WOOD DUCK (AIX SPONSA) NEST SUCCESS AND PRODUCTIVITY IN ARTIFICIAL NEST BOXES ACROSS THE PIEDMONT OF GEORGIA

by

CASSIDY BROWN

(Under the Direction of Susan Wilde)

ABSTRACT

Wood ducks (*Aix sponsa*) are an important game species throughout North America and rely on natural cavities for nesting. Wood ducks face ongoing threats from continued loss of forested and wetland habitats, which has prompted mitigation efforts to provide artificial nesting cavities through the use of nest boxes. However, previous research has shown highly variable nest success in nest boxes, mandating continued studies to document factors influencing the effectiveness of nest boxes to improve productivity. I used game cameras and temperature sensors to track nest progress at 4 locations in the Piedmont ecoregion of Georgia, USA. Nest success was low relative to published studies and could be improved with consistent box husbandry. I detected a novel neurotoxin, aetokthonotoxin, in 12% of collected wood duck eggs, indicating potential risk for vacuolar myelinopathy. Wood ducks act as sentinels when their nests fail in unhealthy habitats, therefore prompting the establishment of management efforts.

INDEX WORDS: aetokthonotoxin, behavior, egg, membrane, nest box, nest success, suboptimal temperature, vacuolar myelinopathy, wood duck.

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CHAPTER 1

INTRODUCTION

Background

Wood ducks (*Aix sponsa)* are important cavity-nesting ducks that breed in eastern North America along wetlands and other small bodies of water. The wood duck, now a common species, faced a profound population decline in the early 20th century. Overharvest and habitat loss led to wood duck declines (Bellrose 1990, Hepp et al. 2020). Concerned conservationists advocating for legislation, pushed for complete protection under the Federal Migratory Bird Act of 1916, and eventually, the Migratory Bird Treaty Act of 1918 with Canada (Bellrose 1976). With the passage of the Migratory Bird Treaty Act, hunting seasons on wood ducks were closed from 1918–1941. During this time wood duck populations recovered, and hunters could once again harvest wood ducks in the Atlantic and Mississippi Flyways in 1942 (Hepp et al. 2020).

Natural cavities are an essential part of the habitat of a wood duck. This species is dependent on pre-existing cavities, expanded through damage or tree decay and excavation by woodpeckers (Zlonis et al. 2020). Therefore, the establishment of nest boxes in agricultural or deforested areas may mitigate the loss of suitable habitat (Lowney and Hill 1989, Davis et al. 2015). Furthermore, nest boxes could increase nest use by southern populations of wood ducks, chiefly if boxes are managed during longer breeding seasons in the southern United States (Lowney and Hill 1989, Davis et al. 2017, Davis et al. 2015). Artificial nest boxes have been shown to support populations of wood ducks by providing available space for nesting (Hepp et al. 1987, 1989; Bellrose and Holm 1994; Kennamer et al. 2016, Dyson et al. 2018). An estimated

100,000 nest boxes in North America yielded approximately 300,000 ducklings annually in the mid-1900s (Bellrose and Holm 1994, Utsey and Hepp 1997, Davis et al. 2015).

Reduced wood duck nesting success in artificial boxes could result from various factors including lack of effective predator guards, crowded locations, inadequate box maintenance, or poor environmental conditions (Hepp, et al 2020, Miller, et al 2024). Wood ducks, and their eggs, can also serve as a bioindicator for the health of the ecosystem, as well as other inhabiting species (Augsperger et al. 2008, Kennamer, et al 2005). Through a diet of aquatic vegetation, many waterfowl species are exposed to a cyanotoxin, aetokthonotoxin (AETX), which induces Vacuolar Myelinopathy (VM) (Haram, et al 2020). Aetokthonotoxin, known as the "eagle-killer toxin", is produced by the epiphytic cyanobacterium (*Aetokthonos hydrillicola*) growing on invasive Hydrilla (*Hydrilla verticillata)* (Wilde et al. 2014, Breinlinger et al. 2021). It was discovered following a mass die-off of eagles in Arkansas impoundments and has now been documented in 25 additional reservoirs across the southeastern United States (Breinlinger et al. 2021). After ingesting the "eagle-killer toxin", eagles, waterbirds, fish and amphibians experience neuropathy, including tremors, and seizures eventually leading to death.

Wood ducks have been included in many contaminant studies due to their extensive geographical distribution in North America and can be easily studied because they are attracted to nest boxes (Kennamer et al. 2005). Lead and mercury are common contaminants in avian eggs. A study in the New Jersey Meadowlands examined lead and other contaminants within tissue, feathers, and eggs of Canada Geese (*Branta canadensis)* in comparison to other native avian species in the area. An instrument with a minimum detection limit of 0.15 ppb was established for lead (Tsipoura et al. 2011). Eggs from Canada Geese at an average of 505 ppb dry weight (161± 36.7 ng/g wet weight) had higher levels of lead compared to other passerine species in the area (Tsipoura et al. 2011). In a study from the U.S. Department of Energy's Savannah River Site in South Carolina, elevated mercury concentrations were found in wood duck eggs. Mercury was most significant in albumen where the average concentration was 0.22 ppm wet mass (Kennamer et al. 2005). Using UGA ICP-MS, mercury sensitivity thresholds for the albumen (0.034 ppm) and yolk (0.007 ppm) were determined; shell limits could not be found as they were low and highly variable (Kennamer et al. 2005). In this study, we investigated the potential negative effect of a prey base contaminated by novel toxin, AETX, a cyanobacterial neurotoxin produced by *A. hydrillicola*.

Study Significance

The purpose of this study is to provide wildlife resource biologists and wood duck managers with an assessment of nest success across Georgia. Site-specific findings can be used to improve or establish management plans leading to higher productivity. For example, boxes that may have had limited success in high-activity recreational or open areas could be removed or relocated to more secluded sites. This study will examine several potential site-specific variables that could contribute to a decline, including box husbandry and the presence of an emerging environmental neurotoxin, aetokthonotoxin, "eagle-killer toxin". The results of this analysis could form the basis for improved box and habitat management by Georgia biologists. Management plans could be implemented to prevent declines and improve nest box productivity for the continued conservation of wood duck populations.

Thesis Structure

This chapter provides a general overview of the life history and habitat requirements of the wood duck. The examination of the number of hatchlings and potential areas of decline can aid in successful wetland management decisions.

The second chapter assesses a 20-year historical data set provided by Georgia Department of Natural Resources (GADNR), an 8-year data set provided by Henry County Water Authority (HCWA), and a 2-year data set provided by the city of Carrollton, Georgia.

The third chapter examines nesting success at 4 sites within the Georgia Piedmont (B.F. Grant WMA, Carrollton, GA subsites, Oconee WMA, and Henry County Reservoirs (Long Branch and Tussahaw). This chapter highlights wood duck nest-specific metrics: proportion of nest success (indicated by the number of hatched eggs), incubation temperature, female behavior (top of the box (TB), perching/presence at box entrance (PE), flying away from the box (FAB), or flying toward the box (FTB)), the potential for disturbance, and relative predation risk.

The fourth chapter investigates an emerging risk to southeastern waterfowl populations using wood ducks as sentinels for contaminant accumulation potential. This chapter describes the methods used to detect the presence of a lipid-soluble cyanotoxin, aetokthonotoxin (AETX) in wood duck eggs from 4 Georgia Piedmont locations.

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CHAPTER 2

PARAMETERS AFFECTING WOOD DUCK NESTING SUCCESS IN ARTIFICIAL BOXES MANAGED BY STATE, CITY, AND PUBLIC UTILITY IN THE GEORGIA PIEDMONT

[1] Brown, C., J.A. Martin, M. Chamberlain, K.C. Callaghan, S.A. McWhorter, and S.B. Wilde. To be submitted to the *Journal of Wildlife Management*.

Abstract

Wood ducks (*Aix sponsa*) in the United States have been under management for decades to increase populations. A well-managed artificial nest box program can enhance nesting opportunities and contribute to overall population growth. Alternatively, poorly managed boxes or those placed in a contaminated habitat could result in poor hatching success rates and mortality. We compared historical nesting data from the Department of Natural Resources in the state of Georgia (GADNR), a public company Henry County Water Authority (HCWA), and Carrollton city-managed sites (Carroll County). Technicians and staff record membranes and unhatched eggs during nest checks, provide location information, and record parameters such as nest competition, predation, and box condition. We determined that nesting success would increase with consistent nest box maintenance and act as an indicator of habitat suitability across state, public, and city-managed sites.

Introduction

Nesting data for wood ducks (*Aix sponsa*) provides managers with species productivity estimates. Since the 1930s, both public and government efforts have led to the installation of thousands of nest boxes helping boost and spread local breeding populations of wood ducks across their range (Kennamer and Hepp 2000). Through the installation of artificial nest boxes, managers can better monitor this cavity-nesting species. Recognizing the link between eggshell membranes and duckling survival, waterfowl researchers have historically counted membranes in nests to gauge reproductive success (Girard 1939, Klett et al. 1986, Brian et al. 1998). Collected nesting data can include various parameters such as the number of membranes (an indication of a successful hatchling), number of unhatched eggs, and evidence of predation. Long-term data collected from 18 national and state refuges across the eastern range of the wood duck revealed substantial discrepancies between the total number of eggs laid and the number of ducklings successfully produced (Semel and Sherman 1995). The Georgia Department of Natural Resources' Waterfowl Committee reports an average of 40% nest success for wood ducks based on collected nesting data (Balkcom et al. [Year unknown]). However, most studies report a higher percentage of wood duck nesting success within nesting boxes. From relevant literature, an average of 66% nesting success in artificial nest boxes was observed (Table 2.1). In contrast, an average of 41% of nesting success within natural cavities was reported (Table 2.1). Year-long nesting data provides an essential means to detect trends in nesting success as well as the overall population productivity of wood ducks.

We evaluated nest success in the Piedmont of Georgia within the Georgia Department of Natural Resources (GADNR) managed sites (provided by Greg Balkcom, former state waterfowl biologist). We listed Public Fishing Areas (PFAs), Wildlife Management Areas (WMAs), and other state-managed sites within each region in addition to the data of the number of boxes found within each site, the number of boxes checked, boxes used by wood ducks, wood duck membrane counts, unhatched wood duck eggs, and boxes used by other cavity-nesting species.

GADNR wildlife technicians checked and cleaned the artificial nest boxes annually and collected data on nest outcomes. Nest box cleaning consisted of removing old nesting material and replacing it with fresh wood shavings. As the boxes were cleaned, technicians counted the membranes found in the boxes, which are used to predict the number of successful hatchlings. Not all boxes were checked during the 20-year cycle; sites left unchecked were removed from the specific years' data set. The data provided included the total counts for a specific site or WMA, but not specific box results. We compared wood duck nesting data in five Henry County Water Authority (HCWA) drinking water reservoirs: Upper Towaliga, Lower Towaliga, Long

Branch, Tussahaw, and Gardner Reservoirs from 2018-2025. Additionally, we evaluated data for two years in four subsites within the city of Carrollton: Curtis Creek, Hobbs Farm, Zyzzx Street, and near the Little Tallapoosa River. Nesting data from these sites included hatched and unhatched counts per box and site from 2021-2022.

We hypothesized that nesting success would differ between the entities: state (GADNR), public corporation and authority (HCWA), and city-managed sites (Carrollton, GA). Differences could be inferred through nest box maintenance, location, husbandry (efficient clean out and timely checks of boxes), and habitat quality. Individual box data was not included in the GADNR data set, but nest box-specific data was collected within both HCWA and Carrollton, GA. Nesting data provides insights for improving habitat and box management for wood ducks within these sites.

Methods:

Data Collection Nesting Parameters

The 20-year dataset included WMAs, PFAs, and other state-managed wood duck sites. We analyzed nest success in 27 state-managed sites within the Georgia Piedmont (Figure 2.1). The following were examined within the data set: the sum of boxes used compared with boxes checked by technicians, the number of boxes occupied by nest competitors within each site (e.g. mergansers, birds, squirrels), the percentage of hatched eggs per site, the percentage of hatched eggs per year, and site-specific membrane and unhatched eggs throughout each year (Appendix A). Site nesting parameters were utilized to isolate areas of high and low success over time. The percentage of hatched eggs was chosen to determine overall nesting success per year and site.

Statistical and Data Analyses

We compared nest success in WMA/sites located within our primary watersheds: Chattahoochee, Savannah, Oconee, Ocmulgee, and Flint. We predicted that hatching success could differ by watershed based on habitat suitability and water quality. The standard error was calculated using pivot tables by dividing the standard deviation by the square root of the sample size (sites in each watershed) (Excel). Watershed significance was measured by implementing a generalized linear mixed model (GLMM) using R version 4.5.0 (R Core Team, 2025). The watersheds were included as a fixed effect, WMAs as a random effect, and hatched versus unhatched as the response variable.

We hypothesized that nest success would be highest in the most well-managed sites unless habitat conditions are unfavorable. We expected success to vary across sites (GADNR, Henry County Reservoirs, and Carrollton, GA) over time relative to husbandry and habitat conditions. We calculated the percentage of hatched eggs or nesting success by taking the total number of hatched eggs and dividing by the total number of eggs/membranes (both hatched and unhatched) found in the nest box. In addition to the percentages of hatched eggs per site, we evaluated yearly nesting success. Finally, we compared sites and years to the average nesting success from relevant wood duck nesting literature.

Results:

Statistical and Data Analyses

We concluded that there was some variability in the hatching rate between different WMAs based on our model. Individual variation in the WMAs did not explain any more of the variation in success beyond the fixed effects (watersheds). Compared to the intercept

(Chattahoochee), the Flint watershed was the only watershed that exhibited a significantly higher probability of nest success indicated by hatching ($p < 0.05$) (Figure 2.2).

GADNR Percent usage of checked boxes

The first aspect of box success was to evaluate wood duck usage compared to checked boxes at each individual site. Allatoona (48 checked boxes), Keg Creek (12 checked boxes), Lake Russell (6 checked boxes), and Wilson Shoals (2 checked boxes) were eliminated from the data set as these sites had no wood duck usage. Based on the percentages of usage and number of sites, these locations were characterized as having low (10-40% usage), medium (50-60% usage), and high (70-100% usage) among boxes checked. Seven sites fell within the low category (Table 2.3), 13 sites were included in the medium category (Table 2.4), and 3 sites fit within the high category (Table 2.5). The lowest-success sites were Pine Log Mountain (9 boxes used from 184 checked boxes) and Phinizy Swamp (1 box used from 12 checked boxes), both of which had less than 10 percent usage.

