

# **FINAL PERFORMANCE REPORT**



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**The Influence of Behavior, Physiology, and Genomics on the  
Reproductive Success of Alligator Snapping Turtles**

**Oklahoma Department of Wildlife Conservation**

**September 1, 2017 – December 31, 2018**

**THE INFLUENCE OF BEHAVIOR, GENOMICS, AND  
PHYSIOLOGY ON THE REPRODUCTIVE SUCCESS OF  
ALLIGATOR SNAPPING TURTLES**



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**State:** Oklahoma

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### A. ABSTRACT

The alligator snapping turtle is considered to be a Species of Greatest Conservation Need in Oklahoma. In 2002, a captive breeding and head-start program was initiated at Tishomingo National Fish Hatchery in Tishomingo, Oklahoma, in an effort to restore this ancient and ecologically important turtle species to those river systems from which it has been extirpated in the state. However, reproductive success has been highly variable for the breeding program and may be indicative of a severely reduced effective population size. For vertebrates, behavior, physiology, and genetics play a substantial role in determining reproductive success, however, little is known about how these factors affect reproduction in turtles. We examined seasonal activity patterns, steroid hormone patterns, and embryonic mortality patterns in the aforementioned captive alligator snapping turtle population to obtain a better understanding of the species' reproductive ecology. We discovered seasonal and gender-specific differences in male and female activity levels during the fall and late spring months. We also detected hourly activity levels congruent with crepuscular, rather than nocturnal or diurnal cycles. As expected, males and females exhibited different seasonal patterns with respect to sex steroid hormone concentrations; however, stress hormone levels did not differ between the sexes. We incubated 582 eggs during the 2017 nesting season, only 49% of which hatched, and hatch success remained highly variable among clutches. Approximately 26% of eggs did not exhibit signs of viable development, suggesting low fertility rates in the captive population. An additional 22% of viable eggs died during the first 10-weeks of incubation, some of which had readily observable developmental deformities.

## B. BACKGROUND

We are presently conducting research to aid in the propagation, reintroduction and reestablishment of self-sustaining alligator snapping turtle (*Macrochelys temminckii*) populations in Oklahoma river systems. **Specifically, our research is focused on understanding the reproductive ecology of this species in order to effectively manage a captive breeding population that forms the nucleus of an alligator snapping turtle conservation program.**

The alligator snapping turtle is a Tier I Species of Greatest Conservation Need in Oklahoma (Oklahoma Comprehensive Wildlife Conservation Strategy 2015). The International Union for the Conservation of Nature (IUCN) lists this species as Vulnerable and it is listed under Appendix III of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Recently, the U.S. Fish and Wildlife Service was petitioned to consider the alligator snapping turtle for federal listing under the Endangered Species Act. Extirpation and declines of alligator snapping turtle populations are primarily due to historical commercial harvest for consumption, take for the pet trade, and habitat loss due to the presence of dams across the species' range (Reed et al. 2002). This species is native to Gulf of Mexico river drainages in the southeastern United States, and the eastern third of Oklahoma encompasses the western-most portion of its range. Alligator snapping turtles may be an important stabilizing species in aquatic ecosystems as they are omnivorous (Sloan et al. 1996, East and Ligon 2013) and serve critical ecological services as both top predators and scavengers.

Tishomingo National Fish Hatchery (NFH) in southeastern Oklahoma maintains a captive population of adult alligator snapping turtles that produce all of the offspring for the propagation/head-start program to reintroduce this species to rivers from which it has been extirpated in Oklahoma (Moore et al. 2013) and other parts of the species' range. Each May at Tishomingo NFH, female alligator snapping turtles leave the water and lay one clutch of eggs on the shore. The eggs are collected from the nests and artificially incubated under uniform conditions that have been optimized in a previous study (Ligon and Lovern 2009). On average, reproductive success rates from this population have been relatively low (average 41.8% from 2002–2011, BM Fillmore, unpublished data) and highly variable among clutches, ranging from 0–95% (DM Thompson, unpublished data). The primary concern with biases in reproductive success is that populations become susceptible to loss of genetic diversity via reduced effective population size (Moore et al. 2008, Miller et al. 2009). The effective population size ( $N_e$ ) refers to the number of breeders in a population that actually contribute to population recruitment. Maintaining population genetic diversity is a major goal of conservation, as a loss of diversity can lead to inbreeding depression, loss of reproductive fitness, and decreased ability to adapt to changing environments, which ultimately inhibit self-sustaining population viability (Frankham et al. 2002).

A comparison of hatch success among clutches from 2012–2014 indicates that reproductive success cannot be attributed solely to maternal identity, as success rates among females have fluctuated annually. An incubation experiment was conducted in 2014 using a randomized block design to determine the effects of clutch (female), incubation box, and position in the incubator on hatching success. Analysis of variance (ANOVA) was used to test for clutch and box effects and a nested ANOVA, with box nested under incubator shelf, was used to test for incubator position effects. Twenty-three clutches and 723 eggs were used in the experiment. Hatch success among clutches in 2014 remained highly variable ( $F_{22, 107} = 9.69$ ,  $P < 0.0001$ ). There was also a strong effect of shelf on hatch success ( $F_{4, 33} = 7.83$ ,  $P < 0.001$ ), with the top shelf in the incubators accounting for approximately 45% of the variance observed in hatch success among shelves. There was no effect of box on hatch success after shelf effects were accounted for by removing boxes on the top shelves of incubators from analyses ( $F_{31, 541} = 0.70$ ,  $P = 0.8833$ ). Tishomingo NFH has retained the randomized block design incubation protocol since 2014 and has discontinued the use of the top shelf in the incubators. Hatch success rates since 2015 have increased to around 75%, rates that correspond with those reported for wild populations (Ewert and Jackson 1994). However, hatch success rates among clutches remain variable (range 0–100%, DM Thompson and BM Fillmore, unpublished data). Variation in reproductive success observed under this experimental design strongly suggests that biological factors are responsible for the intra- and inter-annual variability in hatch success among clutches.

### **C. OBJECTIVE(S)**

1. Determine the reproductive cycles of male and female alligator snapping turtles and quantify levels of hormonal variation among individuals.
2. Quantify seasonal activity patterns of alligator snapping turtles and examine the degree of variation in activity between sexes and among individuals.
3. Assign parentage to every offspring (hatchlings and unviable embryos) produced in 2017. This information will be used to:
  - a) Calculate effective population size using male and female variance in reproductive success.
  - b) Calculate the annual loss of genetic diversity between the adult and offspring population.
  - c) Examine biases in embryonic mortality rates for males and females.
4. Perform pair-wise analyses to quantify the relationships between individual activity patterns, physiological state, genomic profiles, and reproductive success.

### **D. APPROACH**

*Study System* — The data that were analyzed through this study were collected between June of 2015 and November of 2017 in a captive population of alligator snapping turtles maintained under semi-natural conditions at Tishomingo National Fish Hatchery (NFH), located

in southeastern Oklahoma. It is important to note that many of these data were collected prior to the beginning of this grant; therefore, the costs for the collection of these data were not charged to this grant, although the analysis of these data was. No personnel time or expense associated with the data that were collected prior to the start of this grant was charged to the grant. We used existing, unanalyzed data from the Tishomingo NFH to examine the influence of activity patterns on turtle reproduction. We were unable to begin the grant before the 2017 nesting season; therefore the specific activities that were related to the collection and incubation of turtle eggs were not conducted under the grant, but the analyses of those data were conducted during the grant period.

The adult alligator snapping turtles involved in this study are maintained as a single closed population in a fenced pond system. This population contains 24 turtles (19 females, 5 males) that inhabit two bodies of water (**Fig. 1**). They are able to move between the two ponds by crossing a dike, which females have been observed to do during the nesting season (DM Thompson, personal observations). In addition to alligator snapping turtles, the ponds also contain a diverse fish assemblage including dense populations of sunfish (family: Centrarchidae) and minnows (family: Cyprinidae) on which the turtles forage. Turtles are also sporadically provided with supplemental forage, primarily channel catfish (*Ictalurus punctatus*) and shad (family: Clupeidae). The habitat includes abundant aquatic vegetation (*Typha* spp. and *Potamogeton* spp.), algae (*Chara* spp. and *Spirogyra* spp.) and submerged structure (felled trees) throughout the ponds and the water depth ranges from 0.75–2.5 m.

**Activity and temperature data collection** – In June 2015, prior to the initiation of this grant, all of the alligator snapping turtles had been removed from the study ponds and radio transmitters (L.L. Electronics, Mahomet, Illinois, USA) were applied to the posterior portions of their carapaces using epoxy (Marine Epoxy, Loctite, West Lake, Ohio, USA). The turtles were then released back into the ponds and transmitter signals were checked with a hand-held H-type antenna (Telonics, Tucson, Arizona, USA) and receiver (Communications Specialists, Orange, California, USA) to ensure proper functioning. An automated receiving unit (ARU) (JDJC Corp., Fisher, Illinois, USA) was stationed between the two ponds on the far east side of the dike. The ARU was connected to an antenna sitting atop a 9-m tall tower and was programmed to continuously record signal strengths from each radio transmitter, resulting in one datum approximately every 3 minutes for each turtle. An additional radio transmitter was fastened to a concrete block and placed in one of the ponds to serve as a control (**Fig. 2**). Signal strength data were collected from 29 September 2015 through 19 June 2017.

