Scientific, Hampton, NH, USA) and filled to a depth of

1.25 cm with sodium sulfate. The homogenate was extracted twice with the filtrates collected together. The combined filtrate was concentrated to ~2 mL by rotary evaporation, and then further concentrated until solvent-free under a stream of dry nitrogen. The residue was reconstituted in 2 mL of acetonitrile. After vortexing (30 s), the supernatant was applied to a C18 solid phase extraction (SPE) cartridge (pre-conditioned with 3 mL of acetonitrile; Agilent Technologies, Wilmington, DE, USA) and was allowed to pass under gravity. This procedure was repeated twice with the combined eluent collected in a culture tube. After the last addition, the cartridge was rinsed with 1 mL of acetonitrile which was also collected. The eluent was then applied to a 0.5 g NH2 SPE cartridge (Varian, Harbor City, CA, USA), was allowed to pass under gravity, and collected in a graduated conical tube. The cartridge was rinsed with an additional 1 mL portion of acetonitrile which was also collected. The combined eluents were concentrated under a stream of dry nitrogen, to a volume of 300 μL, and transferred to a gas chromatography (GC) vial for analysis.

GC/MS Analysis

Analysis of all samples was performed using a Hewlett Packard HP-6890 gas chromatograph (Wilmington, DE, USA) with a split/splitless inlet operated in splitless mode. The analytes were introduced in a 1 μL injection and separated across the HP-5MS column (30 m x 0.25 mm; 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA) under a temperature program that began at 60º C, increased at 10º C/min to 270º C, was held for 5 min, then increased at 25º C/min to 300º C and was held for 5 min. Detection utilized an HP 5973 mass spectrometer in electron impact mode. Identification for all analytes and quantitation for toxaphene was conducted in full scan mode, where all ions

are monitored. To improve sensitivity, selected ion monitoring was used for the quantitation for all other analytes, except kepone. The above program was used as a screening tool for kepone which does not optimally extract with most organochlorines. Samples found to contain kepone would be reextracted and analyzed specifically for this compound.

For quantitation, a five-point standard curve was prepared for each analyte ($r^2 \geq$ 0.995). Fresh curves were analyzed with each set of twenty samples. Each standard and sample was fortified to contain a deuterated internal standard, 5 µL of US-108 (120 µg/mL; Ultra Scientific), added just prior to analysis. All samples also contained a surrogate, 2 µg/mL of tetrachloroxylene (Ultra Scientific) added after homogenization. Duplicate quality control samples were prepared and analyzed with every twenty samples (typically at a level of 1.00 or 2.50 µg/mL of γ-BHC, heptachlor, aldrin, dieldrin, endrin, and *p,p'*-DDT) with an acceptable recovery ranging from 70 – 130%. Limit of detection ranged from 0.1-1.5 ng/g for all OCP analytes, except toxaphene (120-236 ng/g), and limit of quantitation was 1.5 ng/g for all analytes, except toxaphene (1500 ng/g). Repeated analyses were conducted as allowed by matrix interferences and sample availability.

Nutrient Analysis

Thiamine concentrations were measured in clutches collected during years 2001, 2002, and 2003. For analysis, samples were shipped overnight on dry ice (solid CO_2) to the USGS Leetown Science Center, Appalachian Research Laboratory in Wellsboro, PA. Thiamine concentrations were determined as described in (Brown et al., 1998). Briefly, a known amount of the frozen yolk sample was first placed in 2% trichloroacetic acid (TCA, Sigma, St. Louis, Missouri, USA) homogenization solution. The extract was then

washed with ethyl acetate:hexane (3/2, vol/vol, Sigma) to remove excess TCA. An aliquot of the washed solution was reacted with potassium ferricyanide (Sigma) to produce thiochrome derivatives. The resulting derivatives were separated on a Hamilton PRP-1 column (Alltech, Deerfield, Illinois, USA) and detected with a spectrofluorometer set at 375 nm excitation wavelength and 433 nm emission (Shimadzu, Columbia, Maryland, USA). Authentic standards of thiamine pyrophosphate, thiamine monophosphate and thiamine-HCL (ICN Biomedicals, Montreal, Quebec, Canada) were used to quantify the amount of thiamine in each sample.

In addition to thiamine analyses, samples from selected clutches collected during 2002 were sent to ABC Research Corp. in Gainesville, FL, and analyzed for vitamin A (carotene, retinol, and activity) (AOAC 960.45 and 941.15), vitamin E (tocopherol) (AOAC 948.26), zinc and selenium (AOAC 990.8) using AOCAC methods (Horwitz, 2000).

Data Analysis

For the case-control cohort study, expanded field study, and laboratory experiments ANOVA (PROC GLM; SAS Institute Inc., 2002) was used for inter-site and inter-group comparisons of summary clutch characteristics, with the Tukey test for multiple comparisons among sites and groups ($\alpha = 0.05$). Because relationships between response variables and explanatory variables (Table 6-2) in ecological studies are often complex with interactions occurring, an indirect gradient multivariate analysis method, Detrended Correspondence Analysis (DCA) (ter Braak, 1986) was used to initially evaluate data structure for the case-control cohort study, as well as the expanded field study. Two matrices were constructed for DCA, with the first representing the response variables clutch ID number x clutch parameters) and the second representing the explanatory

variables (clutch ID number x OCP burdens) (Table 6-3). DCA results indicated that a direct gradient, multivariate linear analysis, redundancy analysis (RDA) (Rao, 1964), was appropriate for the case-control cohort study and the expanded field study since the gradient lengths of the DCA ordination axes were never more than (approximately 2 standard deviations (ter Braak, 1995).

For the RDA, similar matrices were constructed with the exception that response variables measured as a percentage (i.e., clutch viability) and response variables measured as a number (i.e., clutch mass) were divided into separate matrices because percentage data were ln(x+1) transformed and not standardized, while continuous data were ln(x) transformed and standardized(ter Braak & Smilauer, 2002).

Automatic forward selection of the best four explanatory variables was for all RDA analyses and Monte Carlo permutation tests were used to determine significance (α = 0.05). DCA and RDA were conducted using the program CANOCO (ter Braak & Smilauer, 2002), and CANODRAW (ter Braak & Smilauer, 2002) was used to construct biplots of environmental variables and response variables to interpret relationships between clutch parameters (response variables) and explanatory factors.

Specific OCP analytes were removed from analysis if measurable concentrations were found in less than 5% of all clutches. Numerical data, such as fecundity, were log-transformed [ln(x)], while proportional data (clutch viability) were arcsine square root transformed to meet statistical assumptions and [ln (x+1)] transformed for RDA analysis .

Results

Field Study

Case-control cohort study

In 2002, 32 clutches were collected from Emeralda Marsh, and Lakes Apopka, Griffin, and Lochloosa. Of the 32 clutches, 20 were selected and each of the 20 was assigned to one of nine categories based on clutch viability and total OCP burdens in eggs (Table 6-1). Only seven categories were filled with six categories being represented by three clutches. The remaining category, "good viability-high OCP burden", was represented by two. Although the number of clutches within each category was not large, clutches assigned to good and intermediate viability categories had significantly greater viability rates compared to poor category clutches, which supports the assignment of these clutches to their respective categories. Similarly, clutches assigned to high, intermediate, and low OCP categories were significantly different from one another with respect to total OCP burdens, further supporting the statistical and biological validity of assigned categories (Table 6-4). Differences among OCP analytes were not determined.

In addition to the somewhat expected differences in clutch viability rates and OCP burdens among categories, significant differences were found with respect to total PCB burdens, total PAH burdens, thiamine monophosphate (TP), and thiamine pyrophosphate (TPP) in eggs (Table 6-4). Although total PCB and PAH burdens differed among categories, levels were below those known to elicit adverse effects on avian development (Summer et al., 1996). Vitamin A was not detected in any of the eggs, with the lack of detection likely due to the relatively higher limit of detection (0.3 ppm) compared to the other nutrients (e.g., 0.1 ppm for vitamin E). Since vitamin A was not detected or

quantified in any eggs, no conclusions can be reached regarding its potential role in embryo mortality in alligators.

No other significant differences were noted for non-OCP variables likely due in part to the relatively small sample sizes and considerable variation in values of clutch parameters. Since the purpose of the study was to develop hypothesis; some important non-significant differences should be pointed out. For example, mean values of total thiamine and free thiamine concentrations of good viability-low OCP clutches and poor viability-high OCP clutches were nearly four-fold those of intermediate viability-low OCP clutches (Table 6-4). This four-fold difference may suggest that reduced viability in clutches with low OCP burdens may be associated with reduced thiamine levels, and that poor viability in clutches with high OCP burdens may not be associated with reduced thiamine levels.

Redundancy analysis (RDA) with forward selection of best four explanatory variables (Table 6-3) provided a way to evaluate the relationships between the non-OCP variables and clutch variables, and allowed each clutch's site to be included in the analysis. Including site in the analysis aided in identifying whether site differences, as opposed to other factors, were related to clutch survival and related parameters. For the 20 clutches included in the RDA, thiamine monophosphate, TP, (lambda A = 26%) and thiamine pyrophosphate, TPP, (12%) were significantly correlated with clutch survival parameters, accounting for 38% of the variation in clutch survival parameters (Table 6-5).

Indeed, TP had a strong positive association with clutch viability and a strong negative association with early embryo mortality, while TPP had a strong negative association with late embryo mortality (Fig. 6-1).

These results are biologically plausible because TP and TPP are the bioactive forms of thiamine needed for the production of metabolic energy and deficiencies have been associated with intrauterine growth retardation in laboratory models (Roecklein et al., 1985). In contrast, the positive relationship between unbanded egg% and TPP (Fig. 6-1) has little biological implications because an embryo must be present for TPP to be produced. Important to note is that PAH and PCB burdens did not appear to be significantly associated with embryo survival parameters.

In contrast to clutch survival parameters, clutch size parameters (e.g., fecundity) appeared to be associated with the site, as three of the four extracted explanatory variables were the nominal site variables. Of these extracted variables, only Lochloosa was determined to be significantly associated with clutch size parameters (Table 6-6), accounting for 27% of the variation. Furthermore, Lochloosa clutches appear to have higher average egg masses and lower fecundity compared to other sites (Fig. 6-2).

Lastly, the relationship between nutrients and chlorinated hydrocarbons were examined via RDA. Interestingly, all four extracted explanatory variables, heptachlor epoxide concentration (lambda A = 18%), dieldrin% (17%), trans-chlordane concentration (15%), Lochloosa (site effect, 9%) were found to be significantly associated with nutrient levels in eggs, accounting for 59% of the variation in egg nutrient content (Table 6-7). Heptachlor epoxide concentrations had a strong negative correlation with thiamine pyrophosphate, but weak positive correlations with the other thiamine forms and nutrients. Dieldrin% had strong negative associations with free thiamine and total thiamine, but strong positive relationships with vitamin E, zinc, and selenium. Trans-chlordane concentrations had strong negative correlations with vitamin

E, zinc, and selenium and little to near-zero correlation with thiamine concentrations. Lochloosa clutches appeared to be associated with increasing thiamine concentrations and decreasing zinc, selenium, and vitamin E concentrations (Fig. 6-3).

In summary, results of the case-control cohort study suggest the main factors associated with reduced clutch viability and increased embryo mortality are decreasing thiamine concentrations (Table 6-5, Fig. 6-1), and that reductions in thiamine concentrations may be associated with organochlorine pesticides (Table 6-7, Fig. 6-3). Therefore, the expanded field study was designed in order to examine how clutch survival parameters vary as a function of OCP burdens and thiamine concentrations in eggs.

Expanded field study

The purpose of the expanded field study was to examine the relationships between thiamine and OCP concentrations in eggs and clutch viability and clutch size parameters. Since consistent methods were used for OCP and thiamine analysis, as well as for egg collections and incubation, data from year 2000 (Sepúlveda et al., 2004), was combined with data from years 2001 and 2002. Using a larger number of clutches (n = 72) over multiple nesting seasons increased ecological validity of conclusions, as well as power in testing the hypothesis that thiamine deficiency and OCP exposure are associated with altered clutch survival parameters and altered clutch size.

Clutches from the Lochloosa-Orange complex (n = 18), Emeralda Marsh (n = 19), and Lakes Apopka (n = 14) and Griffin (n=21). No significant differences were noted among sites with respect to clutch survival parameters, clutch size parameters, or the four thiamine parameters. However, biological significance should be noted in that Lochloosa-Orange complex clutches had mean clutch viability rates that were

consistently greater than all other sites by an average of 18%. Furthermore, Lochloosa-Orange clutches had lower embryo mortality rates that were less than all other sites, by an average of 8%. The paucity of statistically significant differences is likely due to the high variance of clutch survival in OCP-contaminated sites. Significant differences were found among sites with respect to many OCP analyte burdens in eggs. Indeed, mean total OCP burdens and number of OCP analytes detected at quantifiable levels significantly differed among all sites (Table 6-8).

RDA was used to evaluate the relationships between the many OCP and thiamine parameters (explanatory variables) and the clutch survival parameters (response variables) (Table 6-3). Initial RDA showed that embryo age at the time of collection was an important factor, but not a specific factor of interest. Further examination of age effects indicated that for all sites, phosphorylation of free thiamine increased with age (Fig. 6-4).

Therefore, another RDA was conducted using age as a covariate. The best four explanatory variables determined via this RDA accounted for 30% of the variation in clutch survival parameters and consisted of total thiamine concentration (lambda A = 16%), thiamine pyrophosphate (7%), thiamine monophosphate (4%), and methoxychlor% (3%), with all explanatory variables determined to be significant (Table 6-9).

Total thiamine (TT) and thiamine monophosphate (TP) were strongly and positively correlated with clutch viability, and negatively correlated with unbanded egg% and early embryo mortality but showed near-zero correlation with late embryo mortality. Thiamine pyrophosphate (TPP) was strongly and negatively correlated with late embryo mortality and had weak to near-zero correlations with remaining clutch survival

parameters. Methoxychlor% (ME%) had positive correlations with unbanded egg% and early embryo mortality and near-zero correlations with other clutch survival parameters (Fig. 6-5).

In addition to clutch survival parameters, redundancy analysis was used to examine relationships between clutch size variables and explanatory variables. Results of the RDA indicated that two of four extracted variables were found to be significant and explained 15% of the variation in clutch size parameters. Extracted variables found to be significantly associated with clutch size variables included free thiamine (lambda A = 9%) and thiamine pyrophosphate (6%). Site effect may be important regarding variation in clutch size parameters, as the nominal variable "GR" (Lake Griffin) approached significance (Table 6-10).

Interestingly, all thiamine forms were positively associated with egg weight and negatively associated with fecundity. Thiamin pyrophosphate was negatively correlated with Griffin (meaning clutches from Lake Griffin had reduced levels of TPP), and had near-zero correlations with clutch mass. Total thiamine and free thiamine had strong, negative correlations with clutch mass and near-zero correlations with GR (Fig. 6-6).

Lastly, redundancy analysis was used to examine the relationship between the various thiamine forms (response variables) and explanatory variables to see if thiamine deficiency was associated with OCP variables or other clutch variables. Results indicated that four extracted variable were significantly correlated with thiamine concentrations and accounted for 31% of the variation in thiamine levels. Interestingly, lipid content of eggs (%) accounted for 16% of thiamine variation, followed by mirex concentrations (5%), trans-chlordane concentrations (6%), and oxychlordane concentrations (4%) (Table

6-11). Lipid content had strong negative correlations with free thiamine and total thiamine and positive correlations with thiamine mono- and pyrophosphate. Trans-chlordane concentrations were positively correlated with thiamine pyrophosphate and negatively correlated with the remaining thiamine forms. Oxychlordane and mirex concentrations in eggs were positively correlated with thiamine monophosphate, had near-zero correlations with total and free thiamine, and weak negative correlations with thiamine pyrophosphate (Fig. 6-7).