GADNR Non-wood duck nest competition/disruptors

Nest competition/nest disruptors (non-wood ducks) were also examined within each site. A total of 1,240 nest boxes were characterized as used by birds, 146 by squirrels, 47 by "other", and 18 by mergansers. The section "birds" was not specified; it can be hypothesized as any cavity-nesting Passerine species or Eastern Screech Owls (*Megascops asio*) of which are common inhabitants in wood duck nest boxes. The section "other" was used to describe a present occupant of the nest not related to the other categories listed. While the "other" was not specified for boxes and sites, this can be hypothesized to be wasps, bats, snakes/predators, or any other fauna residing within the box.

The most common nest competitors were birds, with four sites exhibiting over 200 instances of bird presence. The corresponding sites were West Point (250 boxes used by birds), Big Lazer (249 boxes used by birds), and Blanton Creek (217 boxes used by birds). It is important to note that these sites also had more boxes checked than the other sites. Therefore, there was a higher potential for the discovery of nest competitors. Five sites exhibited fewer than ten instances of birds. These sites were Dawson Forest (2 boxes used by birds), Hart County (7 boxes used by birds), McGraw Ford (6 boxes used by birds), Paulding Forest (1 box used by birds), and Phinizy Swamp (4 boxes used by birds). These areas also had fewer boxes checked compared to the other sites. The second most common nest competitor was squirrels, with 2 sites exhibiting over 20 instances of presence. The sites were Big Lazer Creek (39 boxes used by squirrels) and Rum Creek (25 boxes used by squirrels). Lake Lanier had the highest number of boxes used by “other” (15). The other sites exhibited fewer than 10 occurrences of other species. Boxes used by mergansers were significantly lower compared to the other nest competitors. The highest number of boxes with mergansers was from the Chattahoochee Fall Line (11 boxes). The remaining sites had 5 or fewer instances of merganser presence.

<u>GADNR Membranes and Unhatched Eggs</u>

There were 10,911 wood duck membranes and 6,992 unhatched eggs observed over 20 years. Three sites contributed to the most successful wood duck nests (over 1,000 membranes counted). These sites included Big Lazer Creek (1,685 membranes), Blanton Creek (1,670 membranes), and West Point (1,397 membranes). From 2004 to 2009, there was a downward trend in the combined total number of successful and unsuccessful wood duck eggs at all sites. In 2005, there was an equal number of membranes and unhatched eggs counted (461 membranes, 460 unhatched eggs). From 2013 to 2020, there was an upward trend in both membranes and unhatched eggs. The year 2009 had the lowest counts (214 membranes and 97 unhatched), while 2019 had the highest counts (1,056 membranes and 610 unhatched eggs).

GADNR, Henry County, and Carrollton, GA Nesting Success

Among GADNR sites, Big Lazer Creek, Joe Kurtz, Lake Lanier, McDuffie PFA, Blanton Creek, and West Point had high and less variable nesting success (> 66%) (Figure 2.3). Sites with consistent nesting success <66% include B.F. Grant, J.L. Lester, Oconee, Redlands, Rum Creek, and Vaughter (Figure 2.3). Three sites were of the greatest concern: Pine Log Mountain (consistent low success), Phinizy Swamp (low success with moderate variability). Clarks Hill (high average, and high variance) (Figure 2.3). The remaining sites were less consistent. The most notable years with high nesting success (above 66%) and low variability were 2001, 2002, 2009, 2010, and 2011 (Figure 2.4). Years with low success (below 66%) and consistent results included 2004, 2008, 2015, 2017, 2018, 2019, 2020 (Figure 2.4).

The average nesting success in Henry County reservoirs was below 66%. Gardner and Tussahaw reservoirs had low, consistent success, while Long Branch and Lower Towaliga had more variation, but again, low overall success (Figure 2.5). Upper Towaliga had consistently poor nest success (Figure 2.5). Over time, steady low nesting success was noted in 2018, 2024, and 2025, with additional years showing more variation (Figure 2.6). Nesting success was at its lowest (~ 20%) in 2018 and at its highest (~ 36%) in 2023 (Figure 2.6).

Carrollton sites included were only evaluated from 2021-2022. In 2021, Hobbs Farm was the only site with high (78%) and consistent nesting success (Figure 2.7). There was low nesting success and more variation in Curtis Creek (55%), Little Tallapoosa River (56%), and Zyzzx Street (51%) (Figure 2.7). In 2022, Curtis Creek was the only site to show both high (100%) and low variability (Figure 2.7). Little Tallapoosa River also had high nesting success (69%), but variance was high (Figure 2.7). Hobbs Farm was consistent with low success (40%), whereas Zyzzx Street was variable with low success (29%) (Figure 2.7).

Discussion

We compared the average nesting success (including the lowest and highest percentages). The average and range were much lower than expected for all sites when compared to observed nesting success in the literature (Tables 2.1 and 2.2). Nesting success was both low overall and highly variable. Carrollton's urban sites had an average of 58% success while GADNR properties had an average of 56% nesting success. Box-specific parameters are needed to determine specific factors inducing low nesting success. Various parameters could have affected success, such as temperature/rainfall, predation, nesting competition, surrounding habitat and box location including the presence or absence of emergent vegetation, and box management. As such with the nest competitor information provided by GADNR, there is not an adequate way to assess if this was a contributing factor to nest success or failure since other parameters were not recorded. Areas of poor nesting success can be reevaluated by technicians to determine if boxes were in poor condition or if box placement should be changed. Inconsistencies such as Allatoona, Keg Creek, Lake Russell, and Wilson Shoals should be further examined. These sites and boxes were checked, but there was no recorded usage by wood ducks. Boxes within the Carrollton sites are close to the shore and were built with PVC pipe predator guards (they lack the traditional funnel guards). Increased predation could be cause for lower nesting success, but this parameter would need to be closely examined to determine significance. Several Henry County boxes had late check/box clean out dates (hens had already begun laying eggs). Therefore, nesting data from the previous year was not adequately recorded.

TABLES

Author(s)	Publication Year	Nesting Success % in Natural Cavities	Nesting Success % in Artificial Boxes
Bellrose et al.	1964	37.4%	71.1%
Kennamer et al.	2016	40%; range 10%–63%	67%; range 44%–80%
Croft et al.	2022	N/A	61.2%
Nielsen and Gates	2007	26%–65%	N/A

Table 2.1: Percentages of wood duck nest success vary considerably between studies conducted in natural cavities and artificial nest boxes.

Site	Nesting Success % in Artificial Boxes
Carrollton, GA	58%; range 39%-76%
GADNR WMAs/PFA	56%; range 35%-76%
Henry County, GA	29%; range 9%-51%

Table 2.2: The average percentage of wood duck nesting success from Carrollton, GA (2021-2022), GADNR (2000-2020), and Henry County (2018-2025) is low relative to literature values. The lowest range (9-51%) within Henry County indicates sites of concern.

Chattahoochee Fall Line	Clarks Hill	Fishing Creek	Lake Lanier	McGraw Ford	Phinizy Swamp	Pine Log Mountain
30%	20%	30%	30%	10%	10%	<10%

Table 2.3: Sites with low wood duck nest usage among checked nest boxes (10-40% usage).

B.F. Grant	Big Lazer Creek	Blanton Creek	Cedar Creek	Clybel	Hart County	J.L. Lester	Joe Kurz	McDuffie PFA	Paulding Forest	Rum Creek	Vaughter	West Point
60%	50%	50%	60%	50%	50%	50%	50%	60%	50%	50%	50%	60%

Table 2.4: Sites with medium wood duck nest usage among checked nest boxes (50-60% usage).

Dawson Forest	Oconee	Redlands
70%	80%	70%

Table 2.5: Sites with high wood duck nest usage among checked nest boxes (70-100% usage).

FIGURES

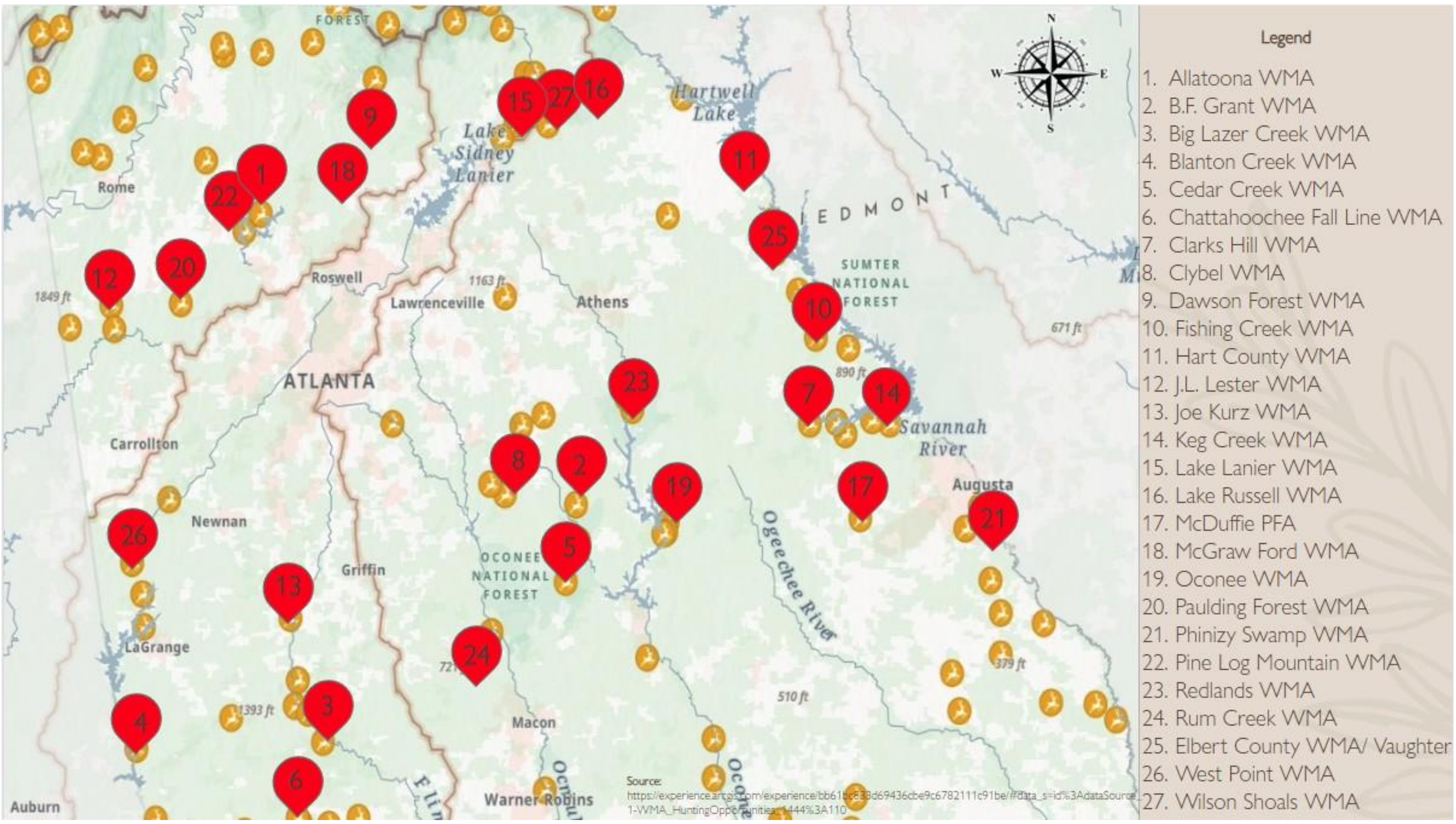

Figure 2.1: A map of 27 state-managed sites included in the 20-year dataset for the Piedmont of Georgia.

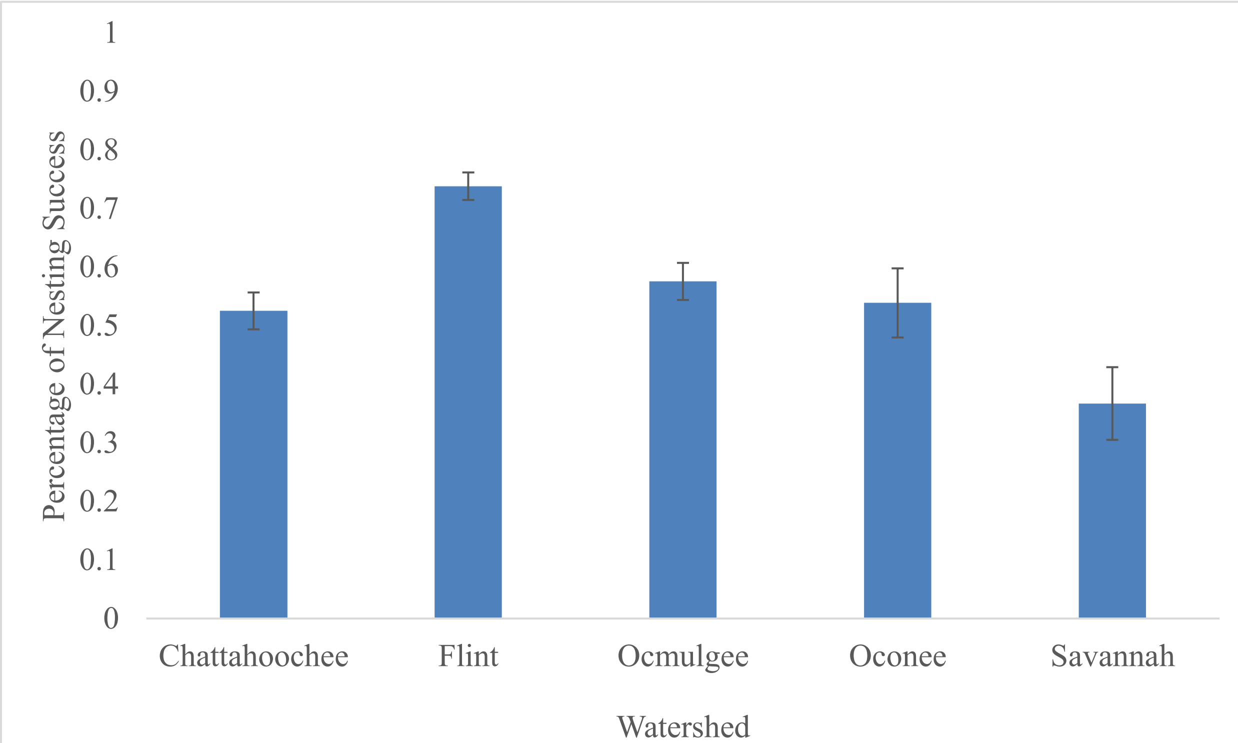

Figure 2.2: Percentage of nesting success (± SE) by site watershed from the Georgia Department of Natural Resources (GADNR) historical nesting dataset (2000-2020). To determine the significance/probability of hatching, data were modeled through a generalized linear mixed model. These watersheds include Chattahoochee, Flint, Ocmulgee, Oconee, and Savannah. The Flint watershed was the only watershed that had a higher probability of hatching ($p < 0.05$).