Eight of the 25 LL Electronics' radio transmitters deployed failed at various times through the study, despite being equipped with a 24-month battery, resulting in data gaps in the annual profiles of some individuals. Failed transmitters were replaced as detected and activity monitoring resumed with the replacement transmitter. By 14 June 2016 only 13 of 25

transmitters remained functional. In June 2016, the turtles were once again removed from the ponds and outfitted with new radio transmitters (Holohil Systems Ltd., Ontario, Canada) (**Fig. 3**), due to high failure rates of the initial radio transmitters. The ARU was reprogrammed with the new transmitter frequencies and signal strength recordings continued as described above. Additionally, there was a 4-week period (14 November – 15 December 2015) in which no activity data were recorded due to storm disruption of the ARU system.

Two temperature data loggers (Thermocron iButtons, Maxim Integrated, San Jose, California, USA) set to record temperatures at different frequencies were affixed to the carapace of each turtle using epoxy (Marine Epoxy, Loctite, West Lake, Ohio, USA). The low frequency data logger was set to record a data point every 4,000 s (66.66 min; selected to ensure adequate memory storage for one year of recording); the high-frequency data logger was set to record a data point every 480 s (8 min) to obtain higher resolution thermal profiles for individuals during the May–June nesting season (Thompson 2013). Two additional low-frequency data loggers were placed in each of the ponds to characterize the thermal habitat of the water. Temperature data loggers were retrieved from the turtles and ponds in June 2016 and the data were downloaded and subsequently relaunched.

***Plasma collection and ultrasonography*** — Blood plasma was collected on 11 sampling occasions from 22 August 2015 – 13 July 2016, at approximately 1-month intervals (excluding the month of January). On each sampling occasion, turtles were hand-captured and brought to the shore for blood collection and sonograms. Hand-held radio telemetry was used to aid in locating individual turtles during sampling, as visibility in the ponds was often low. We recorded turtle ID, pond of origin, time of in-water capture (hh:mm:ss), and time of blood collection (hh:mm:ss) for each capture. A maximum of 3 ml of blood was taken from the caudal vein via the dorsal side of the tail using a 2.54-cm × 21-gauge needle (**Fig. 3**). The blood was immediately transferred to two 1.5-ml labeled microcentrifuge tubes and stored on ice. A second blood draw was obtained from each turtle captured on 19 September 2015 and 10 April 2016 approximately 30 min after the first in order to verify that corticosterone concentrations obtained from the initial samples were representative of baseline concentrations. However, only “30-minute” samples from April were used in analyses because the time elapsed between the first and second blood draws in September were highly variable (*see Appendix A*).

Turtles were retained in individual plastic tote boxes until the last turtle was captured to avoid repeated captures of the same individual. All female turtles were sonogrammed post-capture using a portable ultrasound (Prosound 2, Hitachi Aloka Medical, LTD., Tokyo, Japan) on 10 of the 11 sampling occasions (10 March 2016 was excluded due to a need for equipment repairs). Screen capture images were taken during each sonogram and stored in the ultrasound device for subsequent analysis. Each turtle was then returned to its respective pond. Shortly after the blood draws were collected, the plasma was separated from the red blood cells via

centrifugation, transferred to clean, labeled 1.5-ml microcentrifuge tubes and stored at -20 °C until they could be transferred to storage at -80 °C. Follicle measurements were conducted on images stored in the portable ultrasound using the built-in measurement features.

***Plasma steroid measurements*** — Radioimmunoassay was performed to measure the steroid hormone concentrations of 224 plasma samples for estradiol (E2), progesterone (P4), and testosterone (T), and 263 plasma samples for corticosterone (CORT). Direct assays were used for each hormone and the extraction phase of the assay was split into two groups due to the large number of samples per assay. Half of the plasma samples from each sampling occasion were randomly assigned to each of the two extraction groups. Aliquots of 50 µl of plasma were suspended in 500 µl of distilled water and 20 µl of <sup>3</sup>H Tracer (of either E2, P4, or T) was added to each sample and four standard tubes (50 picogram (pg) to calculate the percent recovery and assess the extraction efficiency. The samples were extracted once with 3 ml of 100% diethyl ether and dried with nitrogen in a water bath at 37 °C. The extracts were then suspended in 1 ml of 95% ethanol and stored at -20 °C overnight. Samples were centrifuged at 0 °C, 2200 rpm for 10-minutes the following day and the supernatant was decanted into a new tube, dried under nitrogen, and resuspended in 300 µl phosphate-buffered saline. The standard curve was run in duplicate for E2, P4, and T (dilution series = 500 – 1.95 pg) and in triplicate for CORT (dilution series = 500 – 1.95 pg).

***Egg collection, incubation, monitoring, and dissection*** — Eggs were harvested from the nests the morning after they were laid. Each egg was rinsed with distilled water (to remove mud), assigned a unique identification code and then weighed and measured. One egg from each clutch was randomly selected to be retain and was stored at -20 °C for a separate study. The remaining eggs were randomly distributed across 32 incubation boxes in two incubators nominally maintained at 27.8 °C to produce a mixed sex ratio. The incubation boxes contained a 2:1 vermiculite to distilled water ratio. Egg viability was assessed every-other-day from 20 May – 31 July 2017 using traditional candling and, when necessary, an embryonic heart rate monitor designed for eggs (Buddy, Avidtronics, Cornwall, England). Daily candling ceased after 31 July because it was an ineffective means of assessing viability, as the embryos were so large that they obscured the passage of light.

Eggs that did not show signs of development or embryos that died during development were removed from the incubator and dissected within 48 hours of removal during the candling period. Egg content condition and embryo condition were recorded during dissections. Embryos, when present, were measured, staged (Yntema 1968), and any deformities were noted. The vitelline membrane surrounding the yolk portion of the egg was collected when available and stored at -20 °C. Tissue samples were collected from dissected embryos, macerated, and stored in lysis buffer at room temperature for genetic parentage analyses.

Approximately two weeks prior to the start of the hatching period, the surviving eggs were moved to new boxes with individual cells and a 2:1 vermiculite to water ratio. These boxes were used to ensure correctly pairing individual hatchlings with their eggs. After hatchlings had absorbed the remaining yolk, they were given a unique identification code, weighed, measured, and randomly assigned to one of 16 indoor rearing tanks.

**Statistical analyses** — Mean hormone concentrations were analyzed separately for each sex across months for the hormones E2, P4, and T using a one-way repeated measure ANOVA. A two-way repeated measure ANOVA was used to test for differences across months and sex in mean CORT concentrations. Monthly differences in mean maximum follicle diameter were tested for using a one-way repeated measure ANOVA. Tukey's *a posteriori* tests were performed when an overall ANOVA significance level reached 0.05. All tests were performed in SAS Version 9.4 (SAS Institute, 2003).

**Personnel** — Individuals who participated in various aspects of this study include Kerry Graves, Brian Fillmore, Ralph Simmons, and Aaron White of Tishomingo National Fish Hatchery; Day Ligon, Sarah Spangler, and Kristen Sardina of Missouri State University; and Ronald Van Den Bussche, Matthew Lovern, and Denise Thompson of Oklahoma State University.

## E. RESULTS

### Activity Indices

Mean weekly activity levels differed by week and by sex (**Fig. 4**). As expected, seasonal activity patterns corresponded with seasonal temperature fluctuations. Generally, as temperature decreased so too did mean activity levels and increased temperatures correspond with increased activity. The lowest relative activity levels occurred during the coldest months of December and January. However, low activity levels also were evident near the time when water temperatures were highest.

The most pronounced deviations in activity levels between sexes occurred in the fall months and during the nesting season. From late September through October, male mean weekly activity levels were higher than those of females and notably males showed a pronounced increase in activity while female activity began to decrease, commensurate with a decrease in mean daily water temperatures. Males also tended to have higher activity levels during the early spring months. The only period for which female relative activity was higher than that of males was leading up to and during the nesting season.

Mean hourly activity levels suggest a crepuscular diel cycle with peak activity occurring near the hours of 15:00 and 19:00 and smaller peaks around 05:00 and 08:00 for most months of the first year of the study (**Figs. 5a – c**). Diel patterns were altered in May and June during the

nesting season as mean hourly activity levels increased from the 22:00 to 06:00 hour in addition to the evening peak around the 19:00 hour that persisted. Differences in male and female activity patterns were most evident from October through December, when seasonal male and female activity levels diverged, and in May and June, when overall female activity levels were greater than those of males.

### **Circulating Steroid Hormone Concentrations**

The number of turtles sampled per month ranged from 19–24 with a mode of 21. Modal number of samples obtained per individual turtle was 10 (range = 7–11). Mean time from capture to blood collection was 2 m 4 s (**Appendix A**).