In summary, results of the expanded field study suggested that thiamine concentrations and certain OCP variables accounted for a significant amount of the variation in clutch survival and size characteristics, supporting the hypothesis that thiamine deficiency and OCP exposure contributes to decreased clutch viability and altered clutch size characteristics (Figs. 6-6; 6-5). Furthermore, decreasing thiamine levels were associated with increasing lipid content in egg yolks, suggesting that alterations in yolk composition are occurring and may be indicative of altered maternal liver function, possibly due to a number of reasons including OCP exposure and female age. In addition, alterations may be related to dietary factors. Indeed, altered liver function, leading to altered yolk composition has been documented in laboratory studies in catfish exposed to similar pesticides (Lal & Singh, 1987), and diets and body condition of alligators have been suggested to differ among two of the lakes included in the present study (Lakes Apopka and Griffin) in central Florida (Rice, 2004).

Laboratory Experiments

Because the case-control cohort and expanded field studies supported the hypothesis that thiamine deficiency and OCPs are associated with altered clutch survival and clutch size parameters, two laboratory experiments were conducted to more directly

test this hypothesis. The purpose of the first experiment was to test the hypothesis that increasing *in ovo* thiamine concentrations would increase embryo survival (thiamine topical exposure experiment) in clutches with high OCP burdens, and the second experiment tested the hypothesis that decreasing thiamine concentrations (via thiamine activity inhibitor) would decrease embryo survival in clutches with low OCP burdens.

A total of 14 clutches were used in the two experiments, with clutches having relatively high embryo mortality, low thiamine levels in eggs, and intermediate (Lake Griffin, n = 5) to high OCP burdens in eggs (Emeralda Marsh, n = 5) used in the thiamine topical exposure study. Conversely, clutches (Lake Dexter, n = 4) having relatively low embryo mortality, high thiamine levels in eggs, and low OCPs were used in the oxythiamine (thiamine-antagonist) topical exposure study. Clutch characteristics differed significantly among sites with respect to fecundity, clutch mass, egg mass, many OCP analytes, total OCP burdens, and number of OCPs detected at quantifiable levels (Table 6-12).

Seven days after topical treatment, three eggs from three different clutches (same clutches sampled for all replicates) were analyzed to determine the amount of thiamine that was transferred into the egg. For Emeralda clutches, results indicated that total thiamine concentrations in egg albumin of the high and low thiamine treatment groups were significantly greater than controls. Indeed, total thiamine concentrations in albumin of the high thiamine and low thiamine treatment groups were over 40-fold and over 30-fold greater, respectively, than those of controls, confirming a significant increase in thiamine levels in these eggs. Thiamine concentrations in egg yolk of Emeralda clutches were also greater in high and low thiamine treatment groups in a dose-dependent manner,

but the difference was not significant, with thiamine concentrations in high treatment groups being only 1.1-fold greater than controls (Table 6-13). Similar results were noted for Lake Griffin clutches, with thiamine treatments showing a dose-dependent, but non-significant increase in thiamine concentrations. High treatment clutches had thiamine concentrations that were 1.2-fold those of controls (Table 6-13). Thiamine concentrations in egg yolk of control groups for both Emeralda and Griffin clutches were high than means reported in the expanded field study but still within respective ranges.

Changes in embryo mortality rates were the primary interest and analysis indicated no significant differences were noted between thiamine treatment groups and controls. However, Emeralda clutches from both thiamine treatment groups had embryo mortality rates which averaged 10% less than those of controls. However, for Lake Griffin clutches, thiamine treatments were associated with a 5-7% increase in embryo mortality (Table 6-13).

For the oxythiamine (thiamine antagonist) study using Lake Dexter clutches, no significant differences were noted between oxythiamine treatment groups and controls. Surprisingly, embryo survival of controls (mean ± standard error: 81 ± 9%) was slightly less than those of the low exposure groups (98 ± 2%), and high exposure groups (88 ± 8%). Oxythiamine concentrations were not measured because oxythiamine has physicochemical properties very similar to thiamine; therefore, transfer rates across the eggshell were assumed to be similar.

Discussion

The present study examined associations between egg nutrients, OCP egg burdens, and clutch survival and size characteristics using a three-tiered approach that identified potentially important associations, and then more rigorously examined hypothesized

associations using large field studies and laboratory experiments. The first tier of the present study, case-control cohort study, suggested that PAH and PCB concentrations , as well as non-thiamine nutrients, were not likely to be the cause of decreased clutch viability as their levels did not show large differences across sites, nor were they significantly associated with alter clutch survival parameters. In addition, the case-control study indicated that thiamine concentrations were significantly associated with clutch survival parameters and that the association suggested decreased thiamine levels in eggs were associated with decreased clutch viability, which is consistent with similar studies involving fish ((Fitzsimons et al., 1999). Lastly, as dieldrin% increased (i.e., the proportion of total OCP burden composed by dieldrin) thiamine levels decreased, suggesting that OCPs may be indirectly involved in decreased clutch viability via thiamine reduction, as OCP exposure has been suggested to decrease thiamine concentrations in laboratory models (Yagi et al., 1979).

Results of the expanded field study provided more support for the hypothesis that thiamine deficiency may be involved in decreased clutch viability and that OCP burdens and lipid content were significantly associated with variation of thiamine concentrations. However, the laboratory experiments, overall, did not support the hypothesis that thiamine is related to embryo viability in alligators as thiamine amelioration or inhibition did not altered embryo mortality rates. One potential reason for the lack of effects is that thiamine levels were already sufficient for adequate embryo survival and therefore increasing concentrations were biologically irrelevant. With the thiamine antagonist experiment, two potential reasons for the lack of effects are that oxythiamine may not have transferred into the yolk compartment and/or the concentration was not high enough

to inhibit thiamine activity to the point that effects were elicited. Although thiamine and oxythiamine treatment experiments were ineffective in the present study, similar studies involving in ovo treatments of fish eggs have been effective in demonstrating the effects of induced thiamine deficiency and thiamine amelioration on embryo and fry survival (Fitzsimons et al., 2001).

In conclusion, decreasing thiamine levels in eggs may be associated with decreased clutch success and lipid content, and OCP burdens may be associated with variation in thiamine concentrations. However, it should be noted that thiamine levels in eggs only explained 38% of the variation in clutch survival parameters in the case-control cohort study and 27% of the clutch survival variation in the expanded field study, which suggest that other factors are likely involved as well. Because of the lack of effects observed in the experimental studies, future studies should try to induce thiamine deficiency in eggs through maternal dietary restriction, especially since embryos are at a relatively advanced stage of development by the time oviposition occurs (Clarke, 1891). A concurrent study involving a captive adult alligator breeding population will be able to control for diet and examine relationships between maternal OCP exposure and thiamine levels in eggs.

Table 6-1. Classification matrix for clutches collected during 2002.

	Total OCP Burden[a]		
Clutch Viability	>3700	3700 ≥ x > 350	≥ 350
(100- 71%)	Good viab./High OCP	Good viab./ Inter. OCP	Good viab./Low OCP
(70-48%)	Inter. viab./High OCP	Inter. viab./ Inter OCP	Inter viab./Low OCP
(47-0%)	Poor viab./High OCP	Poor viab./ Inter. OCP	Poor viab./Low OCP

[a]ng/g yolk wet weight. Good and High = greater than mean + 1 standard deviation, Intermediate = mean ± 1 standard deviation, Low and Poor = less than mean–1 standard deviation.

Table 6-2. Reproductive, morphometric, and contaminant parameters measured on clutches of alligator eggs collected during summer 2000, 2001, and 2002.

Parameter	Definition	Measured as
Response variables		
Fecundity	Total No. of eggs in one clutch	*n*
Clutch mass	Total mass of eggs in one clutch	kg
Ave. Egg Weight	Clutch mass / Fecundity	g
Unbanded eggs% [a]	No. of unbanded eggs / fecundity x 100	Percentage
Early embryo mort.%	No. of deaths < dev. Day 35 / fecundity x 100	Percentage
Late embryo mort.%	No. of deaths ≥ dev. Day 35 / fecundity x 100	Percentage
Clutch Viability	No. eggs yielding live hatchling / fecundity x 100	Percentage
Explanatory variables		
[OCP analyte] in	ng OCP analyte / g egg yolk wet weight	ppb
OCP analyte%	[OCP analyte] / ∑ [OCP] x 100	Percentage
∑[PCBs] in egg yolk	ng PCBs / g egg yolk wet weight	ppb
∑[PAHs] in egg yolk	ng PAHs analyte / g egg yolk wet weight	ppb
Thiamine in egg yolk[b]	Pmoles / g egg yolk wet weight	pmol/g
Zn, Se, Vit. A, E[c]	ng analyte / g egg yolk wet weight	ppb

[a]An egg with no evidence of embryonic attachment. [b]Thiamine was measured in pmoles because various bioactive forms of were measured. [c]These analytes were only measured in clutches collected during year 2002.

Table 6-3. Explanatory variables included in RDA with forward selection of four best variables for case-control cohort and expanded field studies.

Variable[a]	Code
Embryo age at time of collection	Age
Lake Griffin	GR
Lake Apopka	AP
Lake Lochloosa-Orange	LO
Emeralda Marsh	EM
No. OCP analytes at measurable levels	NOC
∑ [OCP]	TOC
% Aldrin	ALD%
[Aldrin]	[ALD]
% *cis*-Chlordane	CC%
[*cis*-Chlordane]	[CC]
% *cis*-Nonachlor	CN%
[*cis*-Nonachlor]	[CN]
% Dieldrin	DL%
[Dieldrin]	[DL]
% Heptochlor epoxide	HE%
[Heptachlor epoxide]	[HE]
%Lipid content	LPC%
% Mirex	MX%
[Mirex]	[MX]
% *o,p*-DDT	ODDT%
[*o,p*-DDT]	[ODDT]
[Methoxychlor]	[ME]
% Methoxychlor	ME%
% *o,p*-DDD	ODDD%
[*o,p*-DDD]	[ODDD]
% Oxychlordane	OX%
[Oxychlordane]	[OX]
% *p,p*'-DDE	PDDE%
[*p,p*'-DDE]	[PDDE]
% *p,p*'-DDD	PDDD%
[*p,p*'-DDD]	[PDDD]
% *p,p*'-DDT	PDDT%
[*p,p*'-DDT]	[PDDT]
% *trans*-Chlordane	TC%
trans-Chlordane	[TC]
% *trans*-Nonachlor	TN%
[*trans*-Nonachlor]	[TN]
% Toxaphene	TX%
[Toxaphene]	[TX]
∑PCBs	[PCB]
∑PAHs	[PAHs]
Free Thiamine	FT

Table 6-3. Continued.

Variable[a]	Code
Thiamine monophosphate	TP
Thiamine pyrophosphate	TPP
Vitamin E	Vit.E
Zinc	Zn
Selenium	Se

[a]For the case-control cohort study, no OCP variables were included in RDA involving clutch survival or size parameters, since clutches were selected *a priori* based on total OCP egg burdens. OCP variables were included in the RDA evaluating the relationship between egg nutrients and chlorinated hydrocarbons. For the expanded field study, only thiamine variables and OCP variables were included after it was determined they were the more important explanatory factors (see results).

Table 6-4. Summary of clutch parameters on clutches collected during 2002.

Parameter[a]	Good-High	Good-Int.	Good-Low	Int.-Low	Poor-High	Poor-Int.	Poor-Low
N$^{o.}$ Clutches	2	3	3	3	3	3	3
Fecundity (*n*)	51 ± 5	49 ± 1.9	41 ± 2	49 ± 5.2	55 ± 5.8	46 ± 4.5	47 ± 0.9
Clutch mass	4 ± 0.5	3 ± 0.7	4 ± 0.2	4 ± 0.4	4 ± 0.4	4 ± 0.6	4 ± 0.1
Egg mass (g)	86 ± 0.8	69 ± 12.5	88 ± 6	86 ± 1.5	76 ± 6.4	78 ± 6.2	79 ± 0.3
Clutch viability	92 ± 3.4 A	80 ± 3.8 A	79 ± 4.3 A	60 ± 5 A	11 ± 9.4 B	15 ± 10.9 B	25 ± 13 B
Damaged eggs	0 ± 0	1 ± 1.3	0 ± 0	0 ± 0	2 ± 1.1	1 ± 1.1	0 ± 0
Unbanded eggs	6 ± 5.7	6 ± 1.5	10 ± 4.9	15 ± 4.2	25 ± 10.5	5 ± 2.7	21 ± 6.8
Early emb. mort.	2 ± 2.3	10 ± 5.1	12 ± 8.1	7 ± 3.3	54 ± 21.6	36 ± 26.9	30 ± 3.9
Late emb. mort.	0 ± 0	3 ± 1.4	0 ± 0	18 ± 4	8 ± 4.3	42 ± 21.8	24 ± 16.9
Dieldrin	248 ± 20.2	72 ± 47.8	6 ± 1.1	9 ± 5	157 ± 66	264 ± 134.8	16 ± 5.4
Hep.Epoxide	3 ± 0.8	11 ± 4.2	4 ± 2.3	2 ± 0.5	6 ± 2.6	4 ± 1.8	5 ± 1.7
cis-Chlordane	161 ± 14.9	15 ± 1.1	4 ± 1.3	7 ± 3.6	145 ± 69.7	25 ± 9.2	13 ± 0.8
cis-Nonachlor	82 ± 11.2	24 ± 4.1	7 ± 1.3	9 ± 4.2	89 ± 38.4	21 ± 6.3	11 ± 0.8
Oxychlordane	23 ± 4.1	25 ± 13.4	8 ± 5	4 ± 1.4	31 ± 6.8	14 ± 5.2	7 ± 2.8
Toxaphene	10289 ± 313.6	0 ± 0	0 ± 0	0 ± 0	4670 ± 1268.9	1928 ± 0	0 ± 0
p,p'-DDD	2614 ± 348.7	10 ± 4.9	3 ± 0.6	4 ± 0.7	897 ± 578.1	18 ± 4.8	4 ± 0.8
p,p'-DDE	19136 ± 3277.6	1167 ± 830.3	139 ± 30.9	117 ± 45.6	9149 ± 3668.7	1019 ± 795	153 ± 28.6
p,p'-DDT	24 ± 0	0 ± 0	0 ± 0	0 ± 0	10 ± 2.1	0 ± 0	0 ± 0
trans-Chlordane	51 ± 1.5	1 ± 0	1 ± 0	1 ± 0	34 ± 15.3	1 ± 0	2 ± 0.5
trans-Nonachlor	251 ± 46	55 ± 13.9	16 ± 4.7	15 ± 5.8	273 ± 141.4	41 ± 15.8	22 ± 2.8
∑[OCPs]	32959 ± 3891.1 A	1391 ± 923 B	188 ± 41.9 C	168 ± 67.9 C	15508 ± 5426.2 A	2057 ± 770.2 B	234 ± 42.5 C
NOC	14 ± 0	11 ± 0	10 ± 0.7	10 ± 0.9	14 ± 0.3	11 ± 0.7	11 ± 0
∑[PAHs]	21 ± 1.3 BC	33 ± 2.8 AB	34 ± 7.6 AB	31 ± 2.9 AB	18 ± 2.5 C	27 ± 3.5 ABC	43 ± 7.3 A
∑[PCBs]	39 ± 0 B	168 ± 28.9 A	83 ± 14.6 A	111 ± 47 A	40 ± 2.5 A	55 ± 10.7 A	92 ± 16 A
Selenium	1000 ± 100	1233 ± 176.4	1067 ± 66.7	1133 ± 166.7	1000 ± 57.7	933 ± 120.2	833 ± 145.3
TP	23 ± 5.7 AB	13 ± 1.6 AB	41 ± 8.4 A	24 ± 1.7 AB	2 ± 1.8 C	12 ± 6.7 BC	13 ± 6.4 AB
TPP	14 ± 3.6 A	0 ± 0.3 C	21 ± 3.1 A	16 ± 2.1 A	18 ± 8.1 AB	4 ± 3.8 BC	7 ± 5.3 AB
FT	463 ± 92.6	719 ± 427.9	868 ± 231.1	238 ± 40.6	891 ± 261.2	553 ± 482.3	326 ± 115.4
∑Thiamine	500 ± 83.3	733 ± 427.7	931 ± 220.0	278 ± 38.9	912 ± 267.4	569 ± 486.5	345 ± 104.3
Vit. E	16287 ± 2389.7	26397 ± 2978.6	19118 ± 7352.9	12255 ± 122.5	21054 ± 1889.2	20025 ± 5831	15221 ± 1784.5
Zinc	15900 ± 300	15433 ± 809	24900 ± 10570.9	15300 ± 750.6	15267 ± 437.2	12367 ± 800.7	12967 ± 788.1

[a]Codes for parameters are listed in Table 6-3.