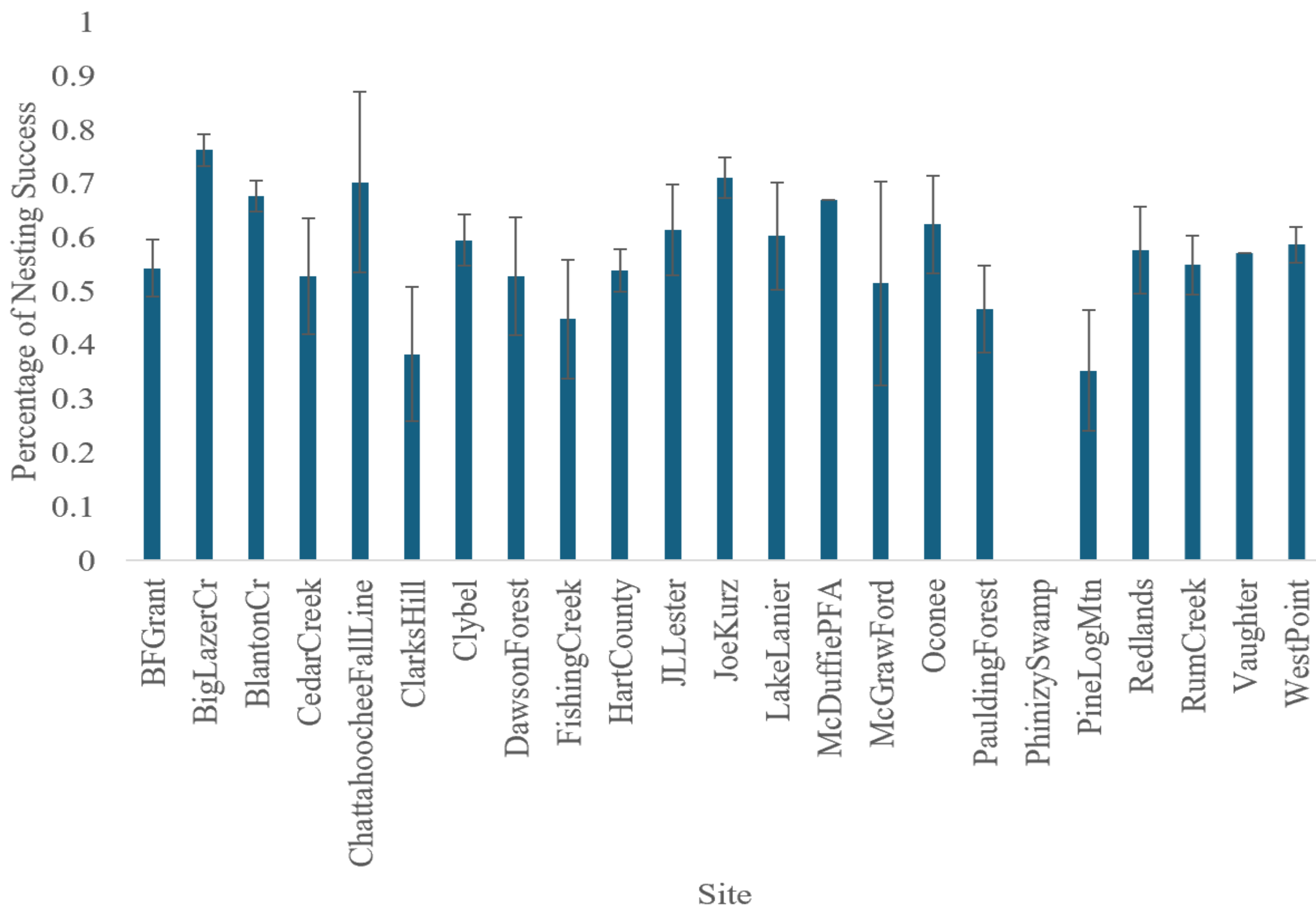

Figure 2.3: The percentage of nesting success (± SE) by sites included in the 20-year Georgia Department of Natural Resources dataset. Nesting success was defined as the proportion of hatched eggs within a given site. Specifically, Big Lazer Creek, Joe Kurz, Lake Lanier, McDuffie PFA, Blanton Creek, and West Point exhibited high nesting success (>66%) with relatively low standard error, indicating consistent and successful outcomes across nests. In contrast, Pine Log Mountain and Phinizy Swamp showed lower nesting success (<50%) with limited variation, suggesting persistently poor performance. Clarks Hill displayed the widest error bars, indicating highly variable nesting outcomes.

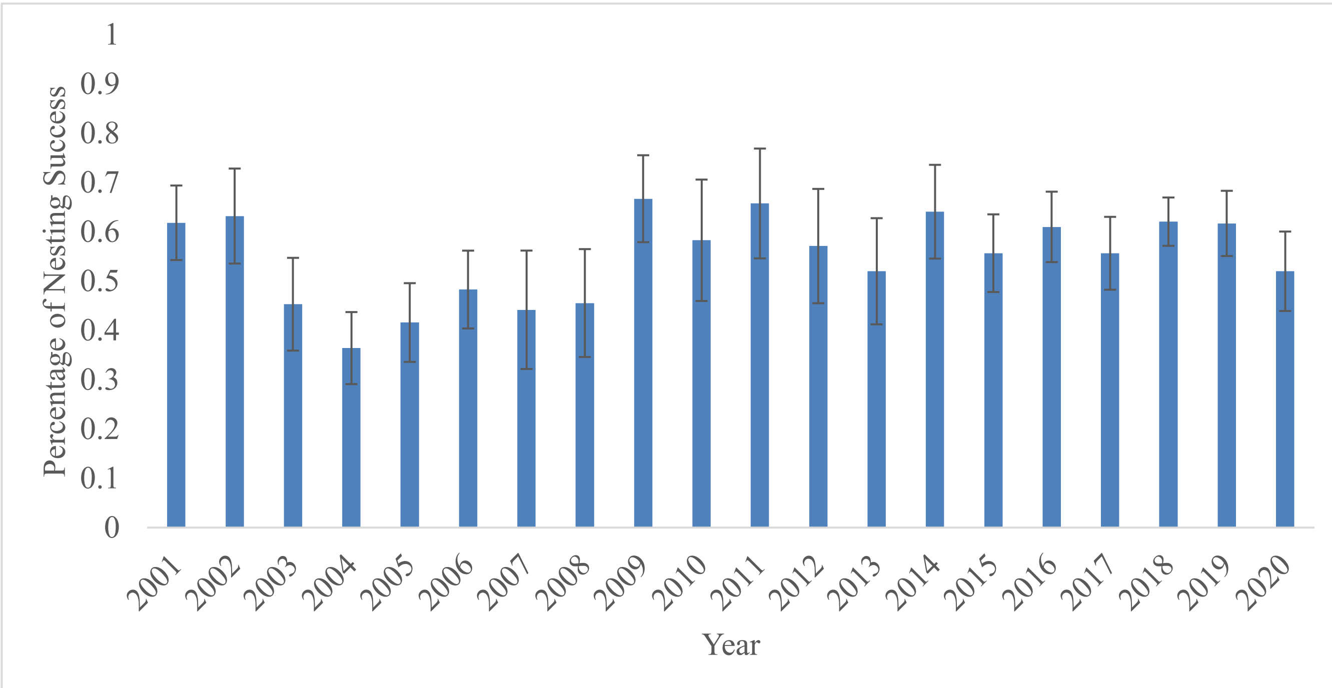

Figure 2.4: The percentage of nesting success (± SE) by year included in the 20-year Georgia Department of Natural Resources dataset. Nesting success was measured annually as the proportion of hatched eggs relative to total eggs laid. Peak nesting success occurred during 2001–2022 and 2009–2011, with averages exceeding 66%, and had widespread and consistent reproductive success. In contrast, the lowest average success was observed in 2004 (~36%), with consistent poor performance across nests. The mid-2000s (2003–2008) were marked by below-average nesting success and high variability, suggesting unstable conditions during this period. Recent years (2015–2020) demonstrated more moderate but stable success rates, remaining below the 66% threshold.

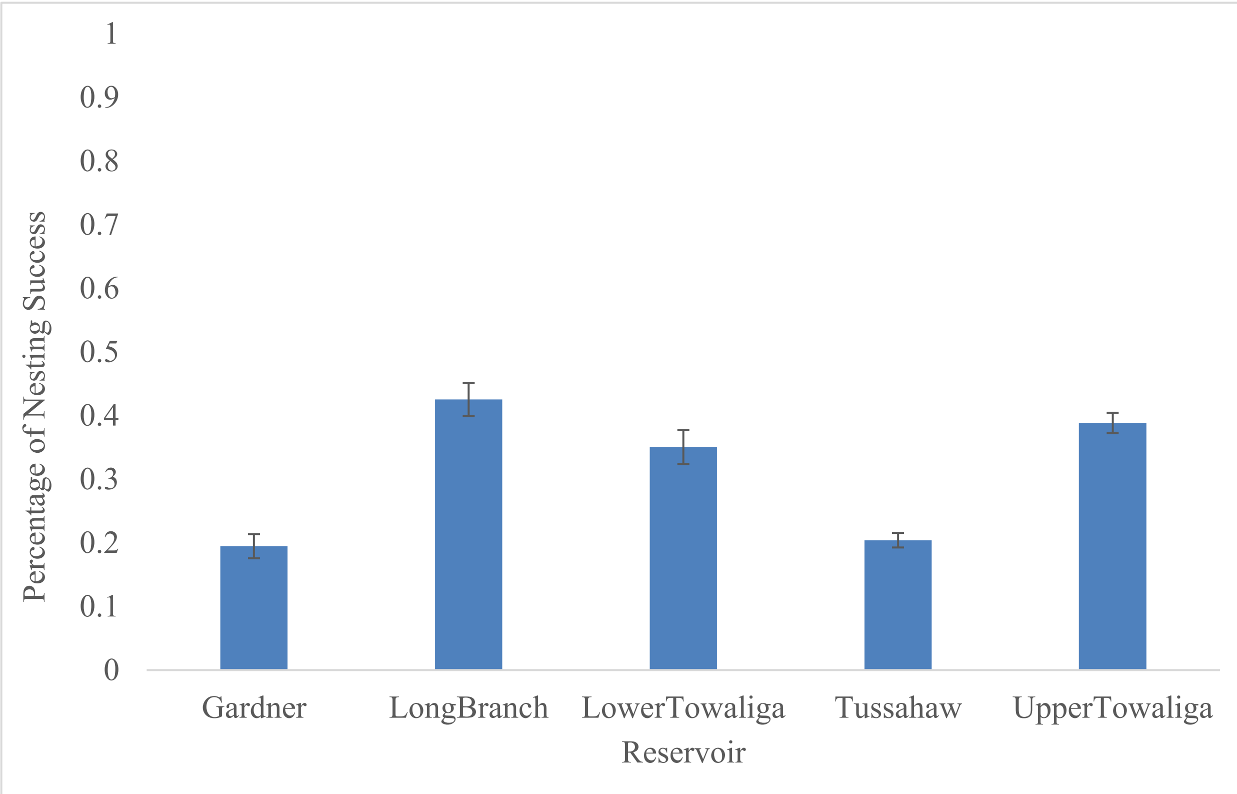

Figure 2.5: Percentage of nesting success (± SE) by site (Gardner, Long Branch, Lower Towaliga, Upper Towaliga, and Tussahaw Reservoirs) from the Henry County Water Authority (HCWA) wood duck nesting database. Nesting success was highest at Long Branch and Upper Towaliga, both approaching 40–43%, while Gardner and Tussahaw exhibited the lowest success rates (~19–20%). All reservoirs fell below the 66% success threshold, classifying them as low success sites, though Long Branch and Upper Towaliga were more consistent in performance based on their relatively narrow error margins.

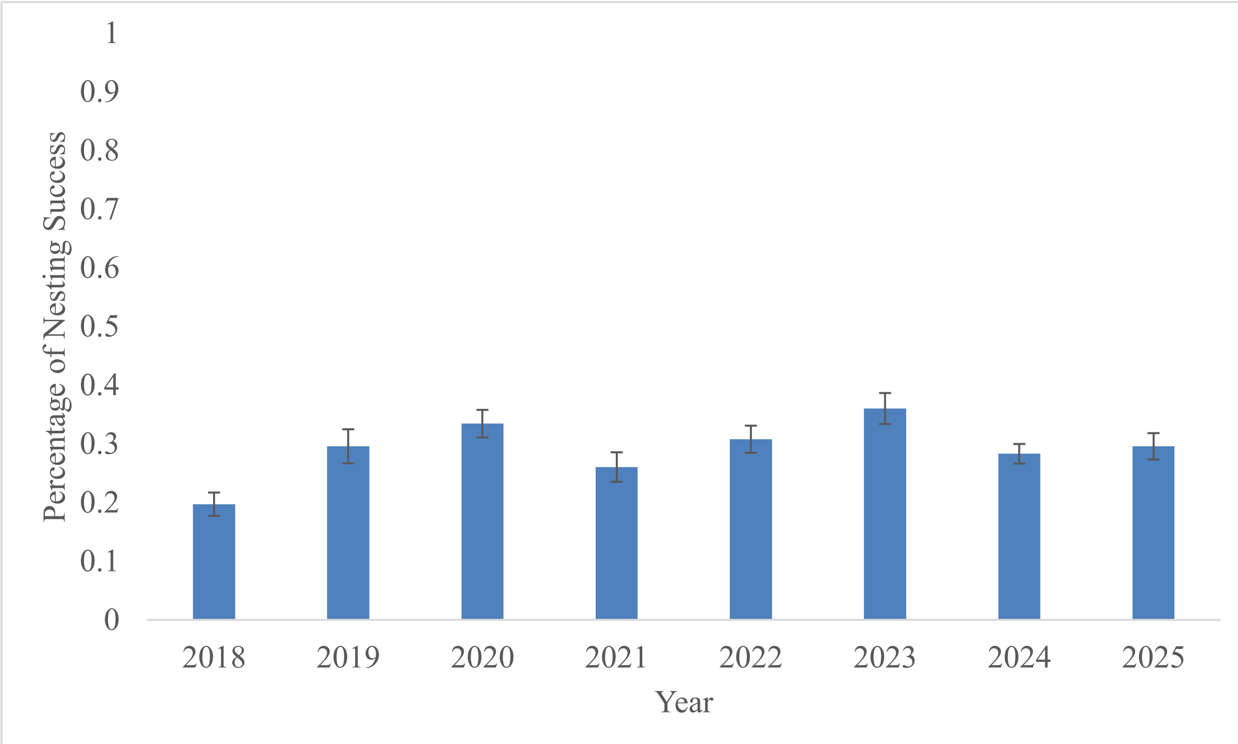

Figure 2.6: Percentage of nesting success (± SE) by year (2018-2025) from the Henry County Water Authority (HCWA) wood duck nesting database. Nesting success increased from 2018 through 2020, peaking at approximately 34%, then declined in 2021 and fluctuated mildly through 2025. Despite some year-to-year variation, no year surpassed the 66% threshold for high nesting success. The most consistent performance was observed in 2020 and 2023, while 2018 and 2021 showed slightly greater variability. Overall, the trend suggests persistently low nesting success across the time series.