*Seasonal and sex effects on plasma sex steroid concentrations* — Female E2 concentrations differed throughout the year ( $F_{10, 164} = 10.12$ ,  $P < 0.0001$ ) (**Fig. 6**). Circulating E2 was lowest during the months of November and December and peaked in mid-July. Significant decreases among consecutive sampling occasions occurred between September and October ( $t = 2.14$ ,  $P = 0.034$ ) and between July and August of the previous year ( $t = 4.76$ ,  $P < 0.0001$ ), while increases were observed between December and February ( $t = -2.05$ ,  $P = 0.0418$ ) and June and July ( $t = 3.75$ ,  $P = 0.0002$ ). Seasonal variation was detected also in female P4 ( $F_{10, 163} = 13.71$ ,  $P < 0.0001$ ) and T ( $F_{10, 164} = 30.88$ ,  $P < 0.0001$ ) (**Fig. 6**). Circulating P4 concentrations remained relatively unvaried throughout the fall and winter then sharply increased between March and April ( $t = -3.55$ ,  $P = 0.0005$ ) and again between April and “May” (i.e., 30 April 2016) ( $t = 3.75$ ,  $P = 0.0002$ ), at which point mean P4 was observed to peak. Levels of P4 then decreased between May and June ( $t = -3.19$ ,  $P = 0.0017$ ) and again between July and August of the previous year ( $t = 2.76$ ,  $P = 0.0065$ ). Female T concentrations steadily increased from August through early April, with significant increases occurring between August and September ( $t = 1.99$ ,  $P = 0.0487$ ) and March to April ( $t = -2.28$ ,  $P = 0.0237$ ), then plummeted from April to May ( $t = -11.87$ ,  $P < 0.0001$ ) where they remained low until presumably increasing again the following August.

No seasonal differences were detected for male E2 ( $F_{10, 38} = 0.77$ ,  $P = 0.6539$ ) or P4 ( $F_{10, 37} = 1.29$ ,  $P = 0.2722$ ). Seasonal variation was observed in male T ( $F_{10, 37} = 3.01$ ,  $P = 0.0069$ ). Mean T concentrations were highest in the fall months of August through December and lowest in the spring from April through June (e.g., September vs. April,  $t = 3.35$ ,  $P = 0.0019$ ), however pair-wise comparisons among consecutive months did not yield any significant differences, possibly due to low power caused by small sample sizes.

*Seasonal effects on follicular development* — Ovarian follicle size varied across months ( $F_{9, 148} = 14.38$ ,  $P < 0.0001$ ) (**Fig. 6**). Vitellogenic follicles were detected from August through February with the steepest growth period occurring between August and September ( $t = -3.82$ ,  $P = 0.0002$ ). By early April follicles had reached their maximum diameter and were considered to be in pre-ovulatory condition. We cannot conclusively determine if vitellogenesis continued

through March or if follicles had already reached maximum size because we do not have sonogram data from the March sampling occasion. Shelled eggs were detected in May, 11 days prior to when the first nest was laid, indicating ovulation occurred between 9 and 30 of April 2016 (**Fig. 7**). Post-ovulatory ovarian condition was observed after the nesting season had ended and was marked by significant decreases in the maximum diameter of ovarian structures between the months of May and June ( $t = -3.28$ ,  $P = 0.0013$ ) and June and July ( $t = -3.44$ ,  $P = 0.0007$ ). Mean maximum diameter also differed between July and August of the previous year ( $t = -2.25$ ,  $P = 0.0261$ ), suggesting follicular regression may continue before the growth phase is initiated again in August and September.

*Seasonal and sex effects on plasma corticosterone concentrations* — Initial plasma CORT concentrations were lower than those obtained “30 minutes” after capture ( $F_{1, 33} = 11.04$ ,  $P = 0.0022$ ) for both sexes ( $F_{1, 33} = 0.03$ ,  $P = 0.8549$ ), indicating that the initial plasma samples obtained are indicative of baseline CORT concentrations (**Fig. 8**). There were no differences between male and female CORT concentrations across months ( $F_{1, 197} = 0.29$ ,  $P = 0.5933$ ); however, seasonal differences were detected ( $F_{10, 193} = 2.00$ ,  $P = 0.0357$ ) (**Fig. 9**). The highest concentrations of CORT were present in August and September, and again from May through July, while the lowest concentrations were observed in March and April.

### **Embryonic Mortality and Reproductive Success**

There were 18 alligator snapping turtle nests produced from the study population ponds between 15 May and 6 June 2017; thus, only a single female failed to nest in 2017. From the 18 nests we collected 600 eggs, 582 of which were incubated for this study. We paired 9 of the 18 clutches with their respective females, five via time-lapse photographs and four via temperature profiles obtained from their data loggers. We also were able to use thermal profiles to identify the single female in the study population that did not nest.

Approximately 69% of eggs showed viable signs of development, such as complete whitening of the shell and visible embryo development ( $n = 392$  and  $n = 400$ , respectively); however, 93 of these eggs stopped developing during the candling period (**Fig. 10**). An additional 54 embryo mortalities occurred after daily candling of the eggs ceased on 31 July 2017. There were 37 eggs of questionable viability, evidenced by abnormal partial whitening patterns, and 152 eggs that showed no signs of development (**Table 1**).

A total of 291 eggs were removed during the incubation period and dissected. Embryos were observed in 113 of the dissected eggs. Developmental stage 8 was the earliest an embryo was identified to stage and there was a slight peak in mortality at stages 14–17 followed by a much larger peak at stage 25 (the stage immediately preceding hatching). One or more deformities were observed in 32 (28.3%) of the embryos in 13 of the 18 clutches and varied in degree of severity from minor to lethal. However, the exact condition of 22% of the embryos

visualized could not be discerned due to varying degrees of decomposition; therefore, the number of deformed embryos reported represents a minimum value.

Hatch success was 49% for the population with a great deal of variation among individual clutches (minimum 4%; maximum 97%) [Table 1]. Nineteen of the 283 hatchlings also had some type of deformity. Most deformities were relatively minor, for example an extra scute or a kinked tail; however, there were several more severe abnormalities including one anophthalmic hatchling. Five of the potentially viable offspring died from maggot infestations that occurred at the end of the incubation period and these were not included in hatch success calculations. Each hatchling was given a unique identification code prior to obtaining morphometric measurements and blood samples for future genetic analyses.

Whole genomic DNA was isolated from 134 embryonic mortality tissue samples and 277 hatchling blood samples (n = 411). Gel electrophoresis was used to assess the quality of each sample and DNA concentrations were recorded for each sample. Approximately 100 single nucleotide polymorphic (SNP) loci were identified for genotyping and parentage analysis, but as described in Section H below, we were unable to complete the development of the primers for these SNPs. Whole Genome Sequencing (WGS) was performed on five alligator snapping turtle samples, one from the Tishomingo NFH and four from across the species' range, to generate additional data needed to extend the sequence length surround the SNPs for future primer development.

## **F. DISCUSSION**

Alligator snapping turtles are commonly referred to and accepted as being nocturnal animals (Ewert et al. 2006). However, our results suggest that this species exhibits a crepuscular diel activity cycle. Throughout most of the annual cycle, male and female turtles show heightened activity levels during the early morning (dawn) and evening (dusk) hours. Nocturnal activity increased during May and June when female turtles are nesting at night under the cover of darkness, but interestingly we also observed males to become increasingly active at night, as well. July diel patterns also deviated from the stronger crepuscular patterns detected in the fall, winter, and early spring, possibly in response to increased water temperatures.

The highest seasonal concentrations in male T were detected in the fall months of 2016, which coincided with increased male activity, despite decreasing temperatures. The same activity and seasonal temperature patterns were also detected for males in 2017. However, we did not see the same pattern with females as their activity levels began to decrease in the fall in correspondence with decreasing water temperatures. These opposite activity patterns displayed by males and females in response to the same thermal environmental conditions suggest that there may be an important relationship between male T concentrations and activity levels, and that this is likely a biologically important time of year for male alligator snapping turtles.

Testosterone plays an essential role in facilitating male spermatogenesis and often is associated with aggression. Increased male activity may be associated with mate searching, male-to-male competition/aggression, or foraging. Males may experience greater mating success in the fall when females are becoming less active and may be less inclined to flee or become more willing to accept male mating attempts.

Successful fall mating would require that female alligator snapping turtles be capable of storing sperm until the following spring when ovulation occurs. Although this remains unknown for alligator snapping turtles, many turtles are capable of long-term sperm storage (Pearse and Avise 2001). We observed that vitellogenesis continues through early spring and follicles are ovulated in April. Increased female progesterone concentrations in early and late April support our sonogram evidence of April ovulation followed by albumen production in the oviducts. Females achieved their highest seasonal testosterone concentrations during the spring months when those of males were steadily decreasing. These opposing patterns in male and female testosterone have been observed also in the stinkpot turtle (*Sternotherus odoratus*) (McPherson et al. 1982, Mendonça and Licht 1986). Testosterone is known to inhibit vitellogenesis; therefore, peak T concentrations occurring in March may serve to halt follicular growth. Females became increasingly active in early March in 2016, or shortly thereafter in 2017, when water temperatures started to rise. Overall, males had higher relative activity levels compared to those of females, especially in 2017. Males and females both exhibit greater overall activity levels in the spring than in the fall and the co-occurrence of high activity could potentially be the result of mating activity or could be the result of both sexes beginning to forage after a relatively inactive overwintering period.