Table 6-5. Evaluation of the relationship between concentrations of nutrients, PAHs, and PCBs in eggs and clutch survival parameters via RDA analysis ($\alpha = 0.05$).

Variable	Lambda A	P	F
Thiamine monophosphate	0.26	0.004	6.28
Thiamine pyrophosphate	0.12	0.014	3.95
Free thiamine	0.08	0.102	2.1
∑Thiamine forms	0.08	0.142	2.08

Table 6-6. Evaluation of clutch size parameters and explanatory factors for clutches collected during 2002.

Variable	Lambda A	P	F
Lochloosa	0.27	0.018	6.77
Apopka	0.06	0.198	1.54
PCB concentrations	0.05	0.272	1.25
Emeralda Marsh	0.07	0.164	1.81

Table 6-7. Evaluation of the relationship between nutrient concentrations and explanatory variables for clutches collected during 2002.

Variable	Lambda A	P	F
Heptachlor epoxide conc.	0.18	0.006	4.07
trans-Chlordane conc.	0.15	0.022	3.67
Dieldrin%	0.17	0.002	5.43
Lochloosa	0.09	0.048	3.13

Table 6-8. Summary and comparison of parameters measured on clutches collected during 2000-2002.

Parameter[a]	Loch.-Orange	Griffin	Apopka	Emeralda
No. clutches	18	21	14	19
Fecundity	40 ± 1.8	45 ± 1.8	47 ± 2	46 ± 1.8
	(26–56)	(24–58)	(31–56)	(34–64)
Clutch mass	3.6 ± 0.18	3.5 ± 0.19	4 ± 0.2	4 ± 0.38
	(2.2–4.8)	(1.8–4.8)	(2.6–4.9)	(2.3–9.2)
Egg mass	90 ± 3.1	78 ± 2.2	86 ± 3.5	87 ± 7.4
	(78–139)	(46–89)	(67–120)	(60–180)
Clutch viability	63 ± 5.6	40 ± 6.7	49 ± 8.6	46 ± 9.1
	(0–95)	(0–87)	(0–80)	(0–97)
Damaged%	4 ± 3.3	5 ± 2.5	2 ± 0.9	5 ± 1.8
	(0–60)	(0–46)	(0–12)	(0–27)
Unbanded%	11 ± 2	12 ± 2.3	13 ± 3.6	12 ± 3.5
	(0–33)	(0–32)	(0–40)	(0–58)
Early Emb. Mort.	13 ± 3	26 ± 6.3	17 ± 6.4	25 ± 6.7
	(0–36)	(0–93)	(0–90)	(0–95)
Late Emb. Mort.	8 ± 2.6	18 ± 4.9	19 ± 6.8	11 ± 3.8
	(0–34)	(0–58)	(0–77)	(0–61)
TP	20 ± 3.7	19 ± 3.8	20 ± 3.9	19 ± 5.2
	(0–52)	(0–72)	(0–60)	(0–67)
TPP	12 ± 2.6	7 ± 2.7	8 ± 3.6	11 ± 3.8
	(0–31)	(0–53)	(0–46)	(0–54)
FT	747 ± 102.8	536 ± 93.1	573 ± 110.8	657 ± 125.9
	(77–1324)	(109–1570)	(50–1412)	(57–2171)
TT	780 ± 102.8	562 ± 94.2	601 ± 111.1	688 ± 128.5
	(77–1364)	(152–1583)	(62–1431)	(57–2212)
ALD	0 ± 0	0 ± 0	3 ± 0.1	3 ± 0.3
	(0–0)	(0–0)	(3–3)	(3–4)
oDD	0 ± 0 C	1 ± 0 B	5 ± 2.3 B	47 ± 5.7 A
	(0–0)	(1–1)	(1–9)	(8–104)
oDT	1 ± 0 B	3 ± 0.4 B	10 ± 2.1 A	301 ± 290.9 A
	(1–1)	(1–6)	(1–29)	(4–4373)
ME	0 ± 0	17 ± 0.3	8 ± 2.6	10 ± 1.9
	(0–0)	(17–17)	(6–16)	(6–18)
MI	2 ± 0.4	2 ± 0.4	4 ± 1.4	3 ± 0.9
	(1–3)	(1–4)	(1–17)	(0–10)
DL	4 ± 0.5 D	20 ± 3.4 C	323 ± 66.8 A	186 ± 25.4 A
	(1–8)	(6–70)	(24–957)	(30–387)
HE	3 ± 0.8 C	6 ± 1.4 B	12 ± 2.2 A	6 ± 1.6 B

Table 6-8. Continued.

Parameter[a]	Loch.-Orange	Griffin	Apopka	Emeralda
	(1–10)	(1–30)	(1–30)	(0–29)
CC	2 ± 0.2 D	11 ± 0.7 C	46 ± 12.2 B	109 ± 15.6 A
	(1–4)	(6–17)	(7–179)	(15–281)
CN	5 ± 0.6 C	18 ± 2.4 B	61 ± 12.4 A	71 ± 8.9 A
	(2–13)	(8–54)	(10–171)	(17–166)
OX	4 ± 1.1 C	11 ± 2.1 B	38 ± 6.1 A	24 ± 3.4 A
	(1–18)	(1–42)	(4–72)	(3–57)
TX	0 ± 0 C	2678 ± 376.5 B	2738 ± 224.5 B	7558 ± 703.6 A
	(0–0)	(1928–3111)	(1896–3809)	(3216–12975)
pDD	2 ± 0.2 D	7 ± 1 C	49 ± 13.3 B	1711 ± 225.1 A
	(1–3)	(3–18)	(11–193)	(10–2963)
pDE	76 ± 12.3 D	283 ± 47.4 C	4576 ± 948.3 B	11304 ± 1872.1 A
	(28–231)	(70–979)	(18–13294)	(36–33555)
pDT	1 ± 0 C	2 ± 0.7 BC	9 ± 3.8 B	15 ± 1.7 A
	(1–1)	(1–2)	(1–46)	(6–25)
TC	3 ± 0.7 BC	2 ± 0.2 C	7 ± 2.2 B	31 ± 4.1 A
	(1–4)	(1–3)	(1–27)	(3–58)
TN	8 ± 1.7 C	37 ± 7.2 B	157 ± 36.8 A	208 ± 30.8 A
	(3–25)	(10–155)	(10–532)	(14–555)
TOC	104 ± 16.2 D	783 ± 264.9 C	6855 ± 1267.2 B	20417 ± 2969.9 A
	(43–289)	(127–4488)	(555–18471)	(672–53560)
NOC	9 ± 0.3 D	11 ± 0.2 C	13 ± 0.4 B	14 ± 0.2 A
	(7–11)	(10–13)	(10–15)	(13–16)

[a]See Table 6-3 for parameter codes. Values = mean ± standard error with range in parentheses.

Table 6-9. Evaluation of the relationships between clutch survival parameters and explanatory variables via RDA using age as the covariate.

Explanatory Variable	LambdaA	P	F
Total Thiamine	0.16	0.002	13.96
Thiamine Pyrophosphate	0.07	0.002	6.14
Thiamine Monophosphate	0.04	0.01	3.56
Methoxychlor%	0.03	0.036	2.71

Table 6-10. Evaluation of the relationships between clutch size parameters and explanatory variables via RDA using age as the covariate.

Explanatory Variable	LambdaA	P	F
Free Thiamine	0.09	0.012	6.52
Thiamine Pyrophosphate	0.04	0.01	4.82
GR	0.03	0.054	3.36
Total Thiamine	0.04	0.06	3.9

Table 6-11. Evaluation of the relationships between thiamine concentrations and explanatory variables via RDA using age as the covariate.

Variable	LambdaA	P	F
Lipid content %	0.16	0.002	13.4
Trans-chlordane concentrations	0.06	0.002	6.5
Mirex concentrations	0.05	0.024	4.23
Oxychlordane concentrations	0.04	0.048	3.62

Table 6-12. Site comparisons of parameters measured on clutches collected during 2003.

Parameter	Dexter	Griffin	Emeralda
N$^{o.}$ Clutches	4	5	5
Fecundity	37 ± 3.8 B	46 ± 1.2 AB	46 ± 1.9 A
Clutch mass	3 ± 0.5 B	4 ± 0.1 A	4 ± 0.1 AB
Egg mass	82 ± 4.1 B	93 ± 2.7 A	80 ± 2.6 B
Unbanded eggs	3 ± 2	6 ± 3	5 ± 3.4
Damaged eggs	0 ± 0	1 ± 0.5	4 ± 3.1
Dieldrin	5 ± 1.4	29 ± 9.3	188 ± 78.2
Hep. Epoxide	2 ± 0.4	8 ± 3.1	4 ± 1.5
cis-Chlordane	1 ± 0 B	1 ± 0 B	8 ± 2.6 A
cis-Nonachlor	6 ± 1.8 B	22 ± 7 AB	60 ± 19.8 A
Oxychlordane	4 ± 1 B	14 ± 5 AB	26 ± 10.7 A
Toxaphene	0 ± 0 B	0 ± 0 B	6765 ± 2240.4 A
o,p'-DDD	1 ± 0 B	0 ± 0 B	13 ± 0 A
o,p'-DDT	1 ± 0	3 ± 0.8	3 ± 0.4
p,p'-DDD	1 ± 0	3 ± 0.4	981 ± 407.8
p,p'-DDE	117 ± 28.1 B	399 ± 114.7 B	13166 ± 5918.5 A
p,p'-DDT	1 ± 0 B	2 ± 0.4 B	16 ± 5 A
trans-chlordane	1 ± 0	1 ± 0	3 ± 0.6
Endrin ketone	0 ± 0 B	0 ± 0 B	3 ± 0 A
Mirex	4 ± 1.3	2 ± 0.3	2 ± 0.5
trans-Nonachlor	10 ± 3.7 B	54 ± 19.9 AB	168 ± 60.3 A
∑OCP burdens	171 ± 38 B	556 ± 159.2 B	21410 ± 8499.4 A
N$^{o.}$ OCP analytes	12 ± 0.5 B	12 ± 0.4 AB	14 ± 0.5 A

Table 6-13. Comparisons of parameters measured on the three thiamine treatment groups during 2003.

			Treatment Group		
Site	Component	Parameter[a]	control	low	high
Emeralda	Albumin	FT (g/ng)	68 ± 9.2 B	2414 ± 464.3 A	3120 ± 56.8 A
			(58–77)	(1950–2878)	(3063–3176)
		TMP	2 ± 0.3 B	7 ± 1.2 A	6 ± 1 A
			(2–3)	(6–8)	(5–7)
		TPP	4 ± 2.5	9 ± 2.5	7 ± 1.2
			(1–6)	(6–11)	(6–8)
		TT	76 ± 5.6 B	2436 ± 459.2 A	3138 ± 53.7 A
			(70–82)	(1976–2895)	(3084–3191)
	Yolk	FT	1047 ± 54.5	1122 ± 80.7	1176 ± 4.3
			(992–1101)	(1041–1203)	(1172–1181)
		TMP	41 ± 5.5	31 ± 4.1	36 ± 3.1
			(35–46)	(27–35)	(33–39)
		TPP	21 ± 0.2	18 ± 2.8	24 ± 0.3
			(20–21)	(15–21)	(24–24)
		TT	1125 ± 60.5	1185 ± 71.8	1254 ± 8.4
			(1064–1185)	(1113–1257)	(1246–1263)
		Embryo Mort.	25 ± 1.5	12 ± 4.3	18 ± 9.5
			(23–26)	(8–17)	(8–27)
Griffin	Yolk	FT	1041 ± 115.9	1215 ± 29.9	1233 ± 18.7
			(925–1157)	(1185–1245)	(1214–1252)
		TMP	6 ± 0.6	9 ± 0.4	11 ± 4
			(6–7)	(9–9)	(7–15)
		TPP	2 ± 0.1	2 ± 0.6	4 ± 0.9
			(2–2)	(2–3)	(3–5)
		TT	1052 ± 116.7	1229 ± 28.6	1252 ± 24.7
			(935–1168)	(1200–1257)	(1227–1277)
		Embryo Mort.	36 ± 2.3	43 ± 10.1	41 ± 3.4
			(33–38)	(33–54)	(38–45)

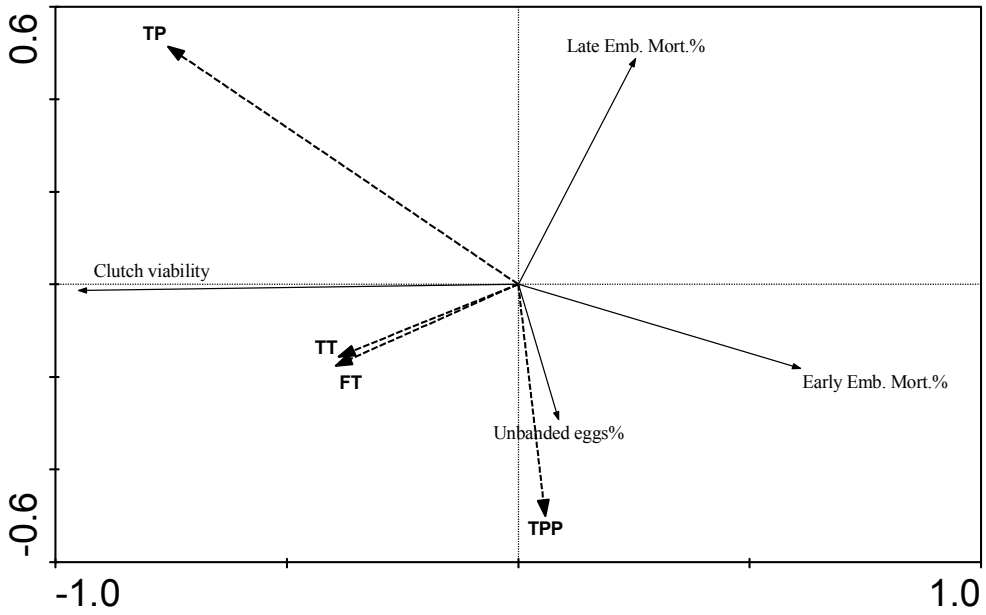

Figure 6-1. Biplot of clutch survival parameters and explanatory factors for clutches collected during 2002.