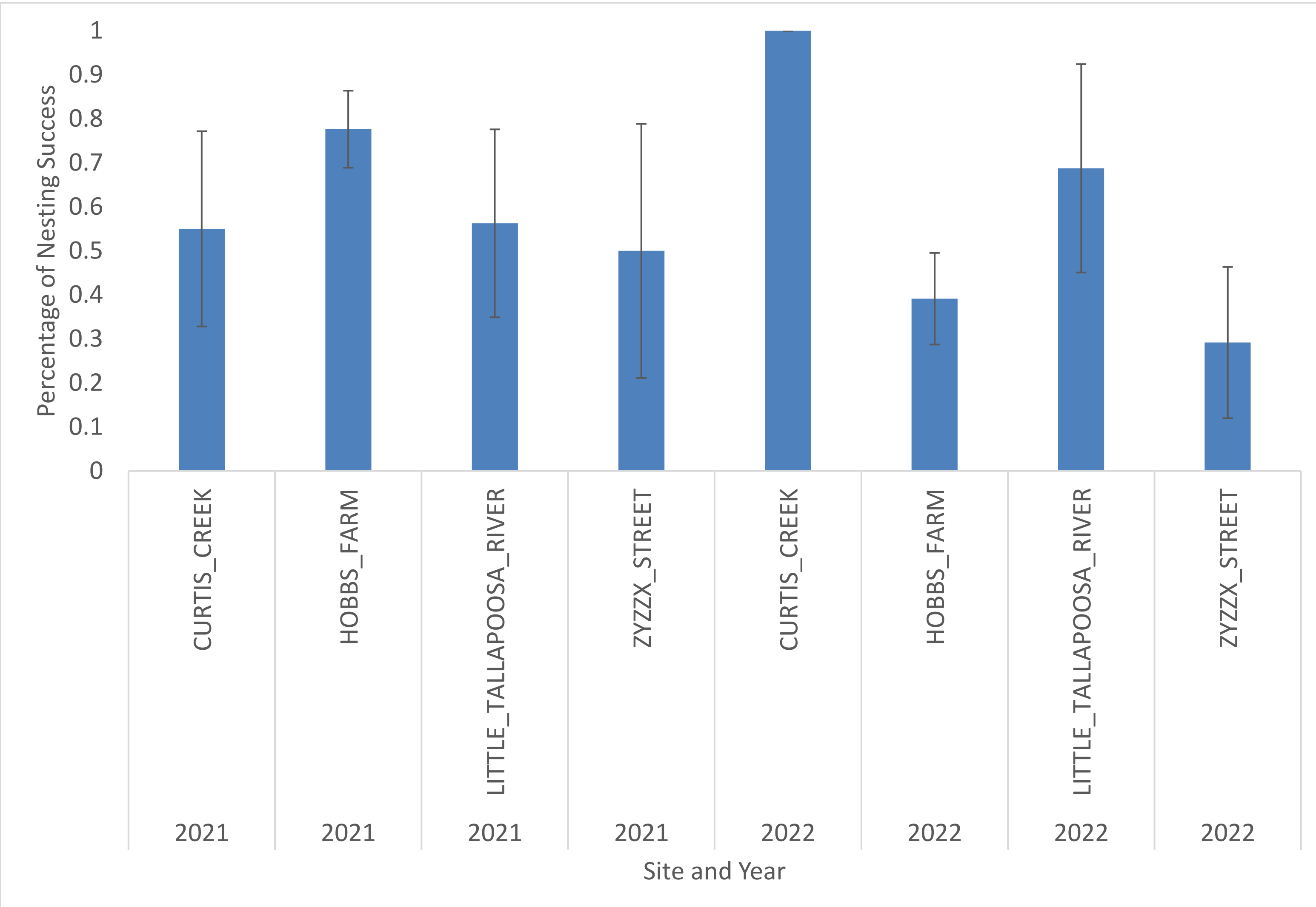

Figure 2.7: Percentage of nesting success (± SE) within four sites in Carrollton, Georgia (Curtis Creek, Hobbs Farm, Little Tallapoosa River, and Zyzzx Street) between 2021 and 2022. Curtis Creek demonstrated a marked improvement, rising from ~55% success in 2021 to 100% in 2022, the highest recorded value in the dataset. Hobbs Farm declined from ~78% in 2021 to ~40% in 2022. Little Tallapoosa River showed moderate improvement but remained variable, while Zyzzx Street maintained consistently low and variable success rates.

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CHAPTER 3

INVESTIGATIONS INTO WOOD DUCK NESTING SUCCESS WITH GAME CAMERAS AND TEMPERATURE SENSORS

[1] Brown, C., J.A. Martin, M. Chamberlain, K.C. Callaghan, S.A. McWhorter, and S.B. Wilde. To be submitted to the *Journal of Wildlife Management*.

Abstract

Wood duck artificial box nesting success and behavior can be used as an indicator of healthy habitat as they are tied to these locations for the breeding season. We choose 4 locations across the Georgia piedmont with a range of nest success and management programs (Oconee and B.F. Grant Wildlife Management Areas), a public company (Henry County Water Authority, Tussahaw and Long Branch Reservoirs), and three Carrollton city-managed subsites (Hobbs Farm, Curtis Creek, and Zyzzx Street water bodies). We installed Exodus Render Trail Cameras close to nest boxes at these sites to examine instances of predation and to assess hen behavior (flying to the box, flying away from the box, perching/presence at the nest box entrance, or on top of the nesting box). Additionally, iButtons (stainless steel computer chips) were installed in nest boxes to measure nest and incubation temperature. Finally, nest fate was recorded at each of the nesting boxes (including number of membranes- indicated as a successful hatchling, number of unhatched eggs, and number of dead ducklings). We determined that there was a significant negative effect of suboptimal nest temperature on nesting success. Our model effectively supports that hens perching at the entrance are associated with successful incubation, and hens standing on top of the box behavior is more frequently observed during nest failure. Hen competition, predators, and nest competitors were also visible and affected individual nest success.

Introduction

Wood ducks (*Aix sponsa)* depend on cavities for nesting. Artificial nest boxes provide additional space when natural cavities are limited. In addition to providing space, well-managed nest boxes could potentially increase nest production in wood duck populations. Prior to the nesting season, boxes cleaned of debris and abandoned eggs in South Carolina produced 35%

more ducklings than boxes managed once during the spring (Utsey and Hepp 1997, Davis et al. 2015). Therefore, effective management of nest boxes is crucial for wood duck recruitment and nest productivity. Nest productivity (success) and recruitment can be determined through the number of successful hatchlings, indicated by the presence of the inner lining of the egg known as the membrane. After hatching, the membrane dries and becomes leathery, persisting within the nesting cavity.

Nest attentiveness and consistent incubation are crucial factors in a successful wood duck nest. Females that have begun incubation spend approximately 87% of the day on the nest and take several recesses each day that average 99 minutes (Manlove and Hepp 2000, Hepp et al. 2005). Before taking a nest recess, the wood duck hen will pluck down from her breast, covering the eggs. This behavior allows the eggs to stay at an ideal temperature while the hen is away. One study found that artificially incubated embryos at temperatures of 35.9 °C developed slowly, expended more energy, and hatched with decreased nutrient reserves compared with embryos incubated at increased temperatures (Hepp et al. 2006, DuRant et al. 2011, McClintock et al. 2014).

While the frequency of nest attentiveness can be related to the behavior of the hen, predation is another factor affecting the nest's success. Camera trap installation, including continuous video recordings, has been used to monitor avian nest predators and is essential in identifying species-specific nest predators (Pietz and Granfors 2000, Richardson et al. 2009, Ball and Bayne 2012, Croston et al. 2018, Ellis et al. 2018, Dyson et al. 2020). Common predators of wood ducks, such as black rat snakes (*Pantherophis obsoletus*) and raccoons (*Procyon lotor*), can be observed through the use of camera traps. Cameras used in monitoring nest boxes can provide a clearer view of the hen’s behavior and external factors causing nest failure.

Methods:

Site Preparation

The study was conducted within four locations containing wood duck boxes throughout the Piedmont of Georgia: Oconee Wildlife Management Area (WMA), B.F. Grant Wildlife Management Area (WMA), two subsites within Henry County (Tussahaw Reservoir and Long Branch Reservoir), and three subsites within Carrollton, Georgia (Hobbs Farm, Curtis Creek, and Zyzzx Street water bodies). Tussahaw Reservoir is approximately 1,466-acres managed by the Henry County Water Authority and located southeast of Atlanta. Long Branch Reservoir encompasses 277 acres managed by the Henry County Water Authority (HCWA) and is located southeast of Atlanta. The Oconee WMA is a 7,400-acre area located in Greene, Hancock, and Putnam Counties in east-central Georgia. The B.F. Grant WMA surrounds a 45-acre waterfowl M.A.R.S.H. (Matching Aid to Restore State's Habitats) project. This location contains an open-water pond southeast of Covington in Putnam County. In Carrollton, GA, three subsites were given names in regard to the closeness of structures or nearby roads. Zyzzx Street site, Curtis Creek site, and Hobbs Farm site were included in the study. These sites are managed by Carrollton City Parks and Recreation, located southwest of Atlanta. Sites were chosen to examine how the state (Oconee and B.F. Grant WMAs), a city (Carrollton subsites), and a public company (Long Branch and Tussahaw Reservoirs) manage wood duck nesting (Figure 3.1).

Seventeen Exodus Render Cell Cameras with solar panels were used on the project. The number of cell cameras installed at each site was dependent on the availability of accessible wood duck nest boxes. The cell cameras were placed on wood duck boxes from February to March 2023 and January to February 2024 at each of the study sites. Four cameras were placed in Carrollton, 4 cameras at Long Branch, 4 cameras at Tussahaw, 3 cameras at B.F. Grant WMA, and 2 cameras at Oconee WMA (Appendix C). The specific boxes to be monitored with cameras were chosen based on several aspects, such as nest box condition (well-maintained boxes in good condition), usage (boxes being actively used by hens), and location (isolated areas with tall vegetation for cover).

iButtons (stainless steel computer chips to measure nest and incubation temperature) were installed at each site. In 2023, a total of 39 iButtons were installed as follows: B.F. Grant WMA (8), Carroll County (9), Henry County (19), and Oconee WMA (3). In 2024, the same number of iButtons were installed and placed as follows: B.F. Grant WMA (7), Carroll County (9), Henry County (20), and Oconee WMA (3). The iButtons were set to collect data at a sample rate of 90 minutes. Two iButtons were placed in each nest box to account for the potential failure of one device. Each set of iButtons was contained within one plastic portion snack bag to keep them secure and dry. By keeping them contained in a bag, it was easier to recover when the chip was full. Bagged iButtons were placed just below the surface under the nesting material. In 2024, the Great Value Portion Snack Bags were put inside brown paper bags, folded over into a square, and duct taped to a small block of wood for ease of removal. During the collection of iButtons, they were observed above the nesting material near the inside top back of the nest box. It was hypothesized that iButtons were moved by the hen as she made a depression within the nesting material. The brown paper bags were chosen as further protection for iButtons, and the wood block to secure them in place from being moved by the hen. Due to the sample rate, iButtons became full within approximately 5 months of activation. Data rollover was not enabled, so an iButton swap was needed at the end of this period as a continuation of data collection.

Camera and iButton Data Analysis

Data from all cameras at each site, including pictures and videos, was recorded in Excel. For each image/video, the site, date, time, camera recorded temperature, species observed, and species behavior were documented. Acronyms were assigned to species sighted within camera data as well as for the species' behavior. Organization of data by species and behavior was considered to examine wood duck behavior, nest recess, hatching/nest success, nest competition, and predation. Compiled data was graphed by the specific factors listed above, e.g., species observed and behavior. Species behavior (flying away from the box (FAB), flying towards the box (FTB), perching/presence at the box entrance (PE), and top of the box (TB)) was observed with field cameras. Behavior frequency was compared to the hours of the day. Actual data values (FAB, FTB, PE, and TB) of female wood ducks were graphed in Excel. It was hypothesized that hen movement, especially (PE), would be observed more frequently in the early morning hours. iButton data for all nests was uploaded into Excel. Time and temperature data from each iButton were graphed and used to estimate nest incubation (Figure 3.2). In comparison with pictures from the nest box of concern, the iButton data aided in a timeline of nest usage. This data was subsequently graphed to show temperature levels of equilibrium, indicating a period of hen incubation in the nest box.

Statistical and Data Analyses

A generalized linear model (GLM) using a Poisson distribution with a log link function was fit to the nesting data in R version 4.5.0 (R Core Team, 2025). Model 1 included the number of membranes (response variable) and the suboptimal nest temperature data recorded by the iButtons. Based on wood duck incubation literature, the suboptimal temperature was characterized as temperatures ≤ 35.9 °C (Table 3.3). It was hypothesized that temperatures falling below this standard exhibit a lower chance of nest success.

Six additional GLMs with a Poisson distribution were generated to test the relationship between the number of membranes and one or more behavioral (species behavior) or environmental (suboptimal temperature) predictor variables in R version 4.5.0 (R Core Team, 2025). The response variable of model 2 was the number of membranes, with the proportion of time spent in PE as the predictor. Model 3 used the proportion of time spent in TB as the predictor of the number of membranes. Model 4 tested the effect of the proportion of time spent in FTB, while model 5 included the proportion of time spent in FAB as the predictor of membranes. To account for environmental conditions, model 6 included both the proportion of time spent in PE and a binary variable indicating suboptimal temperature exposure as predictors. Similarly, model 7 included the proportion of time spent in TB and the suboptimal temperature to assess their combined influence on membrane production.

Results:

Camera and iButton Data Analysis

A model was unable to capture the wood duck behavior frequency (there were mismatch values between predicted and actual values). Therefore, actual frequency values of female wood duck behavior were graphed in Excel to show trends (Figure 3.10). PE demonstrated the highest frequency and observations. Specifically, there was peak activity between 6 and 8 AM (Figure 3.11). Around 6 PM, this behavior also spiked. Similarly, TB conveyed an increase and the highest activity between 7 and 8 AM (Figure 3.11). FTB individuals were observed most frequently around 8 AM (Figure 3.12). FAB individuals were also observed most frequently around 8 AM (in addition to 7 AM) (Figure 3.12).

In 2023, human presence (within about 10 meters of the box) was detected at 5 cameras during the hen incubation. Two of these boxes exhibited no nest activity. In 2024, human presence was detected at 6 cameras during hen incubation. Two of these boxes also showed no nest activity. Compared to the nest fate data (membranes and unhatched eggs) for these boxes, human presence can be hypothesized as not to affect nesting hens. Additionally, hens were not observed flushing from boxes with the presence of humans nearby.