Nesting activity began in May and continued through early June in 2016 and 2017. The nesting season was the only period during the annual cycle in which females were more active than males. We observed a shift in the diel pattern as well during the nesting season in which nocturnal activity was greater than that of diurnal activity, as was expected due to extensive nocturnal nesting activity by females (Thompson 2013). Progesterone levels slightly decreased post-nesting, but remained elevated during June and July, in co-occurrence with the presence of corpora lutea, which synthesize progesterone. Progesterone has been documented to inhibit vitellogenesis, thus suppressing the growth of new follicles. The July spike in female estradiol, which stimulates vitellogen synthesis in the liver, corroborates our observations of early follicle development beginning in August.

The embryonic development and mortality patterns documented in 2017 suggest that low fertilization rates are partially responsible for overall low hatch success rates, as 26% of eggs did not show any signs of development. Low fertilization rates also could explain inter-clutch variability in hatch success. High levels of embryonic mortality equally impacted hatch success rates negatively. Mean mortality rates among developing eggs across clutches was approximately

50% with a considerable amount of inter-clutch variation. While over 25% of embryos visualized at the time of dissection were documented to have some form of deformity, many other eggs contained what superficially appeared to be well-formed, viable embryos. Observations during candling and dissections suggest that extra-embryonic membrane degradation may be responsible for such mortalities. The chorion is a highly vascularized extra-embryonic membrane that facilitates gas exchange across the shell and is vital for maintaining viable embryos. If this structure deteriorates, as we likely observed in some instances, the embryo will die. However, decreased vasculature of the chorion also could occur if the embryo were to die because the heart is responsible for pumping blood throughout this tissue. In many instances, decomposition of the eggs at the time of dissection made it difficult to determine the cause of mortality.

#### **G. RECOMMENDATIONS:**

- 1) Our documentation of crepuscular activity patterns in this population of alligator snapping turtles suggests that trapping efforts may be enhanced by setting nets in the late afternoon and checking traps the same evening they are set, after twilight, as opposed to early in the morning the following day. We predict that employing this strategy would reduce the number of 'lost' captures as it would decrease the amount of time the turtles have to escape from the traps. The loss of captured alligator snapping turtles is a regular occurrence and is frequently evidenced by the disappearance of the entirety of the bait and an intact trap.
- 2) High seasonal activity levels during the fall and spring suggest that greater trapping success might be accomplished by focusing trapping efforts during these seasons, rather than mid-summer when water temperatures are at their highest and alligator snapping turtle activity levels have sharply decreased. September may be an especially good time to capture both sexes as males and females are both active at this time. Trapping throughout October and early November may be productive for targeting male captures due to the increased activity of males and decreased activity of females; however, this strategy could be less effective if male turtles are not motivated to acquire food at this time and are instead more concerned with mate searching or intrasexual competition. March and April also may be productive trapping months due to increased activity levels in both sexes and a presumed interest in nutrient acquisition after the overwintering period. We caution against trapping during the nesting season in May, unless the objective is to target gravid females for research purposes.
- 3) Future studies should investigate the seasonal and diel activity patterns of a wild population of alligator snapping turtles. Although the captive population studied here is maintained under semi-natural conditions and are exposed to normal seasonal changes in day length and temperature, there remains the possibility that the restrictions on movement and potentially "forced" interactions with conspecifics could result in patterns that differ than those found in the wild.
- 4) High concentrations of male T and high male activity during the fall months indicate that male aggression may be especially heightened at this time. We therefore advise against

the introduction of new individuals, especially males, to the captive population during the fall. Spring introductions also may pose a risk to new individuals because of high levels of male and female activity and the possibility that mating and mate competition are occurring at this time. We propose that the introduction of new individuals be made during the summer when male and female activity levels are decreasing and thermal conditions are suitable for exploring a new environment.

- 5) Isolating the mating season(s) remains an important objective for understanding the reproductive ecology of alligator snapping turtles. Future studies should test for effects of isolating different subgroups of male and/or female turtles during different seasons to determine if isolation of the sexes during certain times of the year influences female gravidity and fertility rates. It would be useful to compare the activity and hormonal patterns of the separate subgroup treatments to the baseline data generated in this study to determine if social grouping affects activity and/or hormonal patterns.
- 6) Due to perceived high rates of infertile eggs, we recommend adjusting the sex ratios of the captive population such that there are fewer females per male (2017 sex ratio was 1 male for every 4.75 females). A 1:2 or 1:3 ratio may increase fertilization rates.
- 7) Future studies should investigate the causal nature of embryonic mortality and deformity occurring in this captive population of alligator snapping turtles. Identifying the embryonic conditions of eggs at different stages prior to mortality could provide insights into the causal nature of embryonic deformities and death.

#### **H. SIGNIFICANT DEVIATIONS:**

The grant objectives and methods did not deviate significantly from the approved proposal; however, we were unable to complete the genetic portion of the project within the fiscal year budget constraints of our cooperative agreement with the Oklahoma Department of Wildlife Conservation. The genomics portion of the project was our single greatest expense and the primer development and genetic sequencing had to be contracted to an outside genomics facility. We were able to prepare whole-genome samples during the first year of the grant, but there was insufficient time for our outside contractor to process these samples.

We investigated the use of single nucleotide polymorphism (SNP) genotyping as a means of determining the parentage of the alligator snapping turtle offspring produced by the Tishomingo NFH breeding group. Alligator snapping turtles exhibit relatively little genetic variability, which requires the evaluation of more SNPs to determine parentage than is typical of most species. We selected multiple SNP markers during this study, but the read lengths of the nucleotides surrounding the individual SNP markers were often too short to design effective primers to target these SNPs. This necessarily added the additional step of sequencing the entire genome of several alligator snapping turtles in order to extend the sequence length surrounding the SNPs. We prepared whole-genome samples but the sequencing of these could not be completed by our

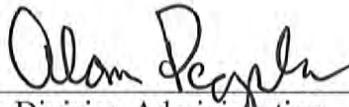
outside contract facility before our budget deadline. Because of this, we were unable to assign both maternity and paternity to each offspring and determine the influence of individual behavior and physiology on reproductive success.

Although the results of the genomic portion of the study were not completed and are not available for this report, we intend to pursue alternative funding to continue this work after the end of the grant. These data should provide important insights that can improve the current alligator snapping turtle breeding program and we plan to provide these results to the ODWC and USFWS as a separate report after completion.

**I. PREPARED BY:** Denise M. Thompson, Oklahoma State University

**DATE:** February 8, 2019

**APPROVED BY:**



Wildlife Division Administration  
Oklahoma Department of Wildlife Conservation



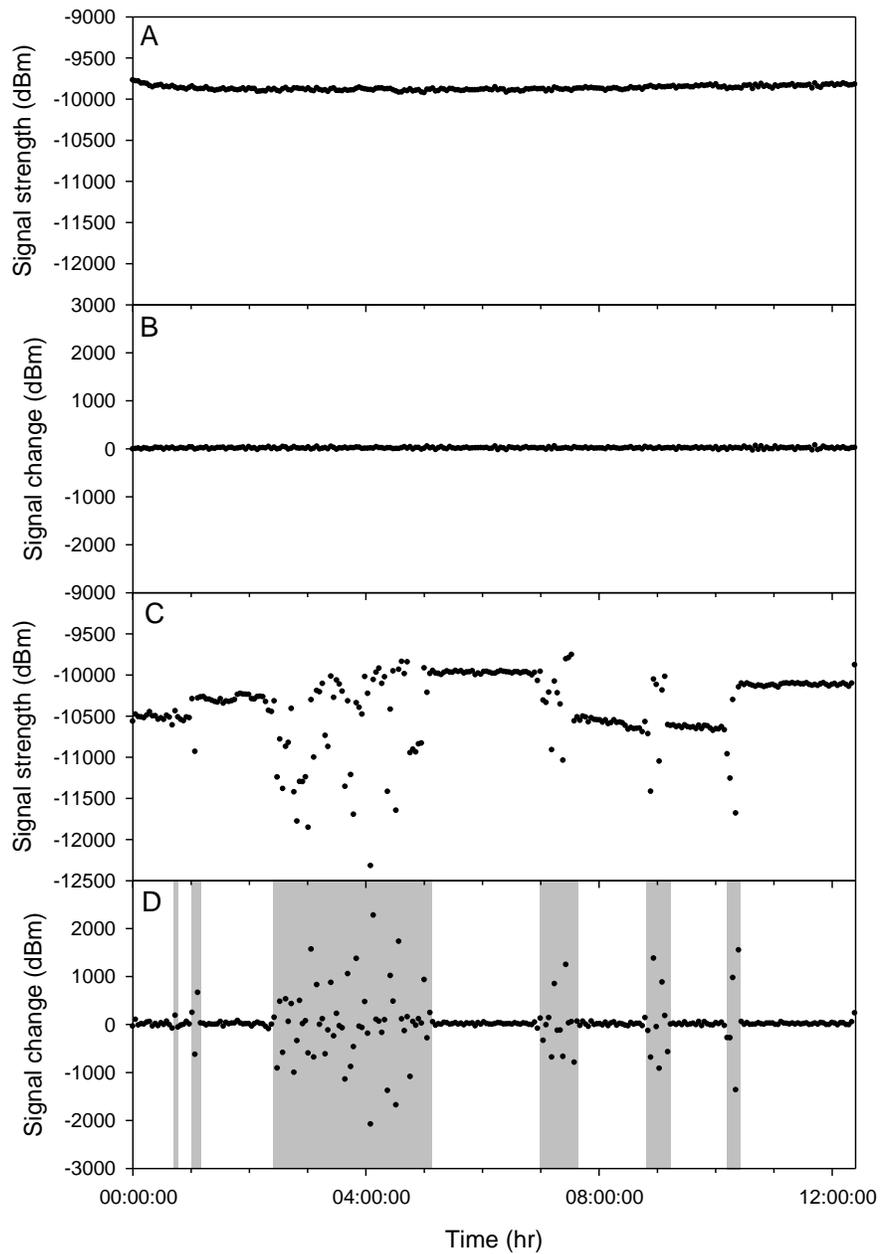
Federal Aid Coordinator  
Oklahoma Department of Wildlife Conservation