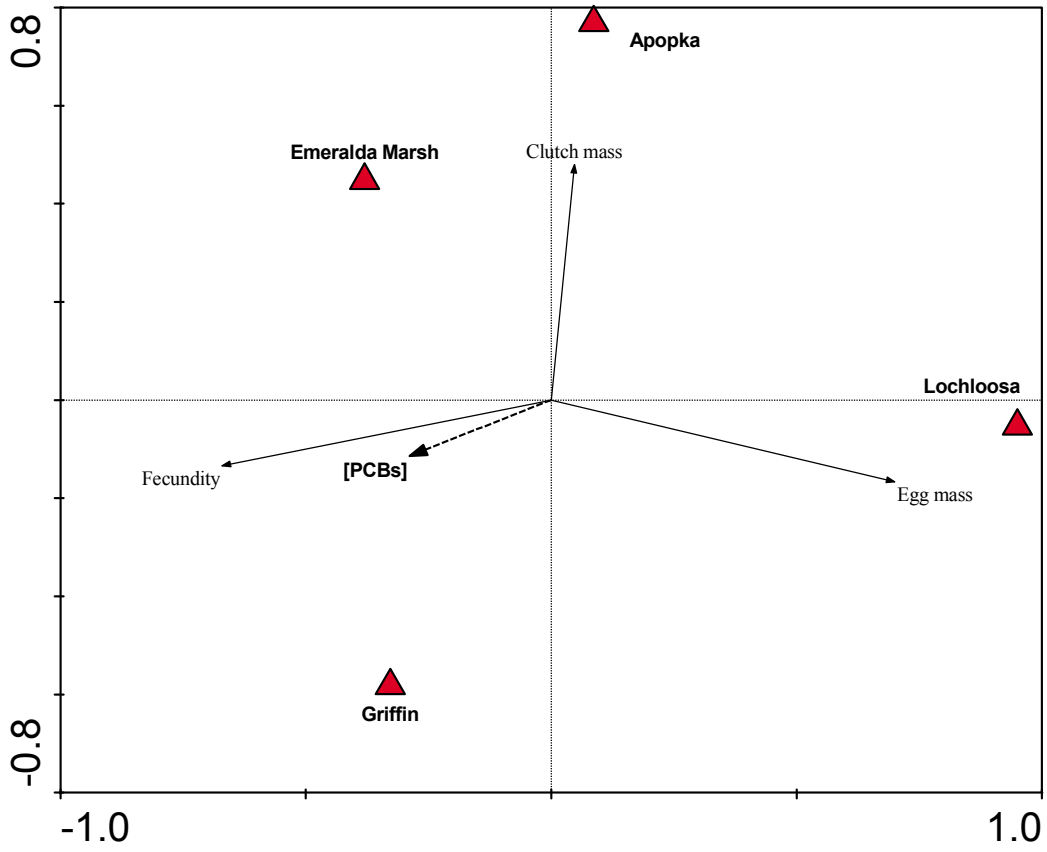

Figure 6-2. Biplot of clutch size parameters and explanatory variables for clutches collected during 2002.

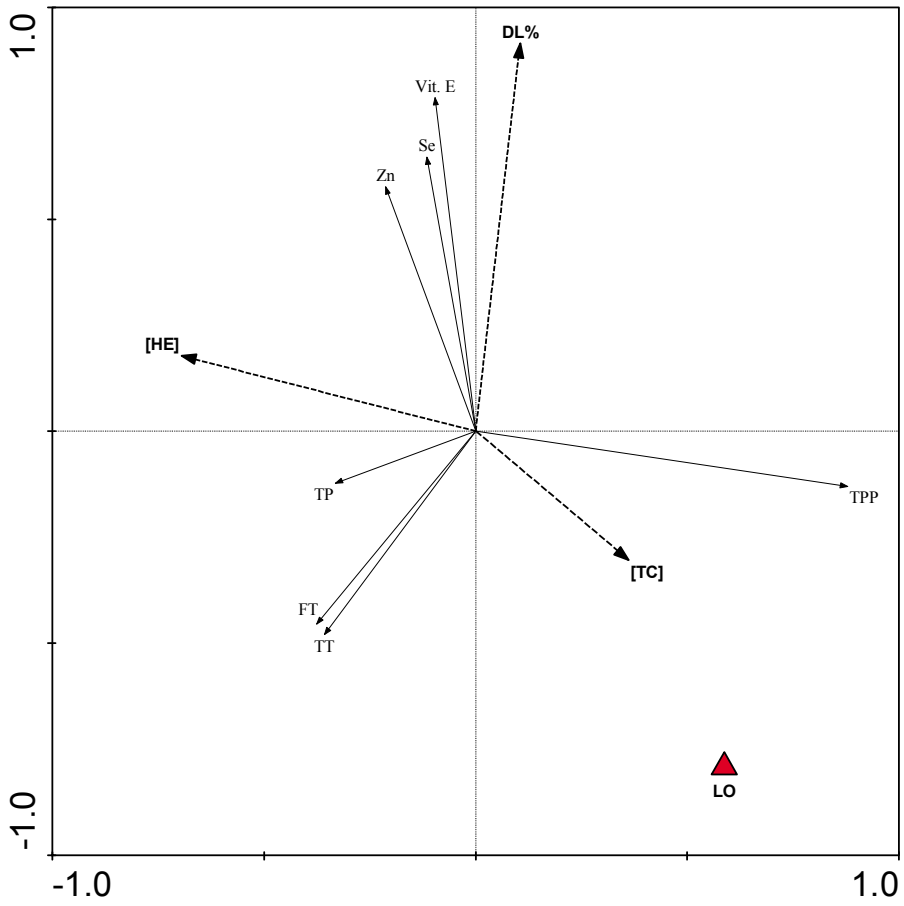

Figure 6-3. Biplot of nutrient concentrations in eggs (solid arrows) and explanatory variables (dashed arrows).

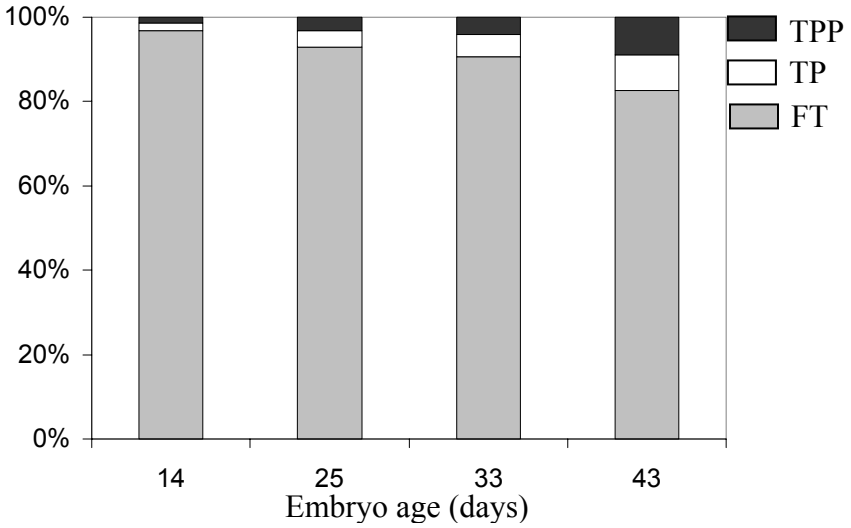

Figure 6-4. Relationships between embryo age and thiamine phosphorylation in egg yolk for 29 clutches collected during 2002 from Lakes Lochloosa (n = 6), Griffin (n = 10, Apopka (6), and Emeralda Marsh (n = 7).

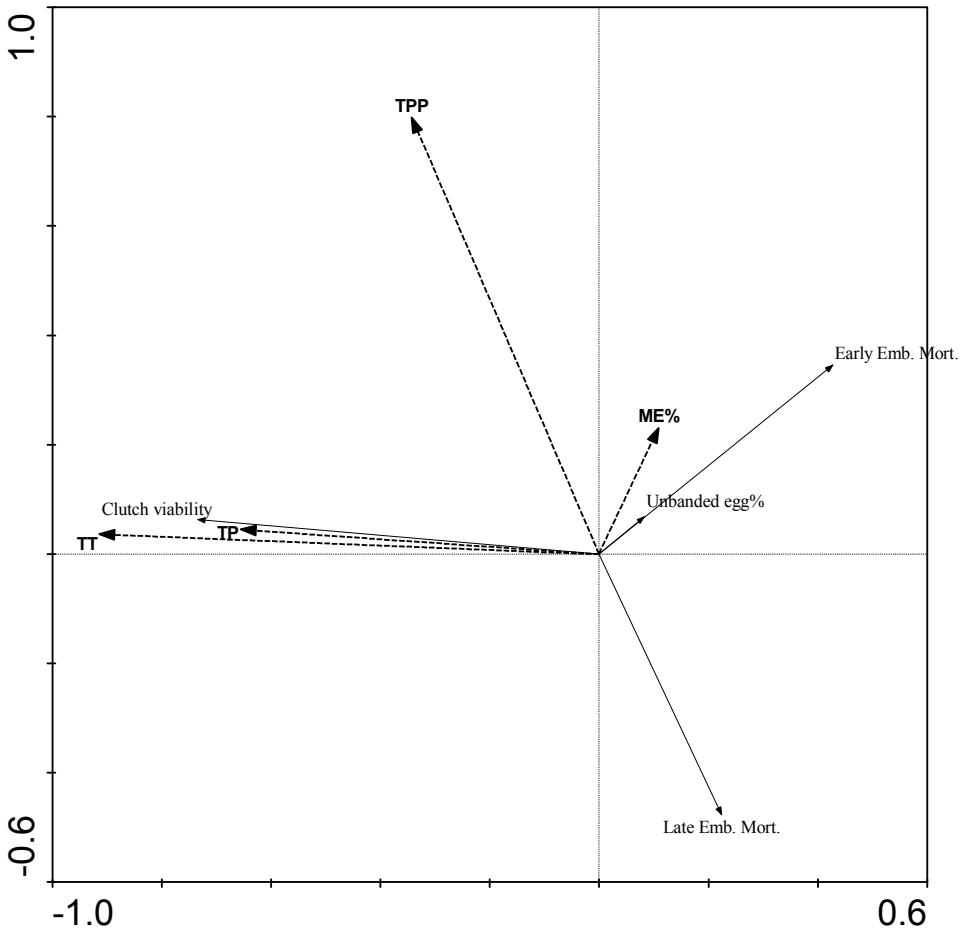

Figure 6-5. Biplot of clutch survival parameters and explanatory variables for clutches collected during 2000-2002. See text and Table 6-3 for definition of explanatory variable codes.

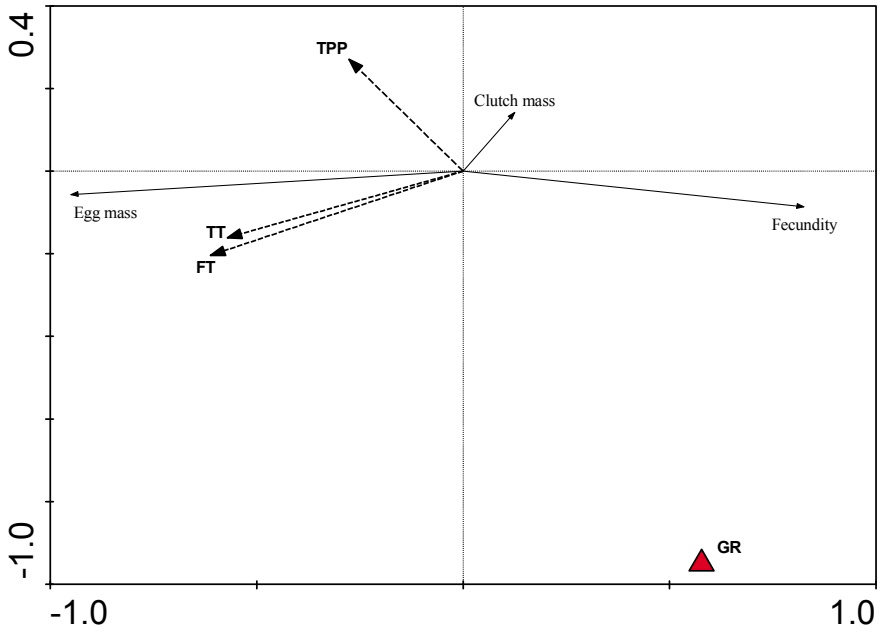

Figure 6-6. Biplot of clutch size variables (solid lines) and explanatory variables (dashed lines) for clutches collected during 2000-2002.

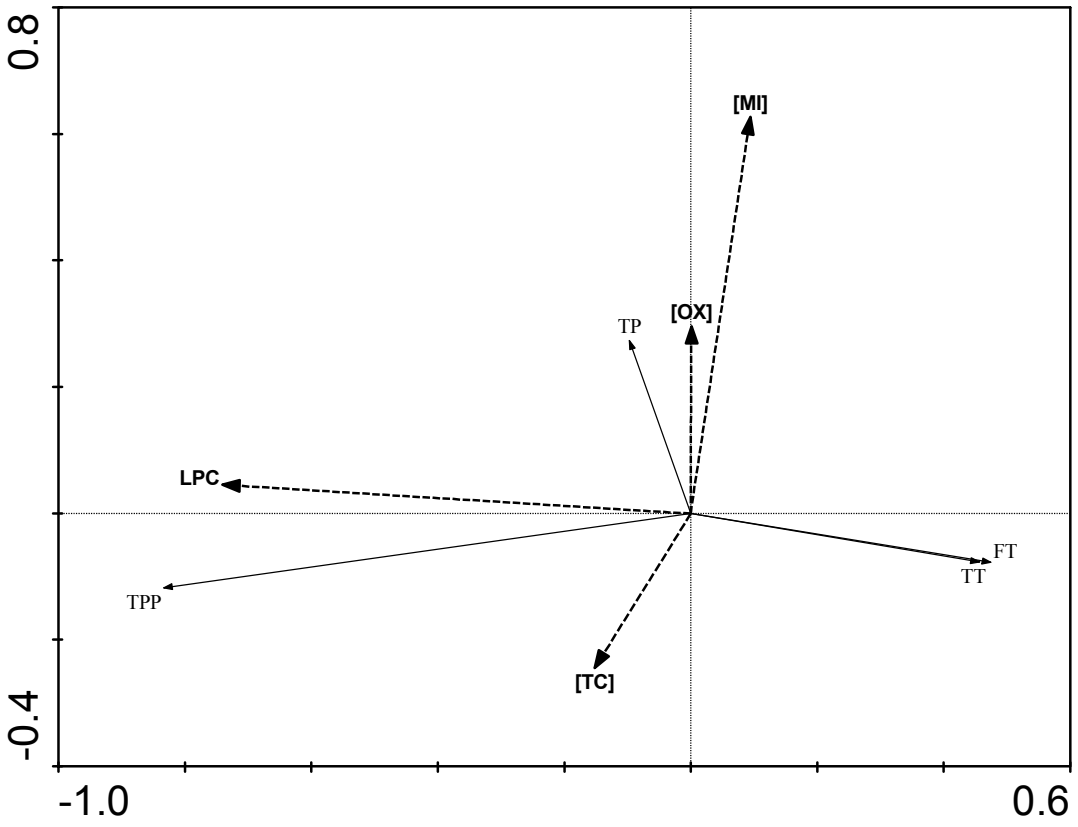

Figure 6-7. Biplot of thiamine egg yolk concentrations (solid lines) and explanatory variables (dashed lines) measured on clutches collected during 2000-2003.