The most common nest competitors were Hooded Mergansers (*Lophodytes cucullatus*), Eastern Bluebirds (*Sialia sialis)*, Great-crested Flycatchers (*Myiarchus crinitus*), and Tree Swallows (*Tachycineta bicolor*). In 2023, 3 cameras captured nest competitors at the box entrance during hen incubation. One of these boxes (Tussahaw Reservoir) showed low success (camera alias: HEN1TUSSA), 2 membranes, and 3 unhatched. Two out of three boxes (B.F. Grant WMA) exhibited no nesting activity (camera alias: BFGR1MP and BFGR2MP). In 2024, 4 cameras captured nest competitors at the box entrance during hen incubation. All boxes had some nest success (presence of membranes) with the exception of a nest box at Long Branch Reservoir (camera alias: HEN4LOBR), which showed no activity. Nest competition could be attributed to a shortened incubation (nest failure) or a deterrence of nesting hens from the box.

Multiple hens attempting to nest in the same box can cause dump nesting, which is defined as a significant number of eggs laid but not incubated (typically over 12). Female competition was identified by a picture or video of one hen in the box and another trying to perch on the box entrance at the same time. In 2023, one instance of hen competition was documented at B.F. Grant WMA. By 2024, two cases were observed, one at Tussahaw Reservoir in Henry County and another at B.F. Grant WMA. Nest fate data from 2023 at the B.F. Grant WMA box showed 16 membranes, 13 unhatched eggs, and one dead duckling. In 2024, nest fate could not be determined for the Tussahaw Reservoir box, while the B.F. Grant WMA box showed decreased nesting success, with only 3 membranes and 1 unhatched egg. The high number of unhatched eggs and reduced nesting success observed at B.F. Grant WMA in 2023 could be attributed to hen competition.

Nest predation was observed within Carroll County sites (Curtis Creek, Zyzzx Street, and Hobbs Farm) and at one B.F. Grant WMA nest box with a snake in 2024. There were only 2 observed nest predators (Eastern Rat Snakes and Raccoons). Except for one instance, predators entered the nest boxes after incubation, and when incubation was not observed. Increased predation can be attributed to the lack of well-constructed predator guards and the distance from the bank (less than 5 meters).

In 2023, 9 out of 16 observed nest boxes exhibited incubation. In 2024, 13 out of 18 observed nest boxes exhibited incubation. This period was considered consistent if the nest temperature was >32 and <38 degrees Celsius for more than 10 days. In 2023, there were 4 cases of observed nest fate (membrane and unhatched eggs counted) on nests where incubation was not identified. Additionally, in 2024, there were 2 cases of observed nest fate on nests where incubation was not identified. These instances in both years were hypothesized to be attributed to an inaccurate temperature read due to movement from the hen. In many cases, iButtons were found in different corners of the top of the nest (moved out from under the nesting material). Some hens were thought to have moved or pushed iButtons out of the way when incubation began.

Statistical and Data Analyses

As expected, results from model 1 indicated that suboptimal temperatures had a negative effect on the number of membranes (Figure 3.3). Increases in suboptimal temperatures led to a decrease in the log of the number of expected membranes. Therefore, the effect of suboptimal temperatures yielded a small p-value ($p < 0.0001$) and was statistically significant.

Across models 2-7, the number of membranes was significantly influenced by specific behavioral and environmental predictors. In model 2, the proportion of time spent in PE had a significant positive effect on membrane number ($p = 0.0139$), indicating that increased perching behavior was associated with more membranes (Figure 3.4). In model 3, the proportion of time spent in TB had a significant negative effect ($p = 0.0151$), suggesting that greater time spent on top of the nest box was linked to fewer membranes (Figure 3.5). Models 4 and 5, which included the proportion of time spent in FTB and FAB, respectively, showed no significant effects on membrane number ($p = 0.452$ and $p = 0.457$), indicating these failed or potentially maladaptive behaviors did not predict membrane output (Figures 3.6 and 3.7). Model 6, which included both the proportion of time spent in PE and suboptimal temperature, showed significant effects for both predictors: the proportion of time spent in PE had a positive effect ($p = 0.0192$), while suboptimal temperature had a strong negative effect ($p < 0.0001$), indicating that suboptimal conditions markedly reduced membrane production (Figure 3.8). Finally, in model 7, the proportion of time spent in TB had a marginally significant negative effect ($p = 0.0501$), while suboptimal temperature remained a strong negative predictor ($p < 0.0001$) (Figure 3.9).

Discussion

In combination, camera data, iButton data, and nest fate results provide insights into factors that potentially affect overall nesting success. From the camera data, the potential for nest disturbance was considered. Nest competition with other species, as well as other nesting wood duck hens, was shown to affect the nest fate. Predation was also observed at several nests. These nests had PVC pipe predator guards instead of the traditional sheet metal funnel guard and were less effective. Human disturbance was also compared to nest fate, but did not show any direct correlation. It can be assumed that nesting females in highly active boating areas were accustomed to any noise or additional disturbance. iButton data showed duration and timing of nest occupation and presented a consistent trend with incubation. Temperature data in combination with nest fate contributed to a timeline of nesting and nests that were used twice in one season. If there is a successful nest indicated by membrane presence, PE is more likely when the temperature is closer to optimal and less likely when the temperature is farther from optimal. As suboptimal temperature increases (nest condition worsens), the probability of TB increases slightly. These findings suggest that the model effectively supports the initial assumption that PE is associated with successful incubation, and TB (when a hen is more likely to be off the nest) is associated with nest failure.

The first model created compared the nest temperature effect (suboptimal) on the number of membranes. The outputs were consistent with the prior expectations, demonstrating a strong negative correlation between suboptimal nest temperature and nest success. The results of the additional models highlight the importance of hen attentiveness (PE) and incubation in determining membrane output. Wood duck behavior and frequency data also suggest nest recess is most frequent in the early morning hours of the day. Earlier nest recesses can be hypothesized as hens avoiding being seen in the day by predators or other nest competitors (attracting other species to the nest box).

TABLES

Author(s)	Publication Year	Nesting Success % in Natural Cavities	Nesting Success % in Artificial Boxes
Bellrose et al.	1964	37.4%	71.1%
Kennamer et al.	2016	40%; range 10%–63%	67%; range 44%–80%
Croft et al.	2022	N/A	61.2%
Nielsen and Gates	2007	26%–65%	N/A

Table 3.1: [1]Percentages of wood duck nest success vary considerably between studies conducted in natural cavities and artificial nest boxes.

[1]This table also appears in Chapter 2, where it is initially introduced and discussed.

Site	Nesting Success % in Artificial Boxes
B.F. Grant WMA	63%
Carrollton, GA	58%
Oconee WMA	44%
Tussahaw Reservoir	41%
Long Branch Reservoir	38%

Table 3.2: The average percentage of wood duck nesting success within sites (B.F. Grant WMA, Oconee WMA, Carrollton, GA subsites, Long Branch Reservoir, and Tussahaw Reservoir) from 2023 to 2024.

Author(s)	Low Temp (°C)	Mid Temp (°C)	High Temp (°C)
DuRant et al. (2010)	35.0	35.9	37.0
DuRant, Hopkins, and Hepp (2011)	35.0	35.9	37.0
Hepp and Kennamer (2012)	35.0	35.9	37.3
Hepp and Kennamer (2018)	34.9	35.8	37.6

Table 3.3: Summary of incubation temperature treatments used in four published studies examining wood duck embryonic development, survival, and recruitment. Each study included distinct temperature groups typically referred to as "low," "mid," and "high." Based on these studies, I calculated average values for each incubation level: Low (35.0 °C), Mid (35.9 °C), and High (37.2 °C). For my analysis, I simplified these categories into two biologically meaningful groups: suboptimal (≤ 35.9 °C) and optimal (> 35.9 °C) incubation temperatures. This threshold reflects the average division between mid and high temperatures across the studies reviewed and is consistent with literature showing that temperatures above ~36 °C produce the most successful developmental outcomes.

FIGURES

Figure 3.1: A state map of major watersheds and study locations; from left to right: Carroll County, Henry County, B.F. Grant WMA (upper right), and Oconee WMA (lower right). Source: https://geology.com/lakes-rivers-water/georgia.shtml

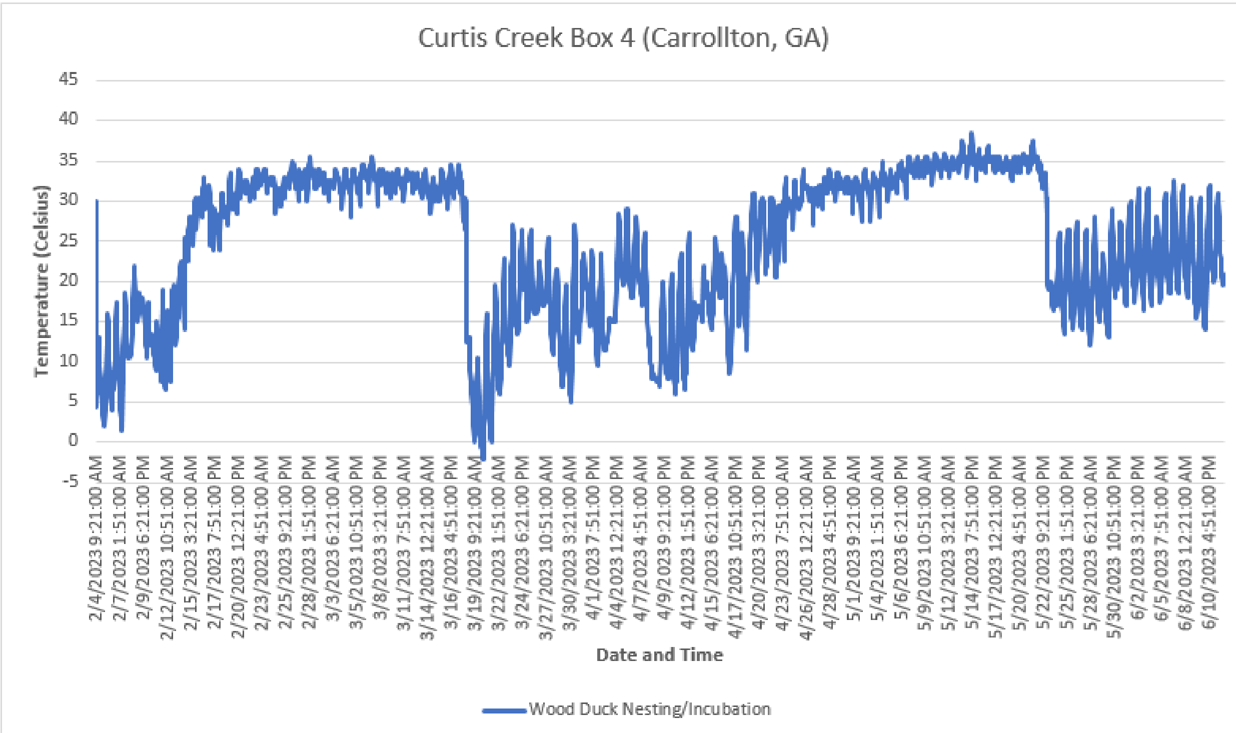

Figure 3.2: Temperature data (Celsius) by date and time graphed from an iButton located at Curtis Creek in Carrollton, GA. Wood duck hen incubation is suspected with nest temperature levels out (> ~32 degrees Celsius) as seen from February to March and April to May.

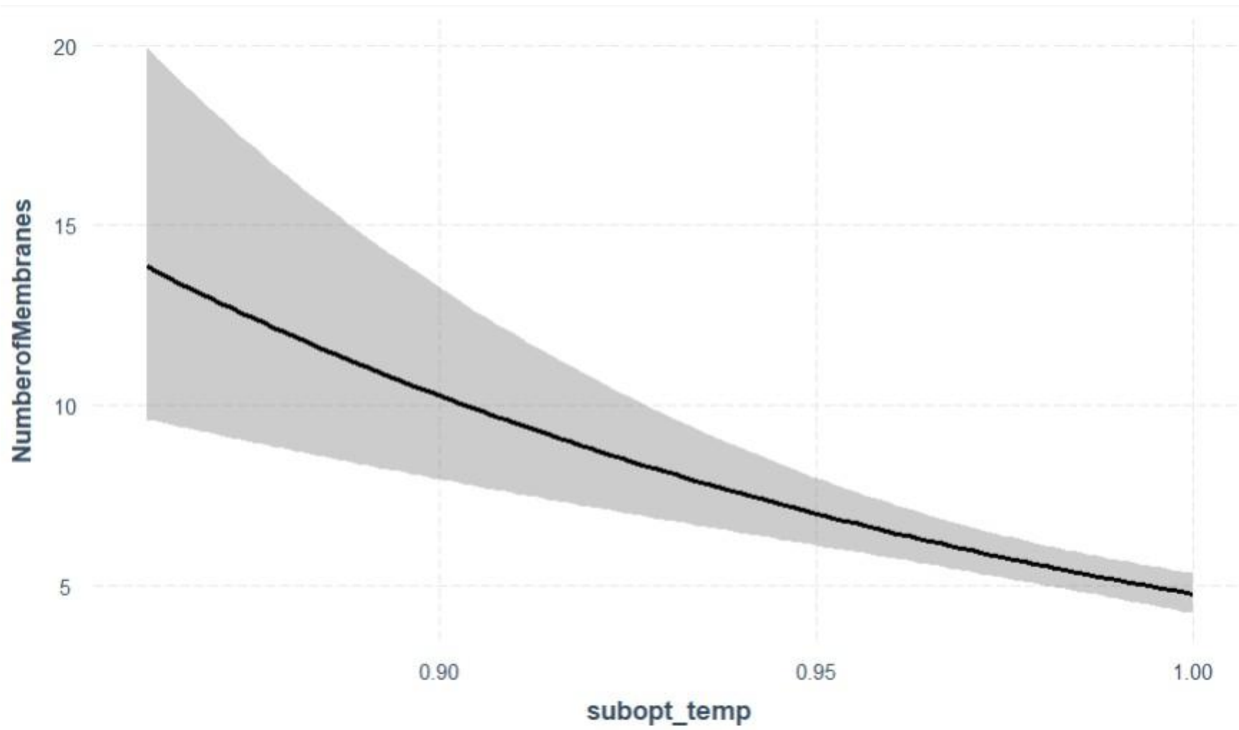

Figure 3.3: Number of membranes compared to suboptimal nest temperature (≤ 35.9 °C) in 2023 and 2024 from nesting data in Carroll County (Curtis Creek, Hobbs Farm, Little Tallapoosa River, and Zyzzx Street), Henry County (Long Branch and Tussahaw Reservoirs), and Putnam County (M.A.R.S.H. Pond, Little Steinbeck Pond, Big Steinbeck Pond, and Lake Sinclair). Data was modeled using a generalized linear mixed model. As suboptimal temperatures ($p < 0.0001$) increased, the number of expected membranes decreased.