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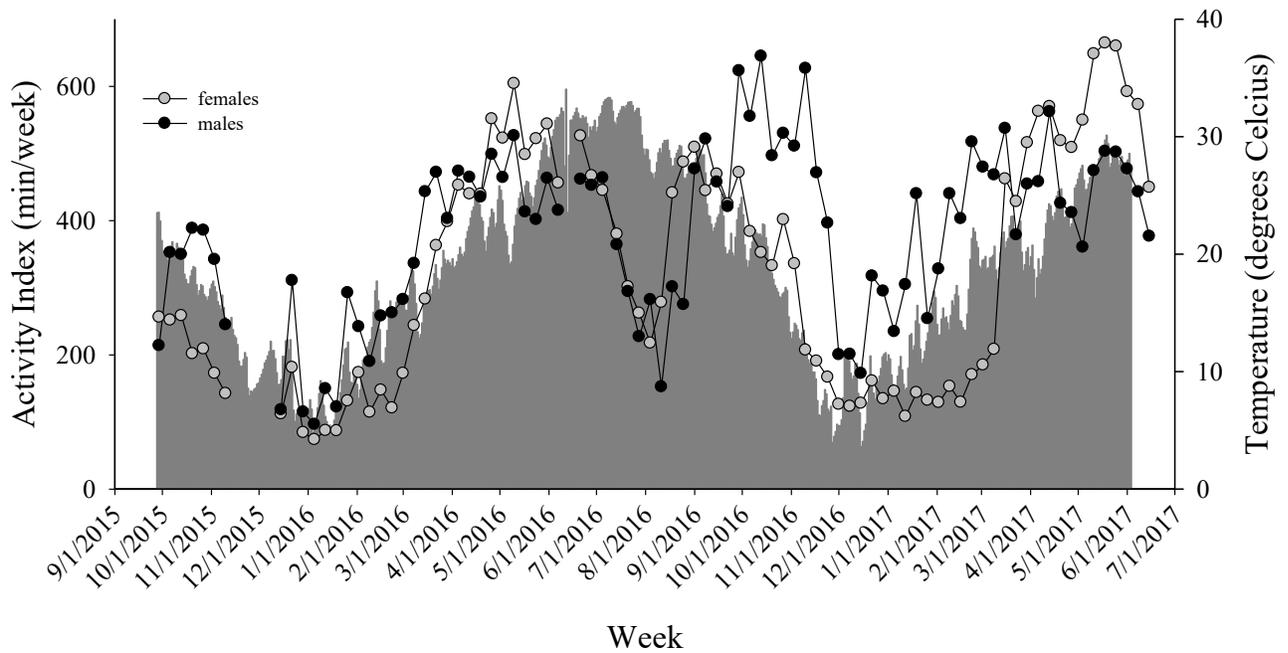
**Figure 1.** Satellite image of alligator snapping turtle ponds at Tishomingo National Fish Hatchery, Tishomingo, OK. The two large ponds are enclosed by a single fence and collectively contain 23 adult turtles. Pond 1 (top) is approximately 2,775 m<sup>2</sup> and Pond 2 (bottom) is approximately 3,600 m<sup>2</sup>. The small pond at the top of the image is isolated from the two large ponds and contains juvenile alligator snapping turtles. Image courtesy of Google Earth.



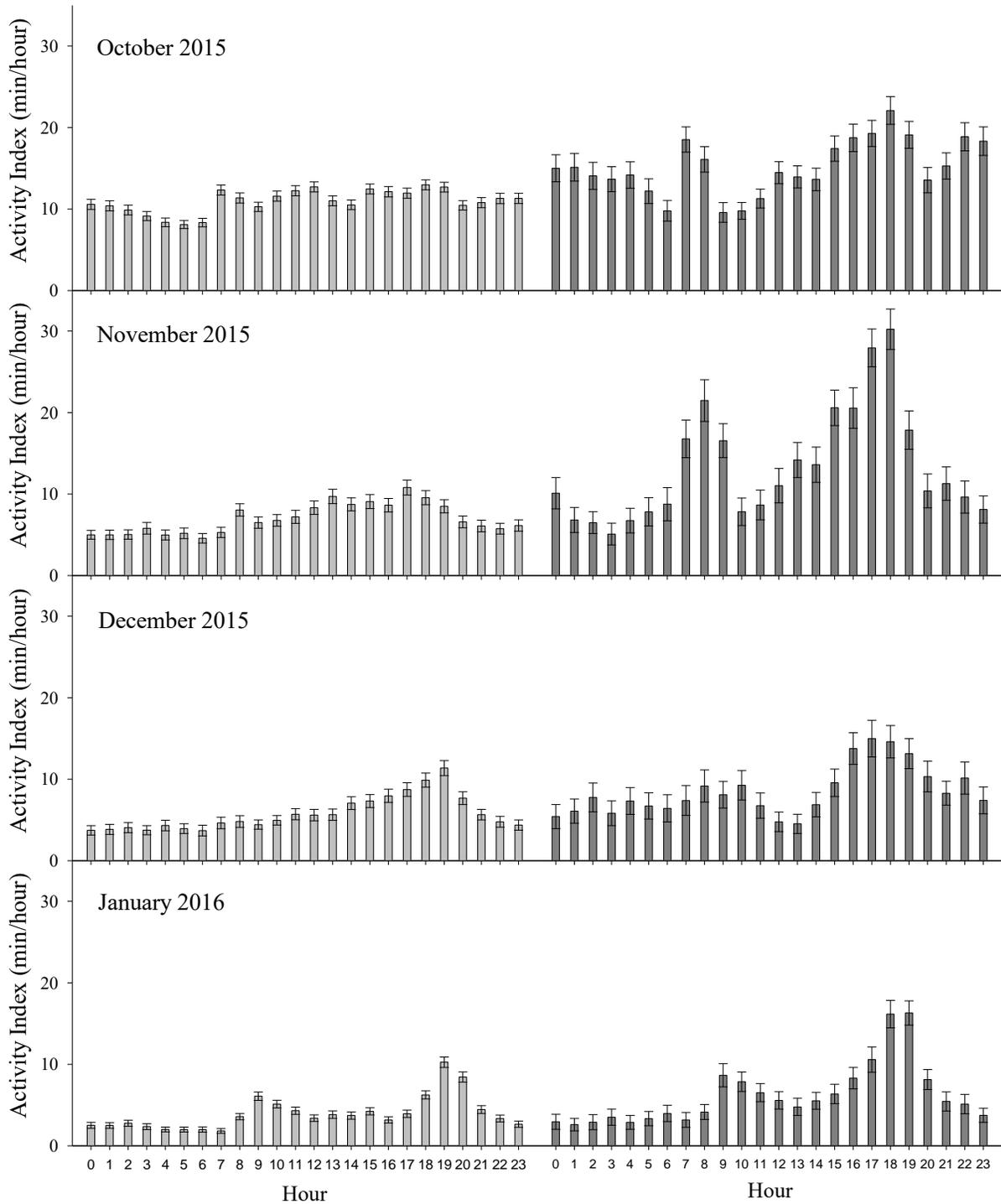
**Figure 2.** Sample signal strength (A and C) and signal change (B and D) plots from the control transmitter (A and B) attached to a stationary cinder block and a transmitter attached to an individual turtle (C and D) over a 12-hour period. Vertical gray bars (plot D) highlight areas of activity generated by changes in position of the transmitter attached to the carapace of a turtle. Note that greater changes in signal strength do not correspond to greater activity or larger movements.