CHAPTER 7
REPRODUCTIVE EFFECTS OF ORGANOCHLORINE PESTICIDE EXPOSURE IN A CAPTIVE POPULATION OF AMERICAN ALLIGATORS (ALLIGATOR MISSISSIPPIENSIS)

In central Florida, American alligator (*Alligator mississippiensis*) eggs collected from organochlorine pesticide (OCP) contaminated sites (Lakes Apopka, Griffin, and Emeralda Marsh) contain total concentrations of OCPs that range from 4,000-30,000 ng/g yolk wet weight. This is several orders of magnitude greater than the reference sites (Lakes Orange and Lochloosa) (Chapter 2). In addition, alligator populations inhabiting OCP-contaminated sites have experienced increased embryonic mortality resulting in reduced clutch success (Masson, 1995; Rotstein et al., 2002; Chapter 2). One possible explanation for these increased rates of embryonic mortality is embryonic exposure to OCPs, as similar effects have been reported in birds (Summer et al., 1996). The present study utilized a population of captive adult alligators to test the hypotheses that maternal exposure to OCPs would increase OCP burdens in egg yolks, leading to increased embryonic mortality and decreased hatch rates.

Materials and Methods

Alligators were obtained from JungleLand Zoo (Kissimmee, FL) and Gatorland Zoo (Orlando, FL). Thirteen male and 14 female adult alligators were randomLy assigned to one of 13 pens (approximately 30 m x 30 m) at a ratio of 1 male: 1 female, except for one large pen which housed two females and one male. Seven pens were designated as treatment pens and six as control. Prior to random group assignment (i.e.,

control vs. OCP group), head length, total length, tail girth, and estimated mass [calculated using total length and tail girth (Woodward et al., 1992)] were determined, with by-sex comparisons (T-test) indicating no significant differences among endpoints ($P > 0.3617$ for all comparisons). Male and female head lengths averaged 35 ± 3 cm (mean ± standard deviation) and 30 ± 2 cm, respectively. Total lengths for males and females were 2.53 ± 0.15 m and 2.23 ± 0.18 m, respectively, and estimated mass was 69.5 ± 13.1 kg for males and 46 ± 13.8 kg for females. In addition to the breeding pairs, two extra treated females were housed separately to monitor bioaccumulation of OCPs and health status via monthly blood assessments of hematocrit, glucose, and total protein (Mader, 1996).

Selection of specific OCP analytes and dose calculations were based on OCP concentrations in alligator yolks collected from contaminated sites in Florida and avian maternal transfer rates (Fairbrother et al., 1999). The dosing regime was designed to coincide with oocyte development and yolk formation (vitellogenesis), which for alligators begins in early fall and continues through late spring (Lance, 1986; Guillette, et al., 1997). Dosing began on 16 and 17 October, 2001. Animals were randomized with OCP-treated individuals receiving one intramuscular (IM) and one intraperitoneal (IP) injection consisting of a mixture of p,p'-DDE (36.5 mg/kg), toxaphene (2.6 mg/kg), chlordane (2.5 mg/kg), and dieldrin (8.4 mg/kg) solubilized in reagent grade olive oil (cumulative injection volume of 40 mL). Control animals received the same volume of olive oil. Animals did not receive oral doses of OCPs until they resumed feeding the following spring. On 16 April, 2002 oral dosing began and continued to 20 October, 2002 when animals went began winter fast. This pattern of animals receiving oral doses

from April to October continued through 2003 and 2004, so animals were exposed for a period of three years. Treated animals received oral doses of p,p'-DDE (0.18 mg/kg), toxaphene (0.13 mg/kg), chlordane (0.014 mg/kg), and dieldrin (0.018 mg/kg). The chemicals were mixed with reagent grade olive oil (total mixture volume per weekly dose = 8 mL). Control animals received the same feed ration minus the OCP mixture.

When females in breeding groups began nesting (24 June–10 July 2002), the two extra OCP-treated females housed in separate enclosures for monthly health status monitoring, and two females which did not produce clutches were sacrificed (via decapitation/cervical dislocation with double pithing) to determine bioaccumulation rates of OCPs. Tissue samples (adipose, liver, and blood) were collected for analytical chemistry, along with one or two egg yolks from each of the females that oviposited, with eggs being collected and incubated using methods described in Chapter 2, except no helicopter or airboat was necessary. Tissues and yolks were screened for 30 OCP analytes by GC-MS according to procedures described in Chapter 3. Lipid content (%) was determined gravimetrically for liver, and adipose tissue, while GC-MS techniques were used for blood (Chapter 3). For 2002 and 2003 clutches, a subset of yolks from five control clutches and four treated clutches were analyzed for thiamine content to determine if thiamine levels were related to OCP exposure and/or clutch viability. Thiamine analysis was conducted using methods described in Chapter 6.

For treated versus control comparisons, T-tests (PROC TTEST; SAS Institute Inc., 2002) and Wilcoxon two-sample tests (PROC NPAR1WAY WILCOXON) were used for parametric and nonparametric clutch parameters, respectively. Numerical data were log-transformed [ln(x)], while proportional data were arcsine square root transformed to aid

in meeting statistical assumptions for parametric tests. Logistic regression (PROC LOGISTIC; SAS Institute Inc., 2002) was used to evaluate associations between clutch survival parameters and potential explanatory factors (i.e., total OCP concentrations, thiamine concentrations, and clutch size parameters).

Results

Nine clutches were collected from the control group and seven from the treated group over a period of three years. Clutch parameters that significantly differed among the groups included clutch viability, incidence of unbanded eggs, lipid content, egg concentrations of seven of eight OCP analytes, and total OCP concentrations in eggs (Table 7-1). Specifically, clutch viability of the control group was 30% higher than the treated group, and the incidence of unbanded eggs was 40% lower in the control group as compared to the treated group. In addition, eggs of the treated group had significantly higher lipid content and total OCP concentrations over those of controls. Importantly, OCP burdens in yolks from the control group (50 ± 3.6 ng/g) were less than those of the reference site (102 ± 15.5 ng/g), and the treated group yielded yolk burdens (13,300 ± 2,666 ng/g) that fell within the range of the mean OCP concentrations (1,169-15,480 ng/g) observed in contaminated sites (Chapter 2). No significant differences were detected with respect to number of clutches produced by each group, fecundity, clutch mass, egg mass, oxychlordane concentrations, or thiamine concentrations, with thiamine being analyzed on five control and four treated clutches during year 2002-2003. Monthly health status assessments on two "extra" females, which were housed apart from breeding females, indicated that blood chemistry values appeared to be within normal limits (Table 7-2). After 10 months of dosing and concurrent with nesting of other captive females (June 2002), the two extra females and two non-reproductive females

were sacrificed and tissues were analyzed for OCP content. Individual chemicals exhibited differing concentrations among tissues, with the differing levels possibly related to varying lipid content of the tissue and the level of the administered dose.

Results of logistic regression indicated that OCP variables and clutch-egg size variables appeared to be associated with clutch survival parameters. Based on the differences between treated and control groups, it was not surprising to find that total OCP concentrations (TOC) in egg yolk was negatively associated with clutch viability and positively associated with incidence of unbanded eggs. Fecundity, egg mass, and clutch mass, which were not correlated with (TOC), but were positively correlated with clutch viability, early embryo mortality, and late embryo mortality, and negatively correlated with unbanded egg incidence. Lipid content in eggs was positively correlated with early and late embryo mortality, and as shown in group comparisons, lipid content was positively correlated with TOC (i.e., significantly higher in the treated group). Thiamine concentrations in eggs were determined to be significantly correlated with one another. In addition, thiamine monophosphate (TP) was found to be positively associated with clutch viability, clutch mass, and fecundity, and negatively associated with unbanded egg incidence. Thiamine pyrophosphate (TPP) was not associated with any of the clutch survival parameters. In contrast, free thiamine and total thiamine were negatively associated with unbanded egg incidence and positively associated with early and late embryo mortality (Table 7-3).

Discussion

The results of this study support the hypothesis that OCPs are maternally transferred to the developing egg, and that maternal exposure is associated with reduced clutch success and increased embryonic mortality. In addition, this is the first study to

develop a method for exposing alligator embryos to endogenous concentrations of OCPs, in contrast to prior studies that have exogenously applied OCPs to eggs to elicit embryonic exposure (Matter, et al., 1998). Importantly, the dosing regime did not induce adult mortality, or alterations in monthly blood chemistry assessments, food intake, weight gain, and behavior (e.g., females fiercely defended their nests). However, subclinical, cytotoxic effects on the liver, gonads, or kidneys may have been undetected. In addition, OCPs may cause functional defects in neural transmission, leading to subtle increases in stress due to sublethal neuronal hyperactivity.

The decreased clutch viability noted in the OCP treated group was due to increased incidence of unbanded eggs. Since unbanded eggs may be the result of embryo mortality occurring prior to embryo attachment (Rotstein et al., 2002) or possibly the result of infertility (or both), and since both parents received similar doses of OCPs and since effects of OCPs vary from species to species and analyte to analyte (Rattner & Heath, 2003), the potential mechanisms by which OCP exposure might possibly induce increased incidence of unbanded eggs are many and may include altered egg quality, direct embryo toxicity, or decreased reproductive function in males.

Other factors besides OCP exposure and egg concentrations that were associated with variation in clutch survival parameters (and that weren't concurrently associated with OCP exposure) included: fecundity, clutch mass (fecundity and clutch mass were collinear), egg mass, lipid content, thiamine monophosphate (TP), free thiamine (FT), and total thiamine (TT) concentrations (all thiamine forms were collinear with one another).

In order to accurately interpret associations, consideration should be given to biological relevance and plausibility, as well as the experimental design and results. Given that the major contribution to decreased clutch viability for both control and treated groups was the increased incidence of unbanded eggs, factors associated with unbanded eggs are due careful consideration. In this respect, concentrations of OCPs in eggs were positively associated with unbanded egg incidence and negatively associated with clutch viability, as expected given the experimental design and results of group comparisons. In contrast to OCPs, clutch fecundity, clutch mass, egg mass, and TP had negative associations with unbanded egg incidence and positive associations with clutch viability. However, none of these explanatory factors were correlated with OCPs, suggesting that they may independently account for a portion of the incidence of unbanded eggs in OCP exposed group and, more importantly, the control group. The biological implications are that in a control situation the incidence of unbanded eggs are related to smaller clutches and lighter eggs, and that the rate of unbanded eggs is basically doubled when captive animals are exposed to OCPs.

Factors that were not correlated with clutch viability were FT, TT, and lipid content. FT and TT were negatively correlated with unbanded egg incidence and positively correlated with early and late embryo mortality. These associations may be a result of the significant correlation FT and TT have with TP, and might not be a result of a direct link with unbanded egg incidence. Positive associations between FT, TT, and early and late embryo mortality, suggest possible thiamine hypervitaminosis. Although unlikely, thiamine concentrations of experimental clutches (both groups) were three-fold to five-fold greater than those of wild clutches, and thiamine hypervitaminosis has been

shown to induce neurotoxicosis in laboratory models (Snodgrass, 1992), which suggests that thiamine toxicity can't be completely discounted. Also correlated with increased early and late embryo mortality were fecundity, clutch mass, and egg mass. Although a biologically relevant reason for this association may be present, the association between embryo mortality and fecundity, clutch and egg mass may be attributed to the fact that as unbanded egg incidence decreases, the more embryos are present and allows the potential for embryo mortality to occur.

In contrast to thiamine concentrations, lipid content was found to be significantly associated only with early and late embryo mortality, and OCP concentrations, which is to be expected as treated groups had significantly higher levels compared to controls. One might conclude that the reasons for the positive association between OCPs and lipid content is simply because OCPs are hydrophobic and lipophilic; however, because lipid content was different between treatment groups, it may be that OCP exposure altered liver and/or follicle function in producing and sequestering yolk components. These results may suggest maternally-mediated alterations in egg quality and resulting decreased clutch viability. Maternally-mediated reductions in clutch viability is a likely scenario as liver is a known target organ for OCP-induced toxicity (Metcalfe, 1998), and exposure to similar organochlorine compounds has been shown to alter liver function (phospholipid production and transfer), vitellogenesis, egg component profiles of other oviparous vertebrates ((Lal & Singh, 1987), and up-regulates biotransformation enzymes that are involved in xenobiotic biotransformation and lipid metabolism (Ertl et al., 1998).

With respect to lipid contents association with embryo mortality, an important finding was that OCP concentrations were not associated with early or late embryo

mortality, suggesting lipid association is not simply due to multicollinearity with OCPs. These results may indicate that maternal exposure to OCPs alters lipid content, leading to increased embryo mortality. This association may be biologically relevant and plausible since differences in egg yolk fatty acid profiles are suggested to be related to reduced clutch viability in captive alligators (Noble et al., 1993; Millstein, 1995).

In summary, results of the present study supports the hypothesis that parental OCP exposure may decrease clutch viability by increasing the incidence of unbanded eggs. These results differ from observations in wild clutches from OCP-contaminated sites in which reduced clutch viability is primarily due to increases in early and late embryo mortality (Chapter 2). However, unbanded eggs may be products of very early embryo mortality (Rotstein et al., 2002), with very early embryo mortality in the captive population being likely related to OCP effects that have been exacerbated by the stress of captivity. Also important to consider is that alligators (less than 50 years old) from OCP-contaminated sites have likely been exposed to OCPs since conception, and therefore may have been reproductively altered during development {Gross et al., 1994} and may respond differently to OCP exposure as compared to previously unexposed adults.
This study confirms, as somewhat expected, that OCPs are maternally transferred in the alligator and that this is likely the major route for embryonic exposure. This study is also the first induce, via maternal OCP exposure, endogenous OCP exposure in developing alligator embryos. Importantly, this ecological relevant experiment demonstrates that parental exposure to OCPs results in decreases in clutch viability similar to what has been observed in wild alligator populations inhabiting OCP-contaminated sites. Lastly, this study provides experimental evidence linking parental OCP exposure to decreased clutch

viability in the American alligator, and suggests a maternally-mediated mechanism may be involved.

Table 7-1. Summary statistics and comparisons of clutch parameters among treated and control groups for years 2002-2004.