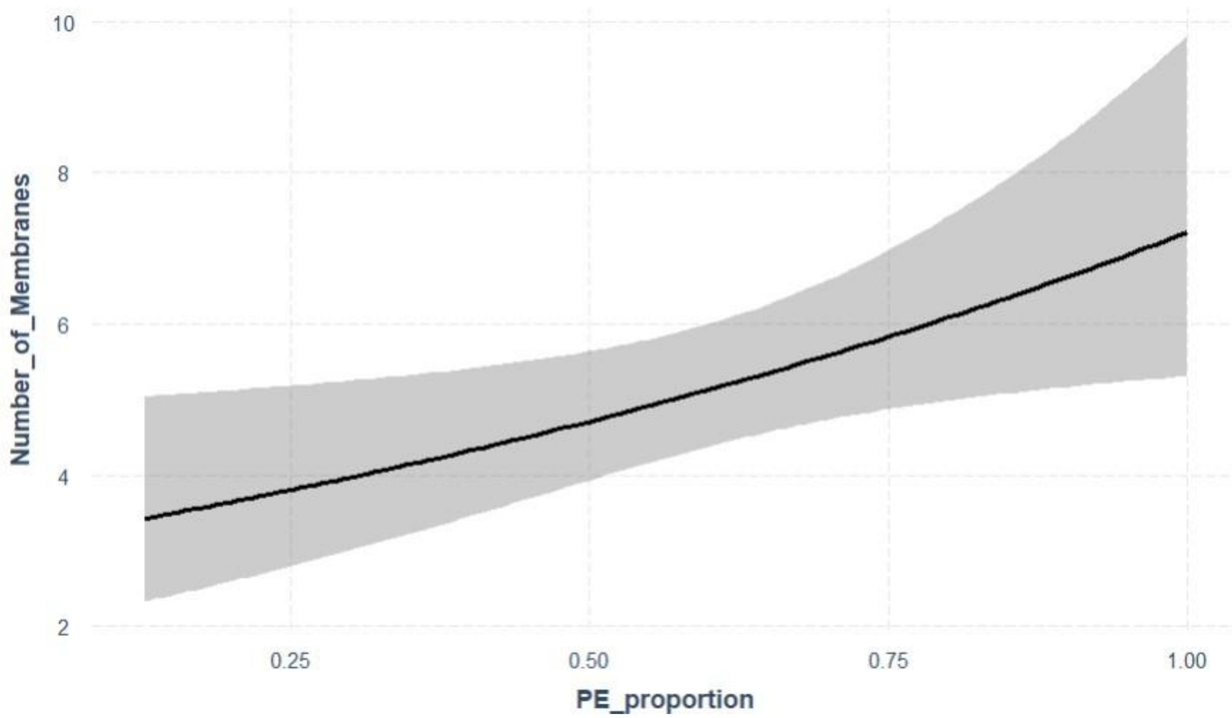

Figure 3.4: Number of membranes compared to the proportion of time spent in PE (perching/presence at nest box entrance). As wood duck hens spent time in PE ($p = 0.0139$), the number of expected membranes increased.

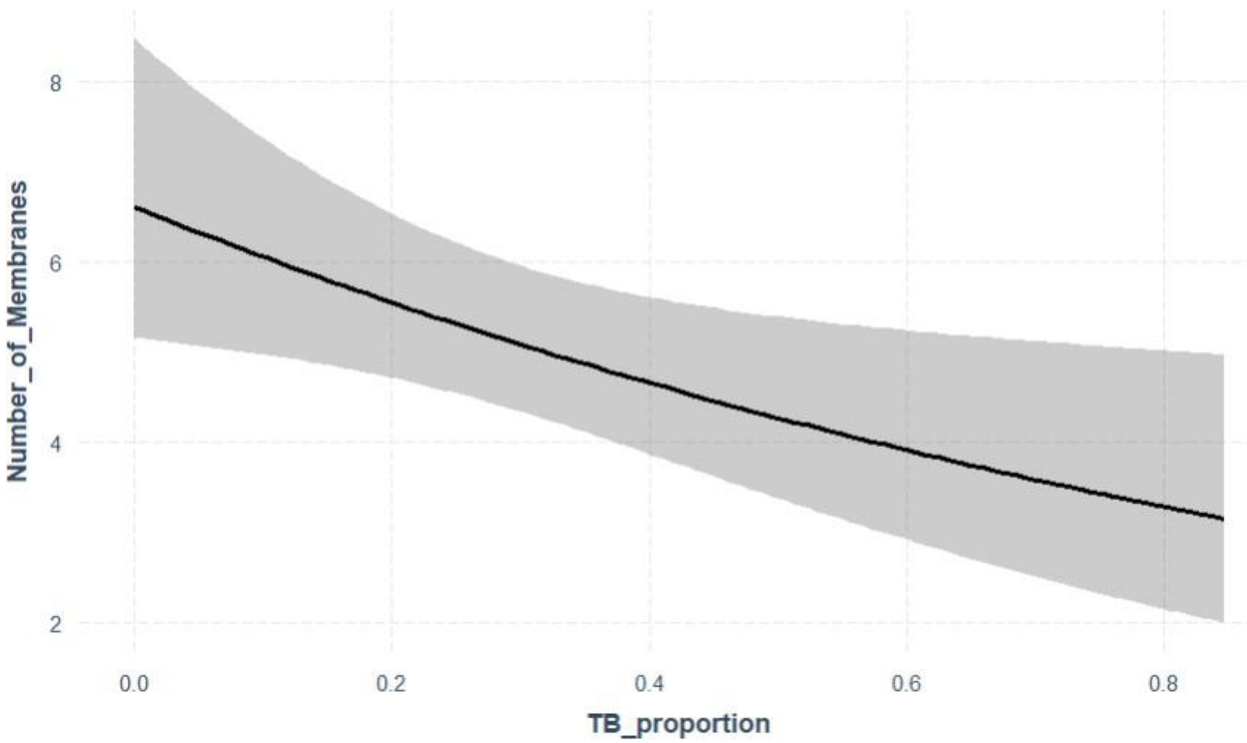

Figure 3.5: Number of membranes compared to the proportion of time spent in TB (top of the box). As wood duck hens spent time in TB ($p = 0.0151$), the number of expected membranes decreased.

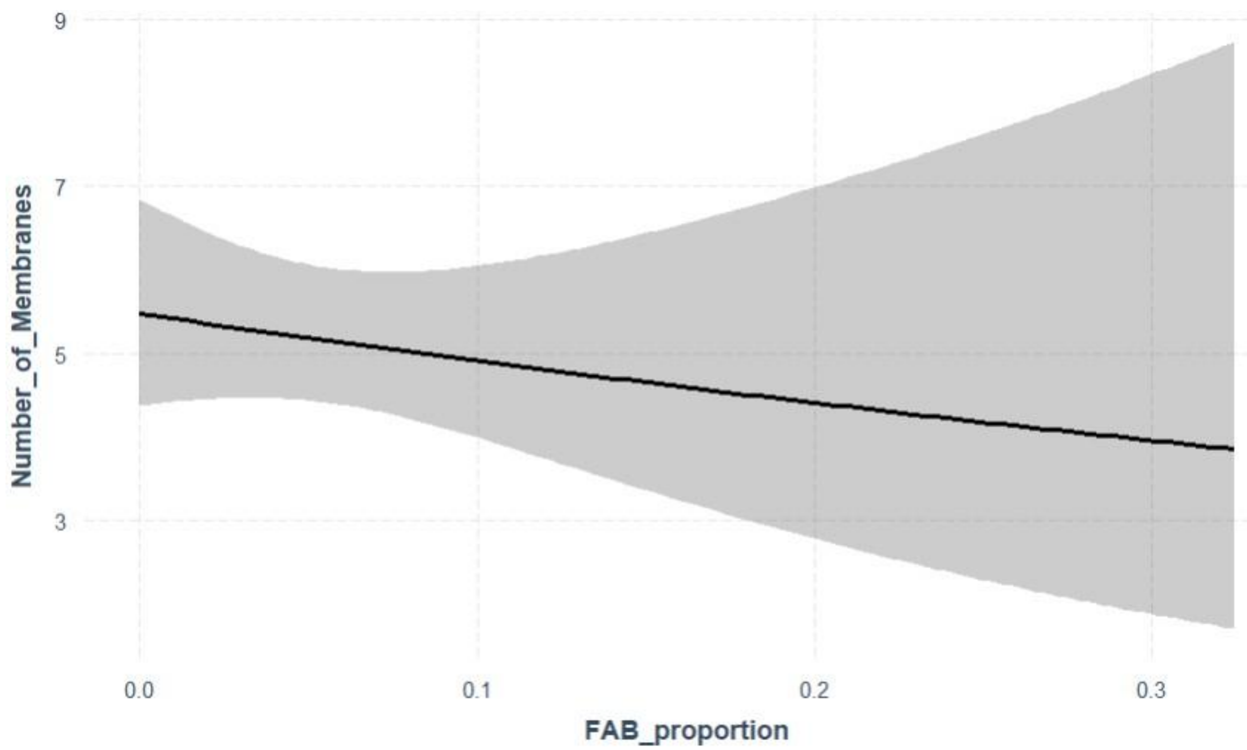

Figure 3.6: There were no significant effects between the proportion of time spent in FAB (flying away from the nest box) on the number of membranes ($p = 0.457$).

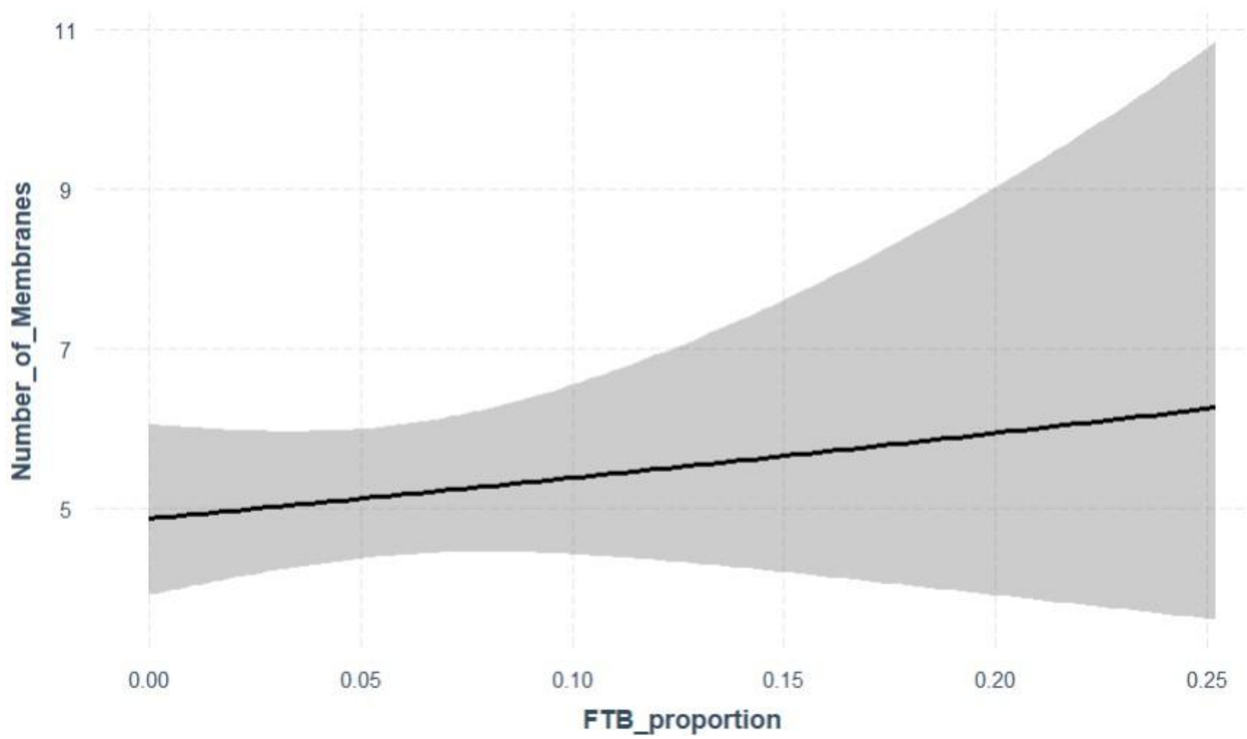

Figure 3.7: There were no significant effects between the proportion of time spent in FTB (flying towards the nest box) on the number of membranes ($p = 0.452$).

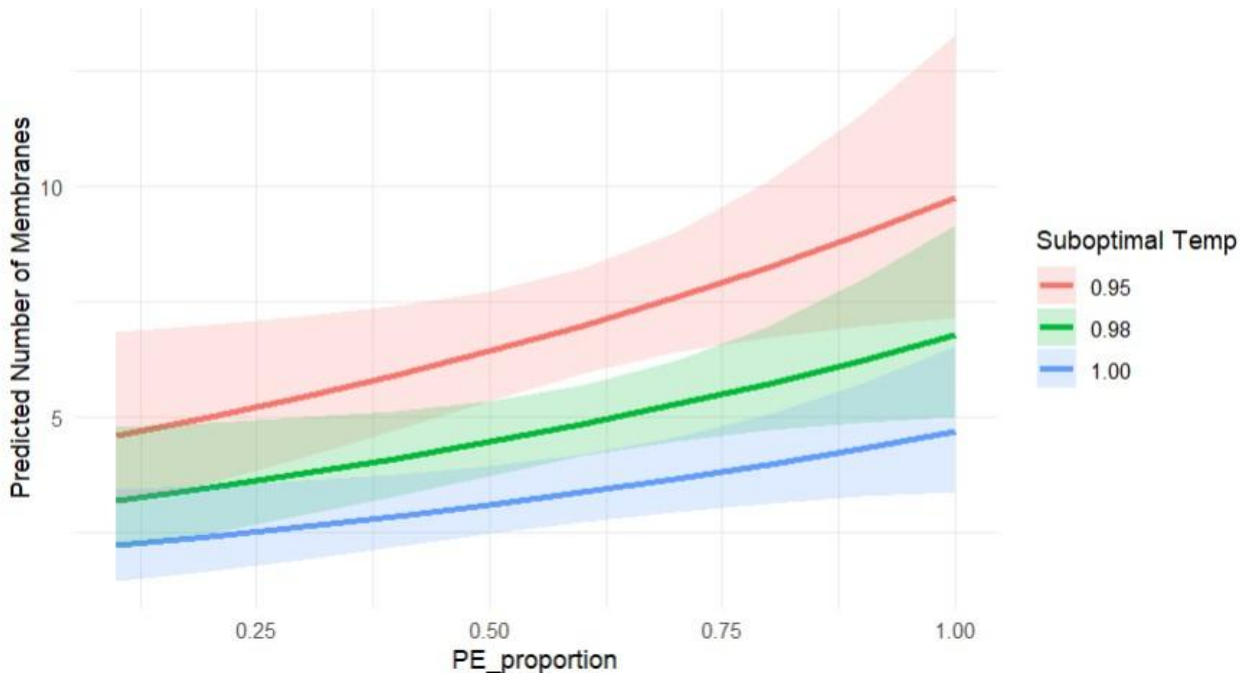

Figure 3.8: Predicted membrane counts from a Poisson generalized linear model (GLM) using the proportion of time a female wood duck was perching or present at nest box entrance (PE) as the behavioral predictor, modeled at three levels of suboptimal temperature exposure: 0.95 (red), 0.98 (green), and 1.00 (blue). These proportions reflect the amount of time during incubation that the nest experienced cold temperatures (≤ 35.9 °C). Confidence intervals (95%) are shown as shaded ribbons. The proportion of time spent in PE (perching/presence at nest box entrance) had a significant effect ($p = 0.0192$) on the predicted number of membranes. However, suboptimal temperatures had a negative effect ($p < 0.0001$).