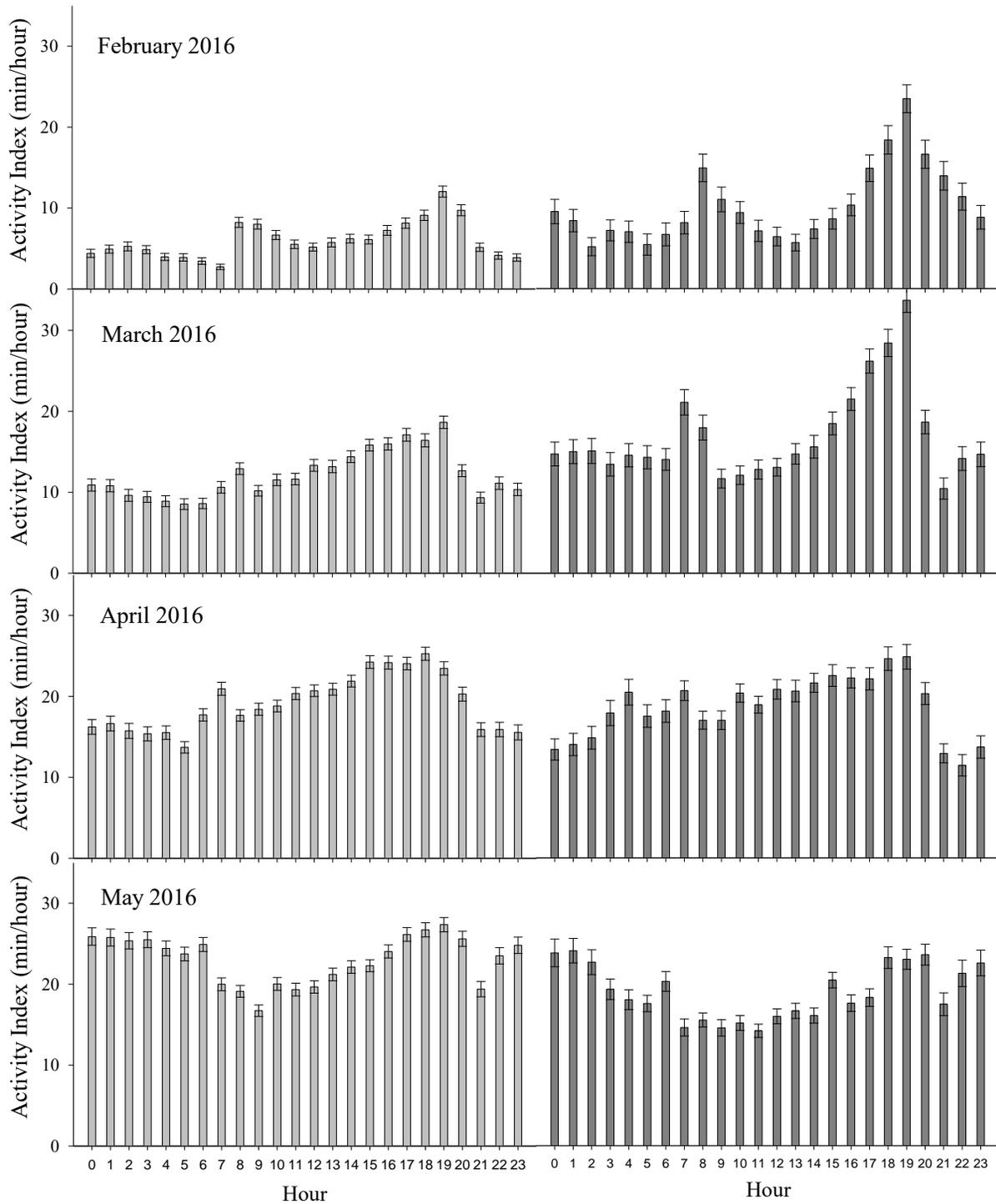
**Figure 3.** Images captured during alligator snapping turtle workup during the 2015 – 2016 field season. A) Female alligator snapping turtle outfitted with temperature data loggers (white spots at top of carapace), a radio transmitter (copper cylinder mid-carapace), and a unique identification number applied with marine epoxy. B) A blood sample being taken from the caudal vein on the dorsal side of the tail. C) Ultrasound probe being applied to the hind-left inguinal fossa to visualize ovarian structures.



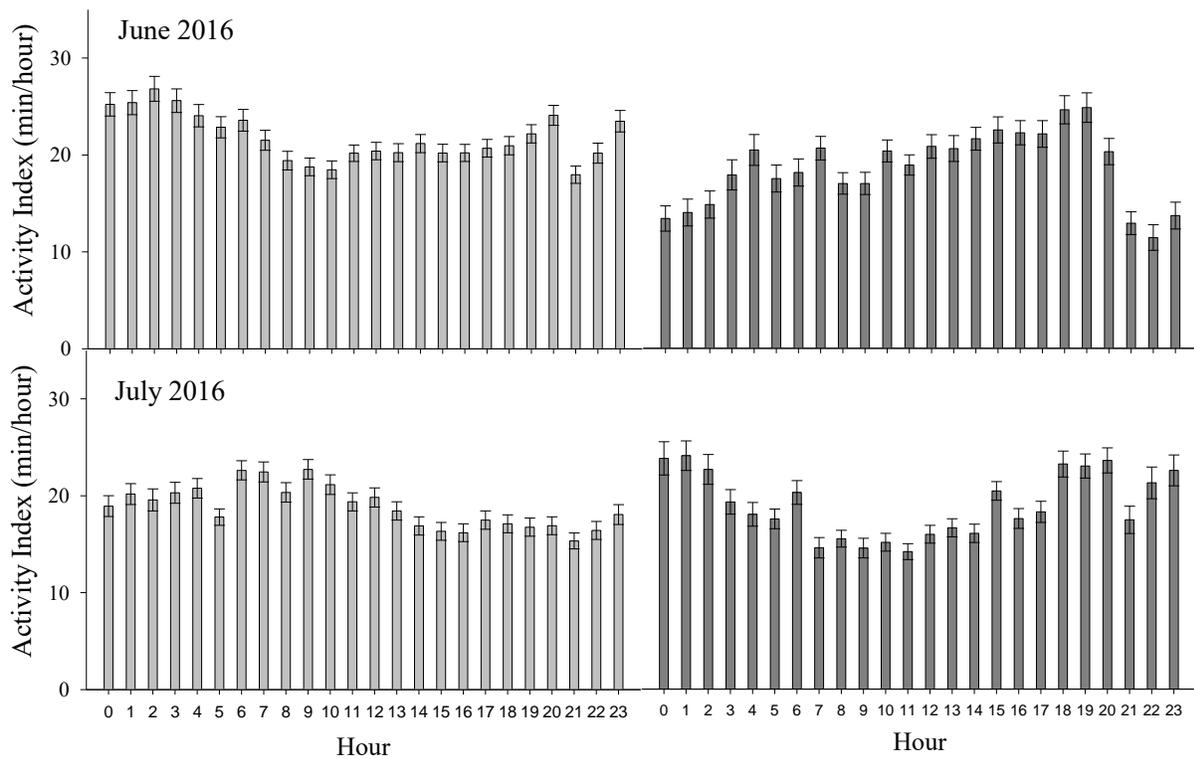
**Figure 4.** An index of mean weekly activity levels (minutes/week) for male (black dots) and female (gray dots) alligator snapping turtles (*Macrochelys temminckii*) from a captive population in southeastern Oklahoma. Activity was recorded over a 90-week period from 29 September 2015 – 19 June 2017. Vertical gray bars represent mean daily water temperatures of the ponds.