Parameter	Control	Treated	Summary
N$^{o.}$ Clutches	9	7	16
Fecundity (*n*)	32 ± 2.4	30 ± 2.5	31 ± 1.7
	(19–40)	(20–37)	(19–40)
Clutch mass (kg)	2.31 ± 0.227	2.22 ± 0.203	2.27 ± 0.151
	(1.33–3.3)	(1.51–2.9)	(1.33–3.3)
Egg mass (g)	73 ± 3	73 ± 1.2	73 ± 1.7
	(53–83)	(70–78)	(53–83)
Clutch viability (%)	44 ± 11*	9 ± 6	29 ± 7.9
	(0–95)	(0–35)	(0–95)
Unbanded eggs (%)	39 ± 12.4*	81 ± 12.3	58 ± 10.1
	(3–100)	(22–100)	(3–100)
Damaged eggs (%)	3 ± 3	0 ± 0	2 ± 1.7
	(0–27)	(0–0)	(0–27)
Early emb. mort. (%)	8 ± 4	4 ± 2.8	6 ± 2.5
	(0–36)	(0–16)	(0–36)
Late emb. mort. (%)	6 ± 2.7	5 ± 5	6 ± 2.6
	(0–20)	(0–35)	(0–35)
Lipid content (%)	19 ± 0.7*	22 ± 0.7	20 ± 0.7
	(15–21)	(20–25)	(15–25)
TP (pmoles/g)	24 ± 6.1	16 ± 8	20 ± 4.8
	(4–39)	(3–39)	(3–39)
TPP (pmoles/g)	21 ± 6	15 ± 3.7	18 ± 3.6
	(11–44)	(6–22)	(6–44)
Thiamine (pmoles/g)	3088 ± 182.8	3035 ± 343.4	3065 ± 170.4
	(2623–3694)	(2576–4045)	(2576–4045)
∑Thiamine (pmoles/g)	3133 ± 182.6	3066 ± 352.8	3103 ± 173.6
	(2640–3731)	(2585–4105)	(2585–4105)
CC (ng/g)	1 ± 0*	24 ± 9.4	14 ± 6.1
	(1–1)	(3–64)	(1–64)
CN (ng/g)	1 ± 0*	10 ± 2.6	7 ± 2
	(1–2)	(1–18)	(1–18)
Dield. (ng/g)	6 ± 1.2*	773 ± 122.1	335 ± 116.4
	(3–11)	(475–1143)	(3–1143)
Oxychl. (ng/g)	1 ± 0.1	2 ± 0.4	2 ± 0.2
	(1–2)	(1–3)	(1–3)
p,p'-DDE (ng/g)	19 ± 2.6*	11729 ± 2200.4	5038 ± 1838.8
	(6–30)	(5801–18448)	(6–18448)

Table 7-1. Continued.

Parameter	Control	Treated	Summary
Oxychl. (ng/g)	1 ± 0.1	2 ± 0.4	2 ± 0.2
	(1–2)	(1–3)	(1–3)
p,p'-DDE (ng/g)	19 ± 2.6*	11729 ± 2200.4	5038 ± 1838.8
	(6–30)	(5801–18448)	(6–18448)
TC (ng/g)	1 ± 0*	25 ± 9.9	17 ± 7.5
	(1–1)	(3–66)	(1–66)
TN (ng/g)	2 ± 0.4*	36 ± 8	17 ± 5.6
	(1–4)	(11–56)	(1–56)
Toxa. (ng/g)	0 ± 0*	2035 ± 720	2035 ± 720
	(0–0)	(1315–2755)	(1315–2755)
∑[OCPs] (ng/g)	50 ± 3.6*	13300 ± 2666.1	5728 ± 2116.4
	(29–60)	(6393–21991)	(29–21991)
No. OCPs (*n*)	5 ± 0.7	7 ± 1.2	6 ± 0.7
	(0–7)	(0–9)	(0–9)

Table 7-2. Organochlorine concentrations and blood chemistry values of captive adult female alligators sacrificed during 2002 (Rauschenberger et al., 2004). Mean ± standard deviation (sample size).

Parameter	Control	Treated
Adipose Tissue (ng/g[a])		
p,p'-DDE	No data[b]	68,315 ± 35,275 (4)
Toxaphene	No data[b]	8,385 ± 1486 (4)
Chlordane	No data[b]	708 ± 200 (4)
Dieldrin	No data[b]	4,372 ± 1,237 (4)
Lipid Content (%)	No data[b]	82 ± 6 (4)
Liver Tissue (ng/g[a])		
p,p'-DDE	No data[b]	8,168 ± 3,750 (4)
Toxaphene	No data[b]	Not detected[c]
Chlordane	No data[b]	23 ± 13 (4)
Dieldrin	No data[b]	143 ± 92 (4)
Lipid Content (%)	No data[b]	4 ± 2 (4)
Whole Blood (ng/g[a])		
p,p'-DDE	No data[b]	179 ± 184 (4)
Toxaphene	No data[b]	Not detected[c]
Chlordane	No data[b]	Not detected[d]
Dieldrin	No data[b]	15 ± 5 (4)
Lipid Content (%)	No data[b]	0.10 ± 0.02 (4)
Hematocrit (%) [e]	20-30	20 ± 4 (2)
Glucose (mg/dl) [e]	74	63 ± 17 (2)
Tot.Plasma Protein mg/dl) [e]	5.1	6 ± 1 (2)

[a]ng chemical/g yolk wet weight (not lipid normalized). [b]No control females were sacrificed. [c]Limit of detection for toxaphene = 230 ng/g. [d]Limit of detection for chlordane = 0.2 ng/g. [e]For controls, blood chemistry values reported by Mader (1996). Values for treated group reflect mean of 10 samples collected evenly over 10 months.

Table 7-3. Explanatory parameters and clutch survival parameters with (±) indicating nature of association and value equal to concordance percentage.

Parameter[a]	Unbanded egg%	Clutch Viability	Early Emb. Mort.	Late Emb. Mort.
TOC[b]	(+) 64	(–) 65	ns	ns
Fecundity[c]	(–) 71	(+) 65	(+) 54	(+) 65
clutch mass[c]	(–) 77	(+) 72	(+) 67	(+) 76
egg mass	(–) 63	(+) 58	(+) 55	(+) 66
lipid %[b]	ns	ns	(+) 58	(+) 68
TP[cd]	(–) 59	(+) 59	ns	ns
TPP[d]	ns	ns	ns	ns
FT[d]	(–) 68	ns	(+) 74	(+) 72
TT[d]	(–) 69	ns	(+) 74	(+) 72

[a]TOC = total OCP concentrations in egg yolk; TP = thiamine monophosphate; TPP = thiamine pyrophosphate; FT = free thiamine; TT = total (∑) thiamine concentrations in yolk. [b-d] Parameters sharing same superscript letters are significantly correlated with each other, those not sharing letters are not correlated. NS = not significant ($P > 0.05$)

CHAPTER 8
CONCLUSIONS

Introduction

The American alligator has a significant role in the ecology, esthetics, and economy of Florida. Thus, maintaining viable populations of alligators is desirable for many reasons. As reproduction is critical for maintaining a species population, any relatively sudden or sustained decrease in reproduction may be cause for concern.

Over the last quarter century, alligator populations in organochlorine (OCP) contaminated lakes in central Florida have garnered intense study and much attention due to their decreased reproductive performance (Woodward et al., 1989; Woodward et al., 1993; Wiebe et al., 2001). Decreased reproductive performance has been attributed to decreased clutch viability due to increased embryo mortality, with mortality typically occurring during the first 20 days of development (Masson, 1995). Furthermore, prior study suggests OCP exposure may be a potential contributing factor to increased embryo mortality since increased mortality had been reported only in sites heavily contaminated with OCPs and since alligator eggs from these sites contained increased levels of OCPs, but a clear relationship was not evident (Heinz et al., 1991).

Understanding biological and environmental characteristics related to embryo development, egg quality, and clutch viability in alligators is necessary in evaluating whether OCPs and/or some other factor(s) may be causally linked to decreased clutch viability. Identifying and understanding factors associated with decreased clutch viability may benefit management of alligator populations and ensure sustainable human use. On

a larger scale, understanding the relationship between OCP exposure and developmental mortality in alligators may provide some insight into the potential impact of organochlorine pesticides on the ecological health of Florida's wetlands.

Evaluating the relationships between clutch viability, OCP exposure, embryo development, and other potentially important biological and environmental factors has been the theme of this dissertation. These evaluations have been accomplished through field studies designed to identify important factors and associations and to test hypothesized associations with laboratory experiments.

Summary of Study's Findings

The first study (Chapter 2) examined clutch viability on OCP-contaminated and reference sites from 2000-2002. Results indicated that clutch viability is significantly lower in contaminated sites, with these sites having higher rates of early and late embryo mortality, and that unbanded eggs also appear to be an important constituent of reduced clutch viability for reference and contaminated sites. In order of importance, major constituents of reduced clutch viability for all sites include early embryo mortality, unbanded eggs, late embryo mortality, and damaged eggs. In addition, clutches from OCP-contaminated sites had an average of 10 more eggs per clutch as compared to the reference site, but average clutch mass was not significantly different, making average egg mass of reference site clutches greater than that of clutches of OCP-contaminated sites. In addition to differences in clutch viability and size, large differences in OCP concentrations in alligator eggs between reference and OCP-contaminated sites were found. Although not surprising given the history of the sites, these results support the continued problems with alligator reproduction in OCP-contaminated sites.

Results of redundancy analyses indicate that for Lake Lochloosa, no significant correlations were determined although significance might have been detected given a greater sample size. For Emeralda Marsh, the weak associations between OCP variables and clutch survival variables suggests that other factors may be involved in reduced embryo survival and increased rates of unbanded eggs. The weak associations for Emeralda Marsh are surprising given that relatively stronger associations were determined for the other high exposure site (Lake Apopka; Table 2-5), as well as the intermediate exposure site (Lake Griffin, Table 2-5), with Emeralda Marsh being separated from Lake Griffin by easily traversable, non-fenced levee. The positive association between early embryo mortality and unbanded egg rates and extracted OCP variables for Lake Apopka clutches suggests that the percentages of dieldrin and *trans*-chlordane in eggs may play an important role in altered egg fertility and/or early embryo survival. For Lake Griffin, the negative to near-zero association between early embryo mortality rates and extracted OCP variables suggests that OCP burdens in eggs may not play an important role in early embryo mortality. However, the positive association between toxaphene burdens and late embryo mortality suggests that as toxaphene burdens increase, so does the risk for increased embryo death during the last 35 days of development. Furthermore, the positive association between *p,p'*-DDT concentrations and unbanded egg rates suggests that these analytes may be involved in altered egg fertility and/or embryo survival (prior to eggshell membrane attachment) (Fig. 2-2).

In summary, the first study suggested that, over all sampled clutches, clutch survival parameters and egg and clutch size parameters vary between the low OCP exposure site (Lochloosa) and the intermediate-high OCP exposure sites. Furthermore,

OCP burdens do not appear to be related to clutch survival for the low exposure site but are associated with clutch survival for the intermediately OCP contaminated site and one of the highly OCP contaminated sites.

The next study (Chapter 3) evaluated the relationship between OCP burdens in eggs and in maternal tissues in order to examine the extent of maternal transfer of OCPs in the alligator and to determine if eggs could be used as predictors of maternal tissue burdens. Major finding of this study were that adipose tissue and yolk burdens were similar when adjusted for lipid content and that yolk was an excellent predictor of adipose tissue burdens. Conversely, blood and yolk burdens were not linearly related. Importantly, liver had higher burdens than yolk after adjustment for lipid content suggesting liver may sequester OCPs, supporting the possibility that liver function may be altered due to chronic OCP exposure. Altered liver function due to OCP exposure may affect lipid metabolism, vitellogenesis, and egg quality (Lal & Singh, 1987), potentially resulting in maternally-mediated embryo mortality. However, non-OCP related maternal factor(s), such as size and age, could also affect clutch viability rates.

The following study (Chapter 4) addressed the potential influence of maternal factors on clutch viability. Results indicated maternal body size was not associated with variation in clutch survival parameters, but moderate associations existed between maternal OCP burdens and clutch survival parameters (18% of variance explained, $P < 0.05$). Specifically, as p,p'-DDE proportions increased in relation to total OCP egg burdens, the incidence of unbanded eggs increased, and as trans-chlordane proportions increased in relation to total OCP burdens in eggs, clutch viability decreased and early embryo mortality increased.

Since increased rates of embryo mortality were the reason for decreased clutch viability, the fourth study (Chapter 5) sought to evaluate the histopathology, growth, and development of embryos, and their associations with OCP exposure (egg burdens). Intra-site comparisons suggested that among all sites and all sampled ages (calendar age = CA), that morphological age (MA) of embryos and embryo mass were greater for live embryos as compared to dead embryos, which suggested that embryos may have been developing normally up to a point at which development stalled and the embryo eventually died, or embryos could have developed at a much slower overall rate until the point at which they perished. Either way it appears that the mass of dead embryos was appropriate for their MA. Morphometry of live embryos did not appear to be significantly related to variation in clutch mortality rates, suggesting that live embryos from clutches with high mortality rates develop similarly to those of low mortality clutches. This finding may suggest a threshold-type response in which embryos exposed to stressors below a certain threshold have the ability to overcome stressors through various cellular homeostatic mechanisms, but above a certain threshold, developmental retardation and lethality occur. Such threshold dose-response patterns have been accepted as a major dose-response pattern in mammalian developmental toxicology (Rogers & Kavlock, 2001).

Furthermore, variation in morphological development of live embryos was significantly associated with variation in the composition and concentration of OCPs in eggs. The strength of the relationships appeared to decrease with the age sampled (CA), with youngest embryos sampled (CA Day 14) showing the strongest relationships between OCP egg burden and morphometric parameters, followed by each subsequent

CA, respectively (Table 5-5). Interestingly, the percentage of the total OCP burden (concentration) composed by an OCP analyte (i.e., HE%), appeared to be more important than OCP analyte concentrations alone. With respect to all sampled ages, except the eldest (CA Day 43), relative proportions of the OCP analyte(s) appeared to be more important than concentrations alone (Table 5-5). For derived morphometric parameters and morphological age (MA), similar patterns were observed in that embryos sampled at younger CA showed stronger relationships with OCP burdens than older cohorts (Table 5-6).

Another important observation was that different cyclodienes appeared to be associated with morphological variation of embryos of different ages (CA). Most important were the components of technical grade chlordane and its metabolites, which include *cis* and *trans*-chlordane, *cis* and *trans*-nonachlor, oxychlordane, and heptachlor epoxide. One or more of these components were found to be significantly associated with variation in embryo morphology for each CA sampled. These data suggest that the chlordane group may merit further study in relation to developmental effects in reptiles, especially considering other studies have suggested that sexual differentiation in turtles may be altered by low dose *in ovo* exposures of these compounds (Willingham, 2004).

In conclusion, the embryo morphology and histopathology study found that embryo mortality occurring in alligator populations inhabiting reference and OCP-contaminated sites was characterized by developmental retardation without gross deformities, or overt presence of lesions to vital organs. However, variation in embryo morphology appeared to be associated with variation in OCP burdens of eggs and the percentage composition composed by an OCP analyte was equally as important as concentration, suggesting the

importance of mixture composition. Younger embryos appeared more susceptible to OCP influence but OCP influence may not necessarily be the result of direct embryo effects. Similar types of embryo mortality, characterized by developmental retardation) has been documented in quail, with embryo mortality determined to be maternally mediated, where maternal liver function was altered, resulting in nutrient deficiencies in eggs that were severe enough to induce embryo mortality (Donaldson & Fites, 1970).

Because nutrition and non-OCP contaminants have been associated with developmental retardation in salmonids (Fitzsimons et al., 1999) and birds (Wilson, 1997; Gilbertson et al., 1991)., the next study (Chapter 6) evaluated embryo mortality in alligators of reference and OCP-contaminated sites as a function of exposure to OCPs, polychlorinated biphenyls (PCBs), and polyaromatic hydrocarbons (PAHs), as well as egg nutrient content. Results of this study suggested that decreasing thiamine levels in eggs may be associated with decreased clutch success and lipid content, and OCP burdens may be associated with variation in thiamine concentrations. In addition, PCBs, PAHs, and non-thiamine nutrients were not found to be significantly associated with clutch viability. Thiamine levels in eggs explained 38% of the variation in clutch survival parameters in the case-control cohort study and 27% of the clutch survival variation in the expanded field study, which suggest that factors in addition to thiamine are likely involved. A lack of effects on clutch viability observed in experiments involving thiamine amelioration and inhibition via topical egg treatments may suggest a number of potential conclusions including that either thiamine has no effect on the embryo or that the embryo was not exposed to enough thiamine or thiamine inhibitor to elicit effects.