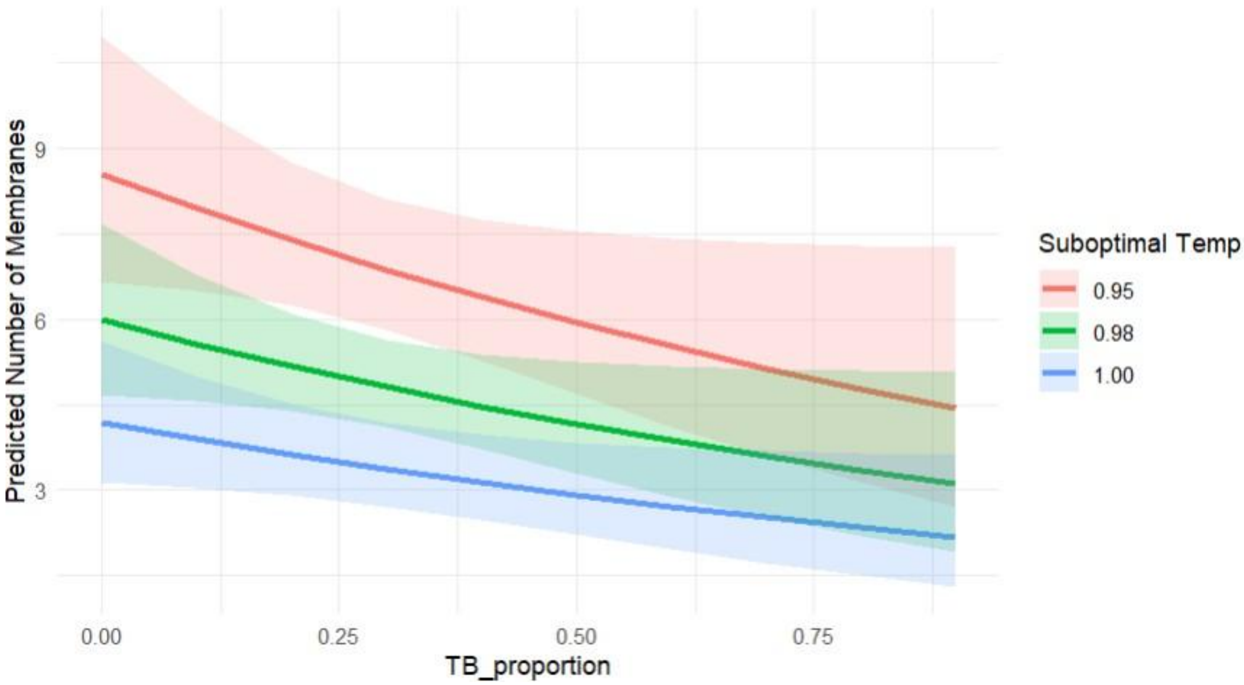

Figure 3.9: The predicted values from a Poisson generalized linear model (GLM) comparing the number of membranes to the proportion of time a female wood duck was observed on top of the nest box (TB) at three levels of suboptimal temperature exposure: 0.95 (red), 0.98 (green), and 1.00 (blue). These values represent nests that experienced suboptimal thermal conditions (≤ 35.9 °C) for 95%, 98%, and 100% of the incubation period, respectively. Shaded ribbons around each line represent 95% confidence intervals. The proportion of time spent in TB (top of the box) had a marginally significant negative effect ($p = 0.0501$), while suboptimal temperature remained a strong negative predictor ($p < 0.0001$).

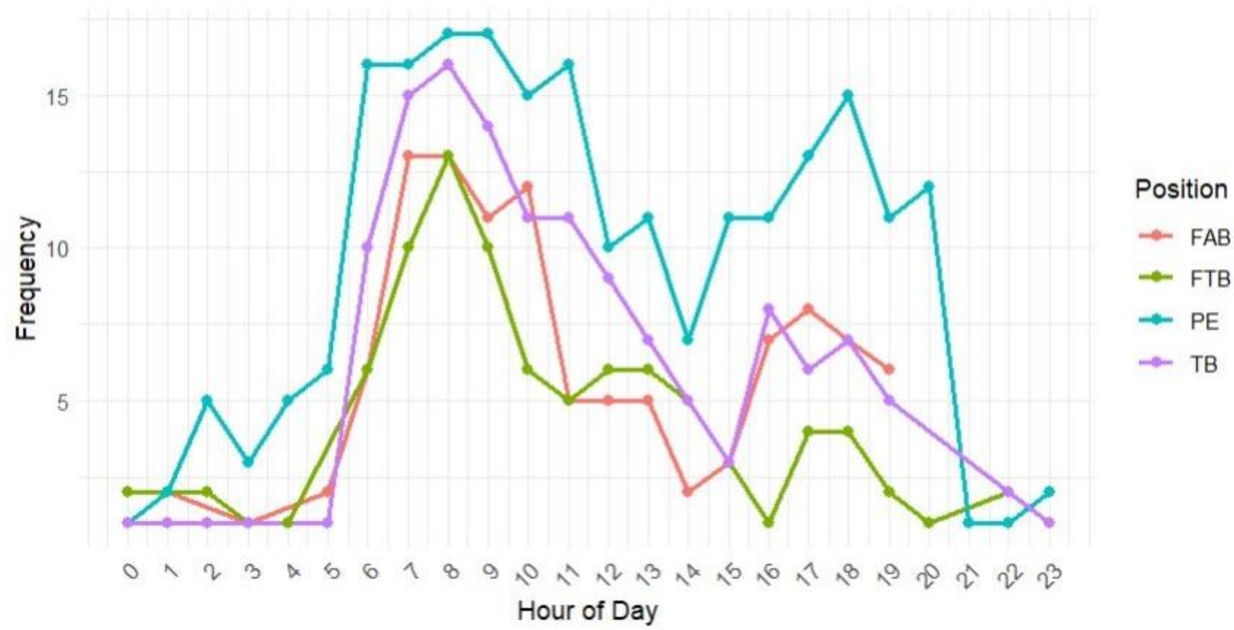

Figure 3.10: The frequency of hen behavior: flying away from the box (FAB), flying to the box (FTB), perching/presence at the entrance (PE), and top of the box (TB) compared to each hour of the day in a combined database (2023 and 2024). Included sites are Carroll County (Curtis Creek, Hobbs Farm, Little Tallapoosa River, and Zyzzx Street), Henry County (Long Branch and Tussahaw Reservoirs), and Putnam County (M.A.R.S.H. Pond, Little Steinbeck Pond, Big Steinbeck Pond, and Lake Sinclair). Behavior was more frequent in the earlier hours of the day, around 8 AM.

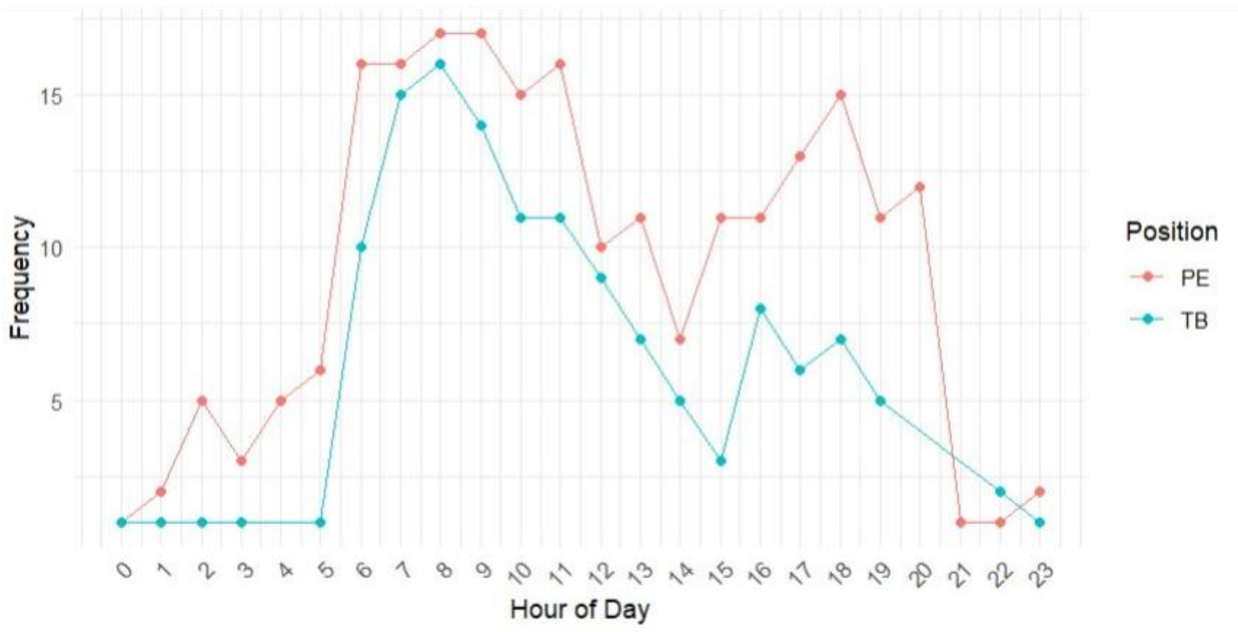

Figure 3.11: The frequency of hen behavior: perching/presence at the entrance (PE) and top of the box (TB) compared to each hour of the day in a combined database (2023 and 2024). Included sites are Carroll County (Curtis Creek, Hobbs Farm, Little Tallapoosa River, and Zyzzx Street), Henry County (Long Branch and Tussahaw Reservoirs), and Putnam County (M.A.R.S.H. Pond, Little Steinbeck Pond, Big Steinbeck Pond, and Lake Sinclair). The two most common behaviors (PE) and (TB) were observed in the early morning hours (around 7 to 9 AM) with a few spikes later in the day.

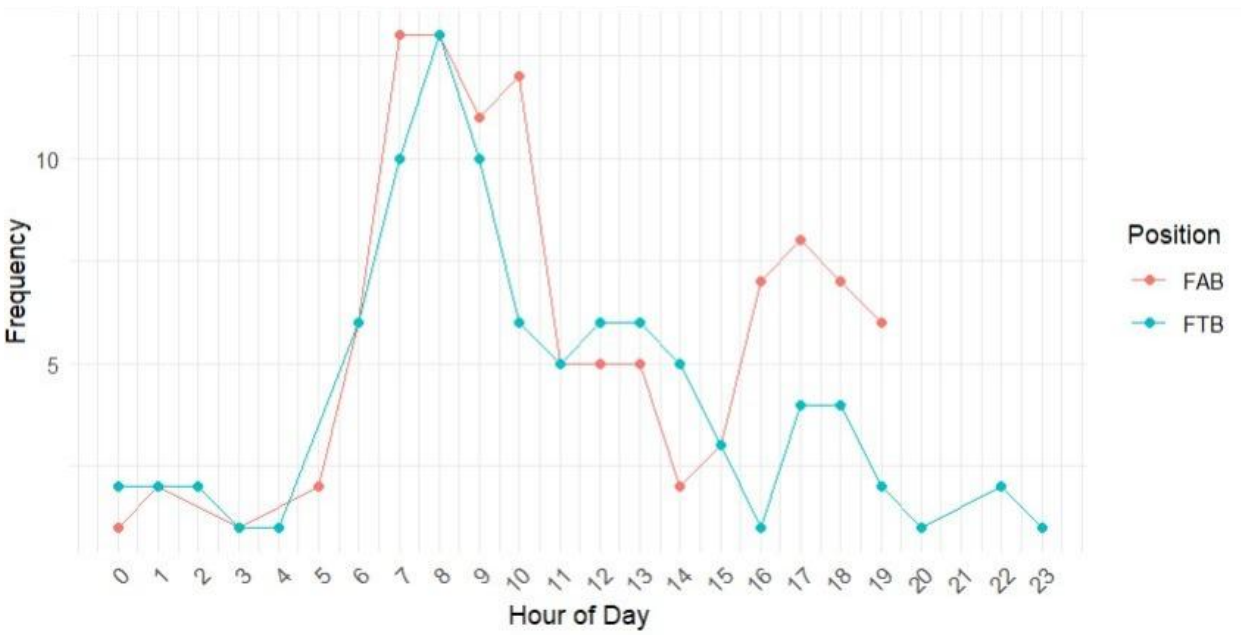

Figure 3.12: The frequency of hen behavior: flying away from the box (FAB) and flying to the box (FTB) compared to each hour of the day in a combined database (2023 and 2024). Included sites are Carroll County (Curtis Creek, Hobbs Farm, Little Tallapoosa River, and Zyzzx Street), Henry County (Long Branch and Tussahaw Reservoirs), and Putnam County (M.A.R.S.H. Pond, Little Steinbeck Pond, Big Steinbeck Pond, and Lake Sinclair). Behaviors FAB and FTB were less common, but like the other behaviors, they were most common in earlier hours, around 7 to 8 AM.

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CHAPTER 4

CYANOBACTERIAL NEUROTOXIN AETOKTHONOTOXIN, "EAGLE-KILLER TOXIN" DETECTED IN WOOD DUCK EGGS FROM ARTIFICIAL NEST BOXES

[1] Brown, C., J.A. Martin, M. Chamberlain, K.C. Callaghan, S.A. McWhorter, and S.B. Wilde. To be submitted to the *Journal of Wildlife Management*.

Abstract

Nest success has been used as a measure of environmental quality in birds. Eggs within a nest offer a glimpse into potential environmental contaminants present in the environment. Wood ducks are a popular game species throughout North America, hence there is keen interest from state and federal wildlife agencies in understanding factors with potential to influence reproduction. I collected wood duck eggs from nest boxes across sites in the Piedmont physiographic region of Georgia, USA. Through toxin extraction methods, I tested for the presence of Aetokthonotoxin (AETX), a cyanobacterial neurotoxin. I discovered the presence of AETX in 12% of collected eggs, which is the first documentation of AETX in wood duck eggs. My findings offer evidence that AETX is a potential factor that could influence reproduction in waterfowl. Future studies should further examine the presence and concentration of AETX found within eggs to better understand the impact on reproductive success.