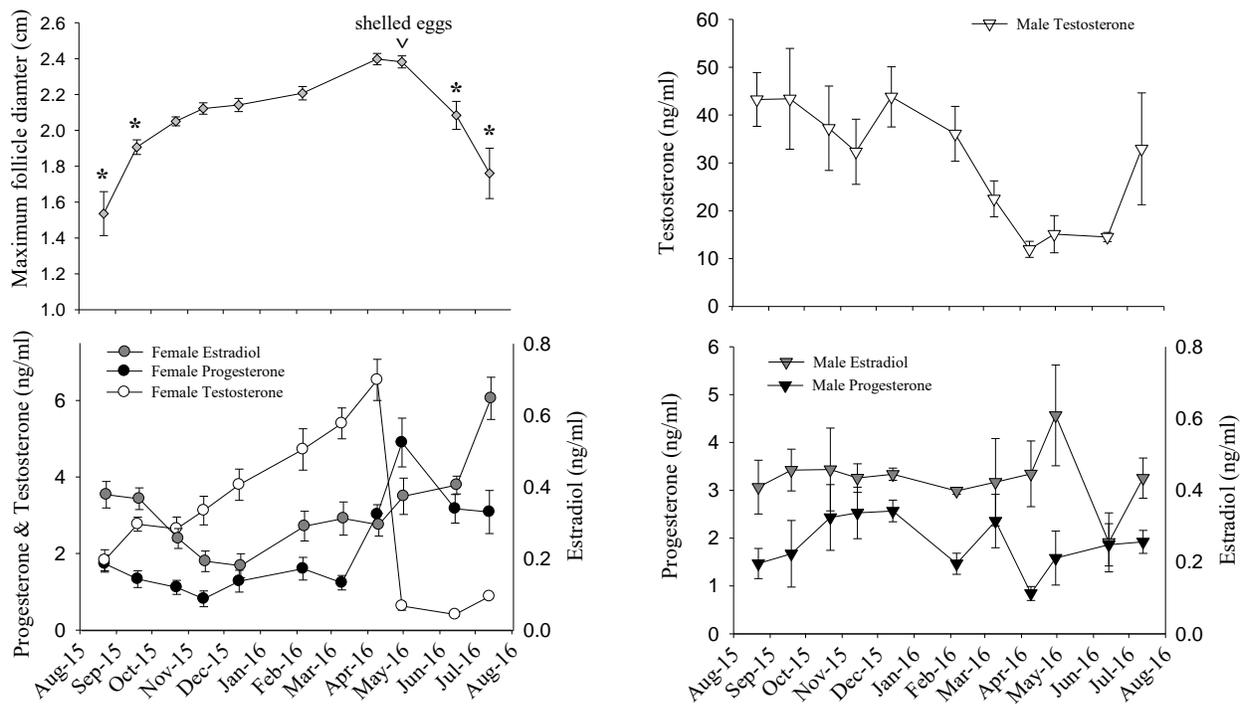
**Figure 5a.** Activity index of mean hourly activity levels (min/hour) by month for female (light gray) and male (dark gray) alligator snapping turtles from a captive population of alligator snapping turtles (*Macrochelys temminckii*). Data presented are from 29 September 2015 – 31 January 2016. Bars represent 1 standard error.



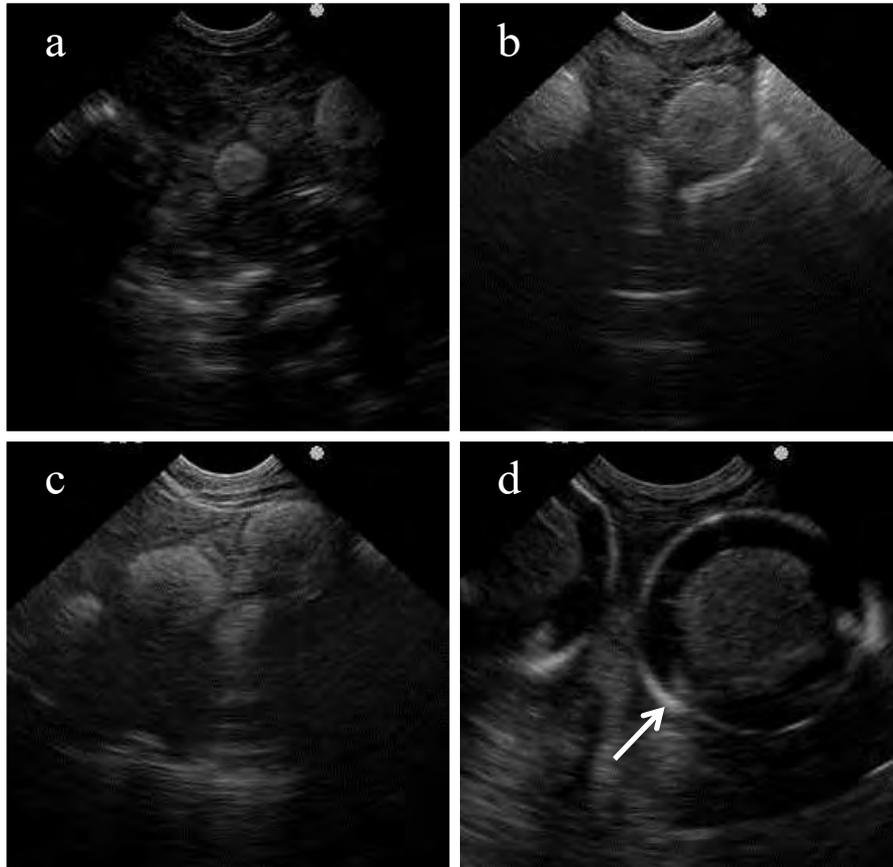
**Figure 5b.** Activity index of mean hourly activity levels (min/hour) by month for female (light gray) and male (dark gray) alligator snapping turtles from a captive population of alligator snapping turtles (*Macrochelys temminckii*). Data presented are from 1 February 2016 – 31 May 2016. Bars represent 1 standard error.



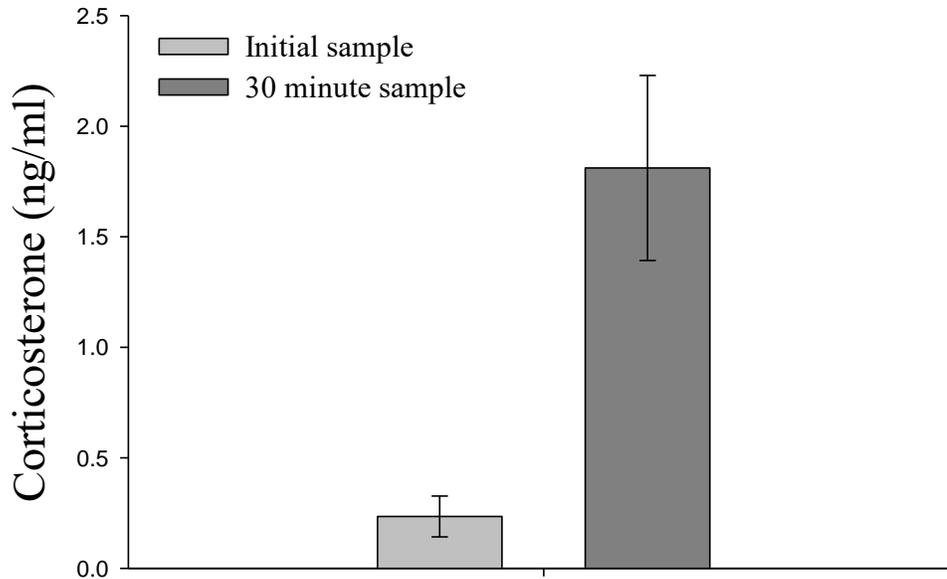
**Figure 5c.** Activity index of mean hourly activity levels (min/hour) by month for female (light gray) and male (dark gray) alligator snapping turtles from a captive population of alligator snapping turtles (*Macrochelys temminckii*). Data presented are from 1 June 2016 – 31 July 2016. Bars represent 1 standard error.



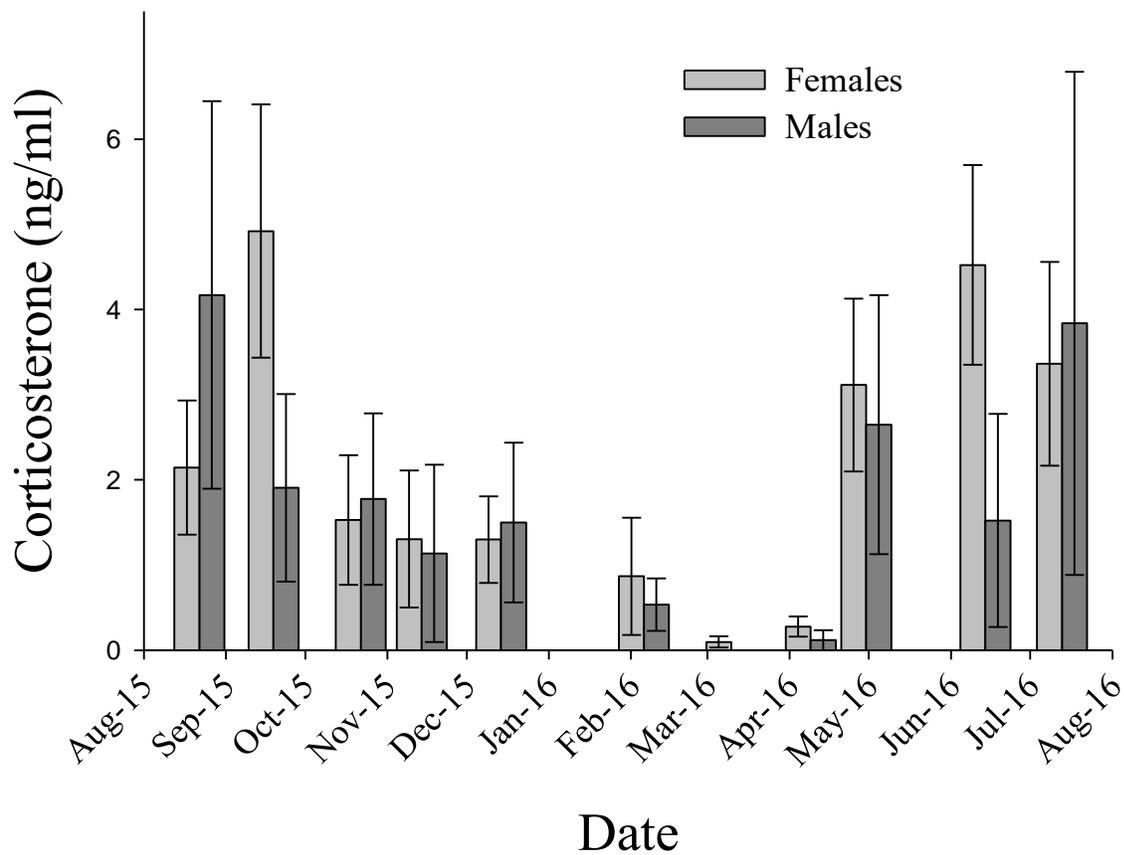
**Figure 6.** Line graphs of follicular development and mean sex steroid concentrations from a captive population of alligator snapping turtles (*Macrochelys temminckii*) over a one-year period (22 August 2015 – 13 July 2016). Each point on the graph corresponds with the sampling date. Top left: mean maximum ovarian maximum follicle diameter (cm). Bottom left: female estradiol, progesterone, and testosterone concentrations. Top right: male testosterone concentrations. Bottom right: male estradiol and progesterone concentrations. Symbols represent mean concentration (or diameter) and bars represent 1 standard error.