The last experiment of this dissertation (Chapter 7) attempted to examine the relationship between OCP exposure and decreased clutch viability in alligators in a more direct way which involved orally dosing a captive breeding population of adult alligators with OCPs to test the hypothesis that adult alligators exposed to OCPs yield clutches with decreased clutch viability as compared to controls. The results of this study found that clutch viability was decreased in the OCP treated group, but that the decrease was due to increased incidence of unbanded eggs and not mortality in banded eggs (i.e., after embryo attachment). However, very early embryo (conceptus) mortality has been documented in unbanded eggs via determination of paternal DNA {Rotstein et al., 2002), so it is likely that a portion of the unbanded eggs were products of early embryo mortality, especially considering captives had not been raised in a contaminated habitat and may have responded more severely to OCP exposure in comparison to their wild cohorts. In addition to OCP factors, the captive study suggested other factors, not concurrently associated with OCP exposure, were associated with clutch viability. These factors included fecundity, clutch mass (fecundity and clutch mass were correlated with each other), egg mass, lipid content, thiamine monophosphate (TP), free thiamine (FT), and total thiamine (TT) concentrations (all thiamine forms were correlated with one another).

In summary, results of the captive parental dosing study support the hypothesis that OCP exposure may decrease clutch viability by increasing the incidence of unbanded eggs and/or early embryo mortality. The biological implications are that in a control situation incidence of unbanded eggs are related to smaller clutches and lighter eggs, and that the rate of unbanded eggs is basically doubled when captive animals are exposed to

OCPs. The study confirms that OCPs are maternally transferred in the alligator and that maternal OCP exposure alters lipid content of eggs and reduces clutch viability.

Overall, our studies (Chapters 2-7) suggest that OCPs may indeed be contributing to the decreased clutch viability in alligator populations inhabiting OCP-contaminated sites. In addition, thiamine levels and clutch size parameters appear to be associated with embryo mortality and decreased clutch viability. These data combined with dead embryos being developmentally retarded suggest that alterations in growth and metabolism are the probable mechanism by which mortality results, as opposed to acute toxicity to organs or specific deformities since these were not greatly observed. Lastly, the captive exposure study provided experimental evidence that parental OCP exposure can reduce clutch viability. Continuing studies beyond this dissertation are investigating the relationship between fatty acid profiles, OCPs, and clutch viability.

Future Considerations and Global Implications

Although difficult and expensive, conducting an expanded captive dosing study is likely the only way to separate which OCPs are actually causing the decreased clutch viability from those that are just collinear. A large number of alligators would have to be involved so that hypothesized OCP analytes could be given individually to determine what component of the OCP mixture was responsible or if decreased clutch viability resulted from some type of mixture effect.

A challenge in trying to relate the present findings to other OCP exposure studies involving birds, mammals, and fish is that the basic metabolic function of an adult alligator is vastly different from most models. For example, blood flow of a 70 kg alligator (0.26 L/min) is less than 8% of that of a 70 kg human, and 0.3% of that of a 70 kg shrew (Coulson & Hernandez, 1983). These differences mean that xenobiotics and

endogenous compounds circulate throughout the alligator at a decreased rate which can affect excretion and elimination, as well as the amount of time target organs are exposed (i.e., decreased blood flow through liver may mean increased OCP exposure to liver). Another factor that may affect OCP toxicity is the temperature of the alligator, as low temperatures have been associated with increased DDT toxicity in exposed fish (Rattner & Heath, 2003). Although it is often believed that being cold-blooded causes low blood flow in alligators, low blood flow is actually related to their relatively small hearts and low blood hemoglobin, and not temperature (Coulson & Hernandez, 1983). Speculatively, low blood flow, seasonally lower body temperatures, and seasonal fasting (possibly resulting in mobilization of lipids and hydrophobic contaminants) may contribute to this species susceptibility to reproductive modulation via OCP exposure.

Another aspect concerning the biochemistry of the alligator is that they are true predators in that they cannot digest complex sugars or starches or plant proteins. Indeed, their sources of glucose are mainly gluconeogenesis, in which the liver uses carbon skeletons of catabolized amino acids to synthesize glucose, and utilization of glucose stores in the carcasses of prey. The implications are the importance of normal liver function in producing energy. Furthermore, liver functions in amino acid storage, in that it has been shown that alligators can store excess amino acids in liver that can be later mobilized for protein production. The ecological trophic level of the alligator obviously contributes to increased organochlorine pesticide exposure via biomagnification, but their apparent susceptibility to maternally mediated development mortality may be exacerbated by the physiological requirements of being a predator. This speculation is supported by studies indicating p,p'-DDE causes eggshell thinning in predatory birds but

not domestic fowl, suggesting predatory species may be more susceptible to the reproductive effects of OCP exposure (Fairbrother et al., 1999). Lastly, alligators are a poikilothermic species that fast for up to six months during a time when vitellogenesis is underway. The concurrent fasting and vitellogenesis means that OCPs are mobilized along with lipid stores to meet the metabolic demands of homeostasis and follicle development, likely increasing risk of OCP-associated alterations in liver function.

Identifying other species that may be susceptible to similar organochlorine-associated embryo mortality is important for both helping to maintain biodiversity and for better understanding of the mechanisms of organochlorine-associated developmental mortality. Key ecological and physiological characteristics to look for in a potential model are that the species be a seasonally fasting, oviparous, highly fecund, poikilothermic predator. Given these attributes, species which may be potential models for examining OCP-induced reproductive toxicity include predatory turtles, such as the common snapper (*Chelydra serpentina*), the softshell (*Apalone muticus*), the alligator snapper (*Macrochelys temminckii)*, water snakes (*Nerodia spp*.), and predatory fish, such as largemouth bass (*Micropterus salmoides*), and bowfin (*Amia calva*). Indeed, alterations in endocrine function and increased developmental mortality have been noted in largemouth bass inhabiting Emeralda Marsh (Sepúlveda et al., 2004). In addition, turtle eggs from Lake Apopka were found to have abnormalities and poor hatch rates around the time reproductive problems with alligators began to be investigated (Franklin Percival, pers. comm..).

The global implications of this dissertation's results and postulations suggest that predatory reptiles and fish inhabiting areas of the world that receive(d) high inputs of

organochlorine pesticides may be at risk of increased rates of embryo mortality or decreased reproductive performance. Many of these areas are in tropical, third-world countries that continue to buy DDT from U.S. manufacturers because it is an economical way to control malarial mosquitoes and crop-destroying pests (Breman et al., 2004). The combination of concern for human health and ecological integrity underscore the exigency for better understanding of the effects associated with OCPs and similar persistent organic compounds, so that best management practices may be developed in order to protect human health and ecological integrity.

LIST OF REFERENCES

Akerman, G., Tjarnlund, U., Noaksson, E., & Balk, L. (1998). Studies with oxythiamine to mimic reproduction disorders among fish early life stages. *Marine Environ Res* **46:** 493-497.

Ashworth, C. J. & Antipatis, C. (2001). Micronutrient programming of development throughout gestation. *Reproduction* **122:** 527-35.

Benton, J., Douglas, D., & Prevatt, L. (1991). Completion report as required by federal aid in fish restoration: Wallop-Breaux project F-30-18, Ocklawaha Basin Fisheries Investigations Study XII. State of Florida Game and Freshwater Fish Commission. Tallahassee, FL.

Billet, F., Gans, C., & Maderson, P. F. A. (1985). Why study reptilian development? In *Biology of the Reptilia. 14. Development A*: 3-25. Gans, C., Billet, F. & Maderson, P. F. A. (Eds.). New York, NY: John Wiley and Sons.

Blus, L. J. (1996). DDT, DDD, and DDE in Birds. In *Environmental contaminants in wildlife*: Beyer, W. (Ed.). Boca Raton: Lewis Publishers.

Breman, J. G., Alilio, M. S., & Mills, A. (2004). Conquering the intolerable burden of malaria: what's new, what's needed: a summary. *Am J Trop Med Hyg* **71:** 1-15.

Brown, S., Honeyfield, D. C., & Vandenbyllaardt, D. L. (1998). Thiamine analysis in fish tissues. American Fisheries Society Symposium 21. 21, 73-81. American Fisheries Society.

Campbell, K. R. (2003). Ecotoxicology of crocodilians. *Applied Herpetology* **1:** 45-163.

Clarke, S. F. (1891). The habits and embryology of the American alligator. *J Morph* **5:** 181-205.

Colborn, T., Von Saal, F. S., & Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* **101:** 378-384.

Coulson, R. A. and Hernandez, T. (1983). Alligator metabolism: studies on chemical reactions in vivo. Oxford, UK, Pergamon.

Deeming, D. C. & Ferguson, M. W. J. (1990). Morphometric analysis of embryonic development in *Alligator mississippiensis*, *Crocodylus johnstoni*, and *Crocodylus porosus*. *J Zool* **221:** 419-439.

de Roode, D. F., Gustavsson, M. B., Rantalainen, A. L., Klomp, A. V., Koeman, J. H., & Bosveldt, A. T. (2002a). Embryotoxic potential of persistent organic pollutants extracted from tissues of guillemots (Uria aalge) from the Baltic Sea and the Atlantic Ocean. *Environ Toxicol Chem* **21:** 2401-11.

de Roode, D. F., Vuorinen, P. J., & Bosveld, A. T. (2002b). Effects of furazolidone, PCB77, PCB126, Aroclor 1248, paraquat and p,p'-DDE on transketolase activity in embryonal chicken brain. *Toxicology* **173:** 203-10.

Donaldson, W. E. & Fites, B. L. (1970). Embryo mortality in quail induced by cyclopropene fatty acids: Reduction by maternal diets high in unsaturated fatty acids. *J Nutr* **100:** 605-10.

Ecobichon, D. J. (2001). Toxic effects of pesticides. In *Caasarett and Doull's toxicology: the basic science of poisons*: 763-810. Klaasen, C. D. (Ed.). New York, NY: McGraw-Hill.

Elsey, R. M. & Wink, C. S. (1985). Femoral bone as a possible source of calcium for eggshell deposition in *Alligator mississippiensis*. *Anat Rec* **211:** A57.

EPA. (2004). http://www.epa.gov/region4/waste/npl/nplfls/towercfl.htm, accessed October 8. US Environmental Protection Agency, Washington DC.

Ertl, R. P., Bandiera, S. M., Buhler, D. R., Stegeman, J. J., & Winston, G. W. (1999). Immunochemical analysis of liver microsomal cytochromes P450 of the American alligator, *Alligator mississippiensis*. *Toxicol Appl Pharmacol* **157:** 157-165.

Ertl, R. P., Stegeman, J. J., & Winston, G. W. (1998). Induction time course of cytochromes P450 by phenobarbital and 3-methylcholanthrene pretreatment in liver microsomes of *Alligator mississippiensis*. *Biochem Pharmacol* **55:** 1513-1521.

Fairbrother, A., Ankley, G. T., Birnbaum, L. S., Bradbury, S. P., Francis, B., Gray, E., Hinton, D., Johnson, L. L., Peterson, R. E., & Van Der Kraak, G. (1999). Reproductive and developmental toxicology of contaminants in oviparous animals. In *Reproductive and developmental effects of contaminants in oviparous vertebrates*: 283-361. Di Giulio, R. T. & Tillit, D. E. (Ed.). Pensacola, FL : SETAC press.

Ferguson, M. W. J. (1981). The value of the American alligator (*Alligator mississippiensis*) as a model for research in craniofacial development. *J Craniofac Genet Dev Biol* **1:** 123-144.

Ferguson, M. W. J. (1985). The reproductive biology and embryology of crocodilians. In *Biology of the reptilia: Vol. 14, Development A*: 329-491. Gans, C. (Ed.). New York, NY: John Wiley and Sons.

Fitzsimons, J. D., Brown, S. B., Honeyfield, D. C., & Hnath, J. G. (1999). A Review of Early Mortality Syndrome (Ems) in Great Lakes Salmonids: Relationship With Thiamine Deficiency. *Ambio* **28:** 9-15.

Fitzsimons, J. D., Vandenbyllaardt, L., & Brown, S. B. (2001). The use of thiamine and thiamine antagonists to investigate the etiology of early mortality syndrome in lake trout (Salvelinus namaycush). *Aquat Toxicol* **52:** 229-39.

Fry, D. M. (1995). Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environ Health Perspect* **103 Suppl 7:** 165-71.

Gadbury, G. L. & Schreuder, H. T. (2003). Cause-effect relationships in analytical surveys: an illustration of statistical issues. *Environ Monit Assess* **83:** 205-227.

Gilbertson, M., Kubiak, T., Ludwig, J., & Fox, G. (1991). Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease. *J Toxicol Environ Health* **33:** 455-520.

Giroux, D. (1998). Lake Apopka revisited: a correlational analysis of nesting anomalies and DDT contaminants. Master of Science Thesis. Gainesville, Florida. University of Florida.

Gray, L. E. Jr., Kelce, W. R., Wiese, T., Tyl, R., Gaido, K., Cook, J., Klinefelder, G., Desaulniers, D., Wilson, E., Zacharewski, T., Waller, C., Foster, P., Lasky, J., Reel, J. G. J., Laws, S., McLachlan, J., Breslin, W., Cooper, R., DiGiulio, R., Johnson, R., Purdy, R., Mihaich, E., Safe, S., Sonnenschein, C., Weshons, W., Miller, R., McMaster, S., & Colborn. T. (1997). Endocrine Screening Methods. Workshop report: detection of estrogenic and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidogenic enzyme mechanisms. *Reprod Toxicol* **11:** 719-750.

Gross, D. A. (1997). Thymus, spleen and bone marrow hypoplasia and decreased antibody responses in hatchling lake Apopka alligators. Master of Science Thesis. Gainesville, Florida. University of Florida.

Gross, T. S., Arnold, B. S., Sepulveda, M. S., & McDonald, K. (2003). Endocrine disrupting chemicals and endocrine active agents. In *Handbook of Ecotoxicology*: 1033–1098. Hoffman, D. J., Rattner, B. A., Burton, G. A. Jr. & Cairns, J. Jr. (Ed.). Boca Raton, FL USA: Lewis Publishers.

Gross, T. S., Guillette, L. J., Percival, H. F., Masson, G. R., Matter, J. M., & Woodward, A. R. (1994). Contaminant-induced reproductive anomalies in Florida alligators. J *Comp Pathol* **26:** 1-8.

Guillette, L. J., Brock, J. W., Rooney, A. A., & Woodward, A. R. (1999). Serum Concentrations of Various Environmental Contaminants and Their Relationship to Sex Steroid Concentrations and Phallus Size in Juvenile American Alligators. *Arch Environ Contam Toxicol* **36:** 447-455.

Guillette, L. J. Jr, Gross, T. S., Masson, G. R., Matter, J. M., Percival, H. F., & Woodward, A. R. (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ Health Perspect* **102:** 680-8.

Guillette, L. J. Jr, Woodward, A. R., Crain, D. A., Masson, G. R., Palmer, B. D., Cox, M. C., You-Xiang, Q., & Orlando, E. F. (1997). The reproductive cycle of the female American alligator (Alligator mississippiensis). *Gen Comp Endocrinol* **108:** 87-101.

Hall, R. J., Kaiser, T. E., Robertson, W. B. Jr, & Patty, P. C. (1979). Organochlorine residues in eggs in the endangered American crocodile (Crocodylus acutus). *Bull Environ Contam Toxicol* **23:** 87-90.

Heinz, G. H., Percival, H. F., & Jennings, M. L. (1991). Contaminants in American Alligator Eggs From Lake Apopka, Lake Griffin, and Lake Okeechobee, Florida. *Environ Monit Assess* **16:** 277-285.