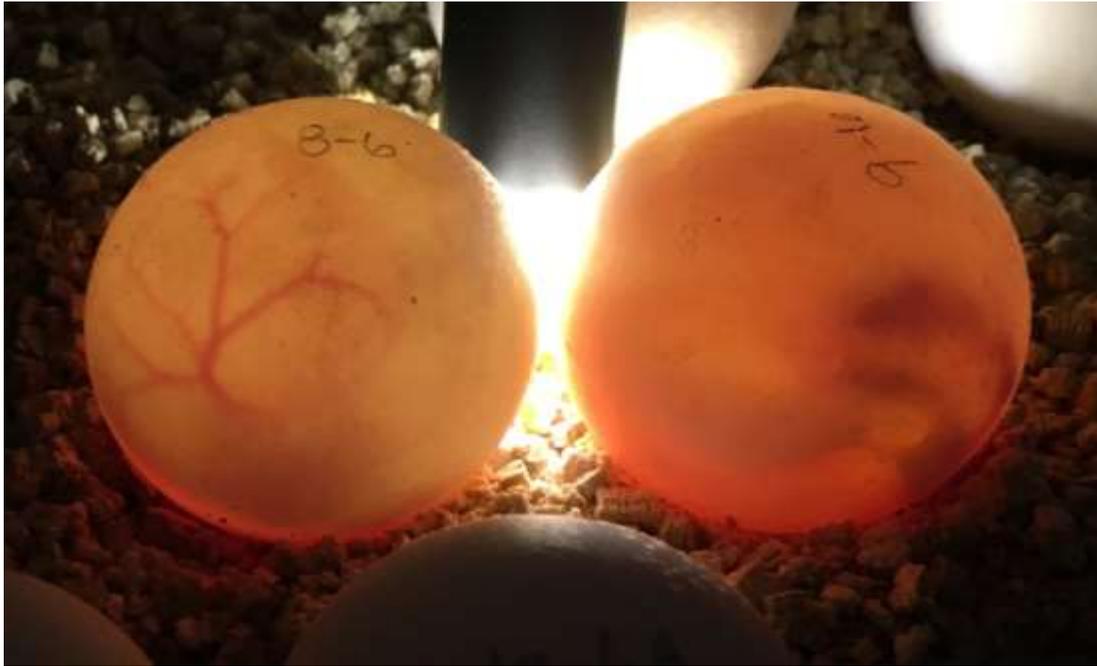
**Figure 7.** Representative sonogram images of ovarian follicular development in the alligator snapping turtle (*Macrochelys temminckii*) over a period of 8 months (late August – early May): (a) early vitellogenic follicles on 22 August 2015; (b) vitellogenic follicles on 14 November 2015; (c) vitellogenic follicles from 6 February 2016; (d) fully developed and ovulated follicle with albumen and shell present (calcified shell indicated by arrow) on 4 May 2016.



**Figure 8.** A comparison of mean CORT concentrations from plasma samples collected at the time of initial capture (n = 19) and 30 minutes after capture (n = 17) from a captive population of alligator snapping turtles (*Macrochelys temminckii*). Bars represent 1 standard error. Samples were collected on 9-April-2016. Mean time from capture to initial blood draw was 1 m 44 s (range 30 s – 6 m 10 s) while mean time from initial to second (30 minute) blood draw was 30 m 37 s (range 28 m 38 s – 35min 51 s).



**Figure 9.** Comparison of female (light gray) and male (dark gray) CORT concentrations obtained from a captive population of alligator snapping turtles (*Macrochelys temminckii*) over a period of one year (22 August 2015 – 13 July 2016). Bars represent  $\pm 1$  standard error.



**Figure 10.** A comparison of viable (left) and nonviable (right) alligator snapping turtle (*Macrochelys temminckii*) embryos during daily candling checks at Tishomingo National Fish Hatchery. Note the vascularization present within the viable egg and contrast that with the lack of distinct vascular structures and dark red blotched appearance of the egg containing an embryo that is no longer viable.

**Table 1.** Variation in early egg developmental patterns, the number of embryo and hatchling deformities, and overall reproductive success among 18 alligator snapping turtle clutches produced in 2017 at Tishomingo National Fish Hatchery.

Clutch ID	Female ID	Eggs Incubated	No Whitening	Partial Whitening	Full Whitening	Embryo Mortalities	Embryo	Hatchlings with Deformities	Eggs Hatched	Percent Hatch Success <sup>+</sup>
							Mortalities with Deformities (minimum)*			
1	F_16	30	14	1	15	4	0	0	12	40
2	F_15	45	29	2	14	9	0	1	7	16
3	F_08	36	0	1	35	11	2	0	21	66
4	F_11	32	2	1	29	4	0	1	26	81
5	F_19	22	14	0	8	4	1	0	4	18
6	Unk.	39	17	9	13	16	2	0	6	15
7	Unk.	26	5	1	20	7	1	0	14	54
8	Unk.	27	2	0	25	7	3	0	18	67
9	Unk.	31	2	0	29	3	0	0	26	84
10	Unk.	34	11	1	22	14	1	0	8	24
11	Unk.	38	0	0	38	1	1	2	37	97
13	F_01	39	3	0	36	5	3	1	31	79
14	F_05	33	18	2	13	9	4	0	6	18
16	Unk.	25	23	1	1	1	0	0	1	4
17	Unk.	40	0	3	37	10	4	8	30	75
20	F_03	31	0	2	29	9	4	3	22	71
21	F_18	13	2	6	5	9	1	1	2	15
22	Unk.	40	10	7	23	19	5	2	11	28
<b>Totals</b>		<b>581</b>	<b>152</b>	<b>37</b>	<b>392</b>	<b>142</b>	<b>32</b>	<b>19</b>	<b>282</b>	<b>49</b>

\* Embryo deformity counts are expressed as minimum values because the embryo condition could not be discerned for a notable proportion of the embryos visualized (22%) due to varying degrees of decomposition.

+ Hatch success rates have been adjusted to exclude mortalities caused by fly and maggot infestation incurred during the last week of incubation. These adjustments only affected the hatch success rate of clutch 3.

**Appendix A.** Descriptive statistics of blood sample collection from a captive population of alligator snapping turtles (*Macrochelys temminckii*), which includes: 1) time between capture and first blood draw (initial sample), and 2) time between first and second blood draws (“30” min. sample time) for the months of September and April.

<b>Sampling Period</b>	<b>Sample Size</b>	<b>Mean</b>	<b>Range</b>
<i>August 2015</i>			
Initial sample	21	00:04:13	00:02:03 – 00:07:38
<i>September 2015</i>			
Initial sample	23	00:02:59	00:01:00 – 00:07:31
“30” min. sample	22	01:04:55	00:31:44 – 02:21:56
<i>October 2015</i>			
Initial sample	24	00:02:09	00:01:04 – 00:06:05
<i>November 2015</i>			
Initial sample	19	00:02:32	00:00:49 – 00:06:59
<i>December 2015</i>			
Initial sample	22	00:01:05	00:00:21 – 00:02:02
<i>February 2016</i>			
Initial sample	20	00:01:10	00:00:25 – 00:02:26
<i>March 2016</i>			
Initial sample	21	00:01:44	00:00:54 – 00:05:09
<i>April 2016</i>			
Initial sample	19	00:01:44	00:00:30 – 00:06:10
“30” min. sample	18	00:30:36	00:28:38 – 00:35:51
<i>May 2016</i>			
Initial sample	22	00:01:17	00:00:21 – 00:04:06
<i>June 2016</i>			
Initial sample	13	00:01:24	00:00:41 – 00:02:42
<i>July 2016</i>			
Initial sample	19	00:02:07	00:00:40 – 00:06:46
	<b>263</b>	<b>00:02:04</b>	<b>00:00:21 – 00:07:38</b>