Henry, K. S., Kannan, K., Nagy, B. W., Kevern, N. R., Zabik, M. J., & Giesy, J. P. (1998). Concentrations and hazard assessment of organochlorine contaminants and mercury in smallmouth bass from a remote lake in the upper peninsula of Michigan. *Arch Environ Contam Toxicol* **34:** 81-86.

Hill, A. B. (1965). The environment and disease: association or causation? *Proc R Soc Med* **58:** 295-300.

Hoffman, D. J. (1990). Embryotoxicity and teratogenicity of environmental contaminants to bird eggs. *Rev Environ Contam Toxicol* **115:** 40-89.

Hoffman, D. J. & Gay, M. L. (1981). Embryotoxic effects of benzo[a]pyrene, chrysene, and 7,12-dimethylbenz[a]anthracene in petroleum hydrocarbon mixtures in mallard ducks. *J Toxicol Environ Health* **7:** 775-787.

Holstege, D. M., Scharberg, D. L., Tor, E. R., Hart, L. C., & Galey, F. D. (1994). A rapid multiresidue screen for organophosphorus, organochlorine, and N-methyl carbamate insecticides in plant and animal tissues. *J AOAC Int* **77:** 1263-74.

Horwitz, W. (2000). Official methods of analysis of AOAC International. Gaithersburg, MD, AOAC International.

James, R. C. Warren, D. A. Halmes, N. C., & Roberts, S. M. (2000). Risk assessment. In *Principles of Toxicology*: 437-478. Williams, P. L. James, R. C. & Roberts, S. M. (Ed.). New York: John Wiley & Sons.

Lal, B. & Singh, T. P. (1987). Impact of pesticides on lipid metabolism in the freshwater catfish, *Clarias batrachus*, during the vitellogenic phase of its annual reproductive cycle. *Ecotoxicol Environ Saf* **13:** 13-23.

Lance, V. (1986). Hormonal control of reproduction in crocodilians. In *Wildlife Management: Crocodiles and Alligators*: 409-415. Webb, G. J. W., Manolis, S. C. & Whitehead, P. J. (Ed.). Chipping Norton, NSW: Surrey Beatty and Sons.

Lance, V., Joanene, T., & McNease, L. (1983). Selenium, vitamin E, and trace elements in the plasma of wild and farm reared alligators during the reproductive cycle. *Can J Zool* **61:** 1744-1751.

Lang, J. W. & Andrews, H. V. (1994). Temperature-dependent sex determination in crocodilians. *J Exp Zool* **270:** 28-44.

Longnecker, M. P., Klebanoff, M. A., Brock, J. W., Zhou, H., Gray, K. A., Needham, L. L., & Wilcox, A. J. (2002). Maternal serum level of 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene and risk of cryptorchidism, hypospadias, and polythelia among male offspring. *Am J Epidemiol* **155:** 313-22.

Mader, D. R. (1996). *Reptile Medicine and Surgery*. Philadelphia, PA: W. B. Saunders.

Marburger, J. E., Johnson, W. E., Douglas, D. R., & Gross, T. S. (1999). Pesticide contamination of fish and sediments in the Emeralda Marsh Conservation Area: relevance to fisheries establishment in flooded muck farms. Technical Memorandum No. 31 to the St. John's Water Management District. Palatka, FL.

Masson, G. R. (1995). Environmental influences on reproductive potential, clutch viability and embryonic mortality of the American alligator in Florida. PhD dissertation. Gainesville, FL, University of Florida.

Matter, J. M., McMurry, C. S., Anthony, A. B., & Dickerson, R. L. (1998). Development and implementation of endocrine biomarkers of exposure and effects in American alligators (Alligator mississippiensis). *Chemosphere* **37:** 1905-1914.

McEvoy, T. G., Robinson, C. J., Ashworth, J. A., Rooke, J. A., & Sinclair, K. D. (2001). Feed and forage toxicants affecting embryo survival and fetal development. *Theriogenology* **55:** 113-129.

Metcalfe, C. D. (1998). Toxicopathic responses to organic compounds. In *Fish diseases and disorders*: 133-161. Leatherland, J. F. & Woo, P. T. K. (Ed.). Cambridge, MA: CABI Publishing.

Millstein, S. R. (1995). Nutrient therapy in the American alligator and its association with egg yolk long-chain fatty acids and egg fertility. Master of Science Thesis Gainesville, FL. University of Florida.

Noble, R. C., Mccartney, R., & Ferguson, M. W. J. (1993). Lipid and fatty-acid compositional differences between eggs of wild and captive-breeding alligators (*Alligator- mississippiensis*)–an association with reduced hatchability. *J Zool* **230:** 639-649.

Rao, C. R. (1964). The use and interpretation of principal component analysis in applied research. *Sankhya A* **26:** 329-358.

Rattner, B. A. & Heath, A. G. (2003). Environmental factors affecting contaminant toxicity in aquatic and terrestrial vertebrates. In *Handbook of Ecotoxicology*: 679-699. Hoffman, D. J., Rattner, B. A., Burton, G. A. Jr. & Cairns, J. Jr. (Ed.). Boca Raton, FL USA: Lewis Publishers.

Rauschenberger, R. H., Wiebe, J. J., Buckland, J. E., Smith, J. T., Sepúlveda, M. S., & Gross, T. S. (2004). Achieving environmentally relevant organochlorine pesticide concentrations in eggs through maternal exposure in *Alligator mississippiensis*. *Marine Environ Res* **58:** 851-856.

Reese, A. M. (1912). The embryology of the Florida alligator (*A. mississippiensis*) . *Proc 7th Int Zool Congr* **1907:** 535-537.

Rice, A. K. (2004). Diet and condition of alligators (*Alligator mississippiensis*) in three central Florida lakes. Master of Science Thesis. Gainesville, FL. University of Florida.

Rice, K. G. (1996). Dynamics of exploitation on the American alligator: environmental contaminants and harvest. PhD Dissertation. Gainesville, FL. University of Florida.

Robson, W. A., Arscott, G. H., & Tinsley, I. J. (1976). Effect of DDE, DDT and calcium on the performance of adult Japanese quail (*Coturnix coturnix japonica*). *Poult Sci* **55:** 2222-7.

Roecklein, B., Levin, S. W., ComLy, M., & Mukherjee, A. B. (1985). Intrauterine growth retardation induced by thiamine deficiency and pyrithiamine during pregnancy in the rat. *Am J Obstet Gynecol* **151:** 455-60.

Rogers, J. M. & Kavlock, R. J. (2001). Developmental toxicology. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*: 351-386. Klaasen, C. D. (Ed.). New York: McGraw-Hill.

Rotstein, D. S. (2000). An evaluation of developmental mortality and fertilization failure in alligators using microsatellite analyses. Master of Science Thesis. Gainesville, FL. University of Florida.

Rotstein, D. S., Schoeb, T. R., Davis, L. M., Glenn, T. C., Arnold, B. S., & Gross, T. S. (2002). Detection by microsatellite analysis of early embryonic mortality in an alligator population in Florida. *J Wildl Dis* **38:** 160-165.

Russell R. W, Gobas, F. P. C., & Haffner, G. D. (1999). Maternal transfer and in ovo exposure of organochlorines in oviparous organisms: A model and field verification. *Environ Sci Technol* 33: 416-420.

Ryberg, W. A., Fitzgerald, L. A., Honeycutt, R. L., & Cathey, J. C. (2002). Genetic relationships of American alligator populations distributed across different ecological and geographic scales. *J Exp Zool* **294:** 325-333.

SAS Institute Inc. (2002). SAS version 9.0. Cary, NC.

Schenck, F. J., Wagner, R., Hennessy, M. K., & Okrasinski, J. L. Jr. (1994). Screening procedure for organochlorine and organophosphorus pesticide residues in eggs using a solid-phase extraction cleanup and gas chromatographic detection. *J AOAC Int* **77:** 1036-40.

Schmidt, R. R. & Johnson, E. M. (1997). Principles of teratology. In *Handbook of Developmental Toxicology*: 3-12. Hood, R. D. (Ed.). Boca Raton, Florida: CRC Press.

Schoeb, T. R., Heaton-Jones, T. G., Clemmons, R. M., Carbonneau, D. A., Woodward, A. R., Shelton, D., & Poppenga, R. H. (2002). Clinical and necropsy findings associated with increased mortality among American alligators of Lake Griffin, Florida. *J Wildl Dis* **38:** 320-337.

Sepúlveda, M. S., Wiebe, J. J., Harvey, A., Basto, J., Ruessler, D. S., Roldan, E., & Gross, T. S. (2001). An evaluation of environmental contaminants and developmental toxicity for the American alligator in central Florida. 21st Society of Toxicology Annual Meeting Abstract Book. March 15-19. San Francisco, CA. pp. 151.

Sepúlveda, M. S., Wiebe, J. J., Honeyfield, D. C., Rauschenberger, R. H., Hinterkopf, J. P., Johnson, W. E., & Gross, T. S. (2004). Organochlorine pesticides and thiamine in eggs of largemouth bass and American alligators and their relationship with early life-stage mortality. *J Wildl Dis* **In press**.

Skaare, J. U., Ingebrigsten, K., Aulie, A., & Kanui, T. I. (1991). Organochlorines in crocodile (*Crocodylus niloticus)*. *Bull Environ Contam Toxicol* **47:** 126-130.

Smith, A. G. (1991). Chlorinated hydrocarbon insecticides. In *Handbook of pesticide toxicology: Vol. 2:* 731–915. Hayes, W. J., Jr. & Laws, E. R., Jr. (Ed). San Diego, CA: Academic Press.

Snodgrass, S. R. (1992). Vitamin neurotoxicity. *Mol Neurobiol* **6:** 41-73.

Spallholz, J. E. & Hoffman, D. J. (2002). Selenium toxicity: cause and effects in aquatic birds. *Aquat Toxicol* **57:** 27-37.

Stenner, R. D., Merdink, J. L., Stevens, D. K., Springer, D. L., & Bull, R. J. (1997). Enterohepatic recirculation of trichloroethanol glucuronide as a significant source of trichloroacetic acid. Metabolites of trichloroethylene. *Drug Metab Dispos* **25 :** 529-535.

Summer, C. L., Giesy, J. P., Bursian, S. J., Render, J. A., Kubiak, T. J., Jones, P. D., Verbrugge, D. A., & Aulerich, R. J. (1996). Effects induced by feeding organochlorine-contaminated carp from Saginaw Bay, Lake Huron, to laying White Leghorn hens. II. Embryotoxic and teratogenic effects. *J Toxicol Environ Health* **49:** 409-38.

Systat, Inc. (1999). SigmaScan Pro. Point Richmond, CA.

ter Braak, C. J. F. (1986). Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. *Ecology* **67:** 1167-1179.

ter Braak, C. J. F. (1994). Canonical community ordination. Part I: Basic theory and linear methods. *Ecoscience* **1:** 127-140.

ter Braak, C. J. F. (1995). Ordination. In *Data analysis in community and landscape ecology*: 91-173. Jongman, R. H. G., ter Braak, C. J. F. & Van Tongeren, O. F. R. (Ed.). Cambridge, UK: Cambridge University Press.

ter Braak, C. J. F. & Smilauer, P. (2002). CANOCO reference manual and Canodraw for Windows Users' guide: software for canonical community ordination (version 4.52). Ithaca, NY, USA, Microcomputer Power.

Voeltzkow, A. (1892). On the oviposition and embryonic development of the crocodile. *Ann Mag Nat Hist (Ser 6)* **9:** 66-72.

Webb, G. J. W., Buckworkth, R., Sack, G. C., & Manolis, S. C. (1983). An interim method for estimating the age of *Crocodylus porosus* embryos. *Aust Wildl Res* **10:** 563-570.

Weed, D. L. (2002). Environmental epidemiology: basics and proof of cause-effect. *Toxicology* **181:** 399-403.

Wiebe, J. J., Rotstein, D., Percival, H. F., Woodward, A., Schoeb, T., & Gross, T. S. (2001). Evidence of developmental toxicity in the American alligator from central Florida lakes. 20th Annual meeting of Society for Environmental Toxicology and Chemistry Abstract Book. November 15-19. San Francisco, CA. pp. 231.

Wiebe, J. J., Sepulveda, M., Woodward, A., Percival, H. F., Abecrombie, A., Wilkinson, P., Harvey, A., Basto, J., Ruessler, D. S., Roldan, E., & Gross, T. S. (2001). An evaluation of environmental contaminants and decreased egg viability in the American Alligator (*Alligator mississippiensis*). 2001 Society of Toxicology Annual Meeting, Nov. 13-17. Baltimore, MD. pp. 160.

Willingham, E. (2004). Endocrine-disrupting compounds and mixtures: unexpected dose-response. *Arch Environ Contam Toxicol* **46:** 265-9.

Wilson, H. R. (1997). Effects of maternal nutrition on hatchability. *Poult Sci* **76:** 134-143.

Wink, C. S. & Elsey, R. M. (1986). Changes in femoral morphology during egg-laying in *Alligator mississippiensis*. *J Morphol* **189:** 183-188.

Woodward, A. R., Jennings, M. L., & Percival, H. F. (1989). Egg collecting and hatch rates of American alligator eggs in Florida. *Wildl Soc Bull* **17:** 124-130.

Woodward, A. R., Moore, C. T., & Delany, M. F. (1992). Experimental alligator harvest. Florida Fish and Wildlife Conservation Commission. Gainesville, FL. pp. 170.

Woodward, A. R., Percival, H. F., Jennings, M. L., & Moore, C. T. (1993). Low clutch viability of American alligators on Lake Apopka. *Florida-Scientist* **56** (1) 52-63.

Woosley, R. L. (1996). Cardiac Actions of Antihistamines. *Annu Rev PharmacolToxicol* **36:** 233-252.

Wu, T. H., Rainwater, T. R., Platt, S. G., McMurry, S. T., & Anderson, T. A. (2000a). Organochlorine contaminants in Morelet's crocodile (Crocodylus moreletii) eggs from Belize. *Chemosphere* **40:** 671-8.

Wu, T. H., Rainwater, T. R., Platt, S. G., McMurry, S. T., & Anderson, T. A. (2000b). DDE in Eggs of Two Crocodile Species From Belize. *J Agric Food Chem* **48:** 6416-6420.

Yagi, M., Kamohara, K., & Itowaka, Y. (1979). Thiamine deficiency induced by polychlorinated biphenyls (PCB) and dichlorodiphenyltrichloroethane (DDT) administration to rats. *J Environ Pathol Toxicol* **2:** 1119-1125.

BIOGRAPHICAL SKETCH

Richard Heath Rauschenberger was born in North Little Rock, Arkansas in 1970, and is the son of Richard Edward and Mary Elizabeth Rauschenberger. Heath graduated from Greenbrier High School in Greenbrier, Arkansas in 1988; and received a BS in wildlife management in 1993, from Arkansas State University in Jonesboro, Arkansas. After gaining professional work experience as a pest control technician and later as a private lands wildlife biologist, Heath returned to Arkansas State in 1999. He entered graduate school and received his MS in biology in 2001. After earning his MS degree, Heath immediately entered the University of Florida College of Veterinary Medicine's doctoral program (under the mentorship of Dr. Timothy S. Gross) and majored in physiological sciences, with a concentration in interdisciplinary toxicology. Heath has held diverse positions such as lifeguard, veterinary technician, grocery store clerk, pest control technician, wildlife biologist, and currently, research graduate assistant, which have aided in rounding out his professional experience. Heath is married, has two sons, and enjoys spending time with them and the rest of his family. He also enjoys outdoor activities and is an active Christian and member of a local church.