Introduction:

Modern research increasingly emphasizes the widespread acknowledgment that neurotoxins are prevalent throughout the environment (Hidayat et al. 2025, Starr et al. 2025). Domoic acid [DOM], a neurotoxin produced by marine organisms such as red algae *Chondria armata* can contaminate shellfish as well as other seafood types (Pulido 2008). As was the case in 1987, when DOM was identified as a human poisoning agent through the consumption of blue mussels (Pulido 2008). Additionally, mercury is a bioaccumulated neurotoxin that can pass through marine food webs, leading to a conversion to monomethylmercury (MMHg) (Starr et al. 2025). As such, marine seafood consumption is one of the most common routes and causes of concern for humans.

In relation to waterbirds, studies have shown that neurotoxins can directly affect bird populations, often leading to mortality. Neurotoxins such as Clostridium botulinum (BoNT) are transferred to birds through the ingestion of contaminated prey (Essian et al. 2016). After eating prey such as fish and invertebrates, the toxin is passed to the birds, resulting in the death of many species of waterfowl and shorebirds. Therefore, the research on potential toxins is essential in the management of healthy bird populations.

Conversely, relatively little research has focused on the presence of toxins in eggs, despite the fact that the egg stage is the most vulnerable stage of a bird's life cycle (Giglio et al. 2022). Eggs have a highly uniform structure, are produced by a known segment of the population (e.g, females), and can be easily collected and handled (Furness et al. 1993, Kennamer et al. 2005). Examinations of egg characteristics, such as mass, facilitate inferences about nest viability and female health, and can also be used as metrics of population productivity. Likewise, assessments of contaminants within eggs, such as *Escherichia coli*, *Salmonellae*, fungi, and *Staphylococci,* allow an improved understanding of factors that may be influencing reproduction in birds (Ricke et al. 2001, Awny et al. 2018). Contaminants such as DDE have been shown to reduce successful hatching (nesting success) and cause shell thinning in avian eggs (Wiemeyer et al. 1984). Therefore, examining contaminants in avian eggs is essential to understanding the effect on successful reproduction. Research is warranted to better understand the potential presence of various neurotoxins in eggs.

Aetokthonotoxin (AETX), a cyanobacterial neurotoxin, originates from the cyanobacteria (*Aetokthonos hydrillicola)*, which grows on the leaves of the aquatic plant hydrilla. This cyanobacterium was discovered by scientists in DeGray, Arkansas, when a high number of Bald Eagle (*Haliaeetus leucocephalus*) deaths were reported (Thomas et al. 1998). This disease, known as Vacuolar Myelinopathy (VM), causes brain and spinal cord lesions resulting in neurological impairments, such as motor and cognitive functions in individuals that lead to death. Researchers conducted studies on the tissue of American Coots (*Fulica americana*), common prey of Bald Eagles, and documented Aetokthonotoxin in the American Coot breast and thigh tissue (Wilde et al. 2014, Breinlinger et al. 2021). American Coots are widespread in reservoirs across the United States, as are numerous other waterfowl species. This raises the possibility that AETX may also affect other species that inhabit the same water bodies. Notably, many of these species are game birds that are harvested for sport and human consumption. As such, research is needed to evaluate the potential presence of AETX in other waterfowl species.

In Georgia, wood ducks (*Aix sponsa*) are the most harvested waterfowl species, making up roughly 60% of the state's annual duck harvest (Kirby 2006). Breeding success and survival of species like wood ducks is essential to understand and enhance population growth (Hepp et al. 2020). Therefore, agencies like the Georgia Department of Natural Resources aim to establish effective management plans to conserve our native game species populations. To assess the possibility of reproductive risks, I collected and evaluated wood duck eggs for the potential of AETX. The presence and concentration of AETX has not been researched within avian eggs. Thus, the unknown effect of possible transfer from the female hen to the egg prompted this study. A series of trials were performed to determine the best methods for revealing and isolating (AETX) which can be attributed to (VM).

Methods:

Egg Collection and Processing

Egg collection and processing were allowed under a U.S. Fish & Wildlife Service - Migratory Bird Permit (permit number MBPER0050092) and a Georgia Department of Natural Resources - Scientific Collection Permit (permit number 1001592281), which permitted the collection of wood duck eggs from each study site. I excluded B.F. Grant WMA because researchers were collecting wood duck eggs at that site as part of another ongoing research project. In February 2023, I collected 5 eggs in Carroll County and a total of 17 from both Long Branch and Tussahaw Reservoirs. In March 2023, I collected 3 eggs at Oconee WMA (See study area description in the introduction of chapter 3). After collection, the eggs were placed in a freezer awaiting processing.

In April 2023, I thawed frozen wood duck eggs and subsequently opened each into a separate petri dish. To avoid contamination, gloves were changed between each egg processed. Additionally, with each new egg sample, utensils and petri dishes were disinfected with Microban and then rinsed with soap and water.

I used a scalpel to cut the yolk into small pieces (enough to fit within 1.5 mL Eppendorf tubes for approximately 1.0 g measurement), whereas I used a transfer pipette to transfer the albumen into separate tubes. I partially crushed the entire eggshell and placed it in a Whirl-Pak sample bag. Eggs were partially crushed for ease of egg extraction; this was prep for step 5 of the Duck Egg Tissue Extraction Protocol (Appendix B).

I transferred any remaining yolk, albumen, or shell (combined) from each sample into one 50 mL centrifuge tube. After processing, I placed all samples in the freezer until the extraction process. I conducted 2 trials to test for the presence of AETX, as the first trial yielded indeterminate results.

Egg Extraction

Trial 1

I filtered all eggs through an extraction process to test for AETX (Appendix B). Wood duck eggshells, yolks, and albumens, along with a chicken thigh, were ground in a mortar with sodium sulfate anhydrous. I used the chicken thigh as a blank control and a spike control (with ATEX), because the tissue showed good recovery from the toxin. I subsequently developed this positive control by adding the ground chicken thigh to 10 mL of methanol and adding 10 µL of 1 part per million (PPM) AETX to the solvent. The addition of 5 µL of triclosan (C12H7Cl3O2) in each sample was used as an internal standard to account for any loss of AETX during sample processing. I then vortexed, sonicated, and filtered each sample into new, clean 50 mL centrifuge tubes. These liquified samples were put into an N-EVAP nitrogen evaporator (2-3 L/min, 60-90 psi) (Organomation, Berlin, MA) to completely dry them.

After the samples were dry, I conducted the solid-phase extraction (SPE), which was implemented to reconstitute the dry samples within a manifold to remove any remaining impurities or compounds that could interfere with the sample. Oasis PRiME HLB 6 cc Vac Cartridge, 500 mg Sorbent per Cartridge, was used in SPE to “cleanup the samples” (Waters Corporation 2023). This sorbent enables reversed-phase cleanup of basic, neutral, and acidic compounds from complex sample matrices. After samples went through this phase, they were allowed to dry again using the aforementioned nitrogen evaporator. When dry, samples were transferred to the Environmental Protection Agency (EPA) in Athens, GA to be run through high-performance liquid chromatography (HPLC) with mass spectrometry (MS) to determine the toxin percentage. HPLC isolates the components of compound mixtures present in a liquid phase. MS is used to measure the mass of gas-phase ions and their fragments. Analytes are ionized and separated according to their mass-to-charge ratio, detected as ions, and their neutral mass is then calculated.

Trial 2

The egg yolk samples were removed from the freezer and moved to the refrigerator to thaw one day before processing. Twenty microliters of yolk sample and 100 µL were measured and added to a 1.5mL vial. Two stainless steel beads were added to the vials, and the samples were vortexed until the yolk and acetone were homogeneous. Subsequently, the samples were processed using Matrix Assisted Laser Desorption/Ionization (MALDI) in negative ionization mode on a Bruker Solarix XR 12 T FTICR MS at the Proteomics and Mass Spectrometry (PAMS) facility at the University of Georgia (NIH Grant S10 OD025118). For a sample to be considered positive on the high resolution MALDI, the peaks should be within 0.001 of the simulated AETX results. The primary indicator peaks for AETX are (649.636, 650.640, 651.634).

Results:

The results from Trial 1 were indeterminate, so I conducted a second trial. For Trial 2, I categorized results as positive, negative, or inconclusive for AETX (Figures 4.1, 4.2, and 4.3). Of 3 eggs collected at Oconee WMA, 2 tested negative, and one was inconclusive. Likewise, the 7 eggs collected at Tussahaw yielded 4 negatives and 3 inconclusive results. Conversely, of 10 eggs collected at Long Branch Reservoir, 5 tested negative, 3 were inconclusive, and 2 tested positive. The 5 eggs collected in Carrollton yielded 3 negative, 1 inconclusive, and 1 positive test.

Discussion

Neurotoxins are recognized for their detrimental effects on various species, including birds (Harem et al. 2020). There is also increasing concern about the potential transfer of neurotoxins from birds to the human food chain, as well as the mechanisms behind this transmission (Cullen, 2025, UGA Dissertation). My research revealed the presence of AETX in 12% of wood duck eggs collected from impoundments in Georgia, USA. These findings represent the first evidence that AETX can contaminate wood duck eggs, further contributing to the growing body of literature that highlights concerns regarding the presence of AETX and other neurotoxins in the environment (Breinlinger et al. 2021).

Trial 1 was implemented to determine the concentration of toxin found within the different parts (shell, yolk, and white) of the eggs. The results were indeterminate, so it was concluded that this trial should not be used to determine whether an egg showed the presence of AETX. For Trial 2, results were categorized as being positive, negative, or inconclusive for AETX. The results from Trial 1 indicated that the yolk conveyed the highest potential for toxin presence. This second trial was more sensitive and conveyed "yes or no" polar questioning. By initiating this trial, eggs with no traces of AETX could be easily determined. More research is needed to determine the optimal methods for extracting and quantifying the AETX in wood duck eggs.

While there has not been any historical research on hydrilla presence/densities within B.F. Grant WMA, Oconee WMA, or Carrollton, GA sites, Henry County reservoirs have long been part of observation for management of Hydrilla (Table 4.1). VM was confirmed at the earliest of 2010 at Upper Towaliga and 2011 at Long Branch. More recently, in 2021 it was confirmed at Tussahaw. Continuous data collection of unhatched wood duck eggs has been mapped with ArcGIS. High-density areas of unhatched eggs can be seen at reservoirs (indicated by lighter colored data points) (Figure 4.4). It is hypothesized that these areas of confirmed VM correlate with the failure of many of the wood duck nests. Researchers confirmed AETX on hydrilla and fish samples collected from October 2021 to August 2022 at Tussahaw Reservoir. The average AETX concentration from hydrilla was 855.68 ± 78.52 ppb, and the average AETX concentration from fish was 51.29 ± 28.65 ppb (Cullen, 2025, UGA Dissertation). To mitigate this issue, Grass Carp (*Ctenopharyngodon idella)* was introduced to these reservoirs as a biological control method. Since Grass Carp are herbivorous, their implementation has been commonly used to aid in the control of aquatic vegetation in small impoundments and reservoirs (Kirk et al. 2001). In 2015, Grass Carp were able to eliminate hydrilla in Upper Towaliga, and more recently in 2024, Long Branch and Tussahaw.

TABLES

Reservoir	VM confirmed	Hydrilla Confirmed	Management	Hydrilla Controlled
Long Branch	2011	Yes	Grass Carp	2024
Tussahaw	2021	Yes	Grass Carp	2024
Upper Towaliga	2010	Yes	Grass Carp	2015
Lower Towaliga	N/A	Yes	N/A	N/A
Gardner	N/A	Yes	N/A	N/A

Table 4.1: Confirmed cases of Vacuolar Myelinopathy (VM) within Henry County Reservoirs. Date of confirmed VM discovery, hydrilla presence, management plan (grass carp implemented to eliminate hydrilla abundance), and date of hydrilla elimination (density low enough to not be of concern).

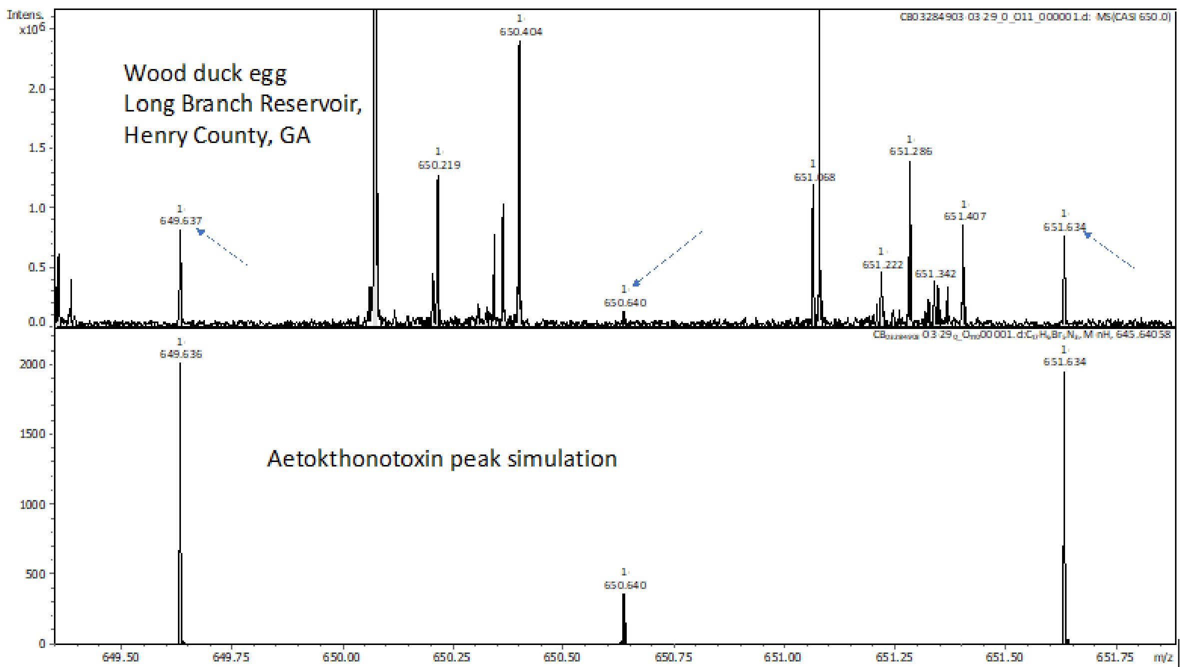

Figure 4.1: Aetokthonotoxin (AETX) was confirmed in a wood duck egg from Long Branch Reservoir, Georgia, USA, using high-resolution MALDI (Matrix-assisted laser desorption/ionization). The bottom portion of the graph is the AETX simulation for comparison with the egg yolk sample on the top portion of the graph.

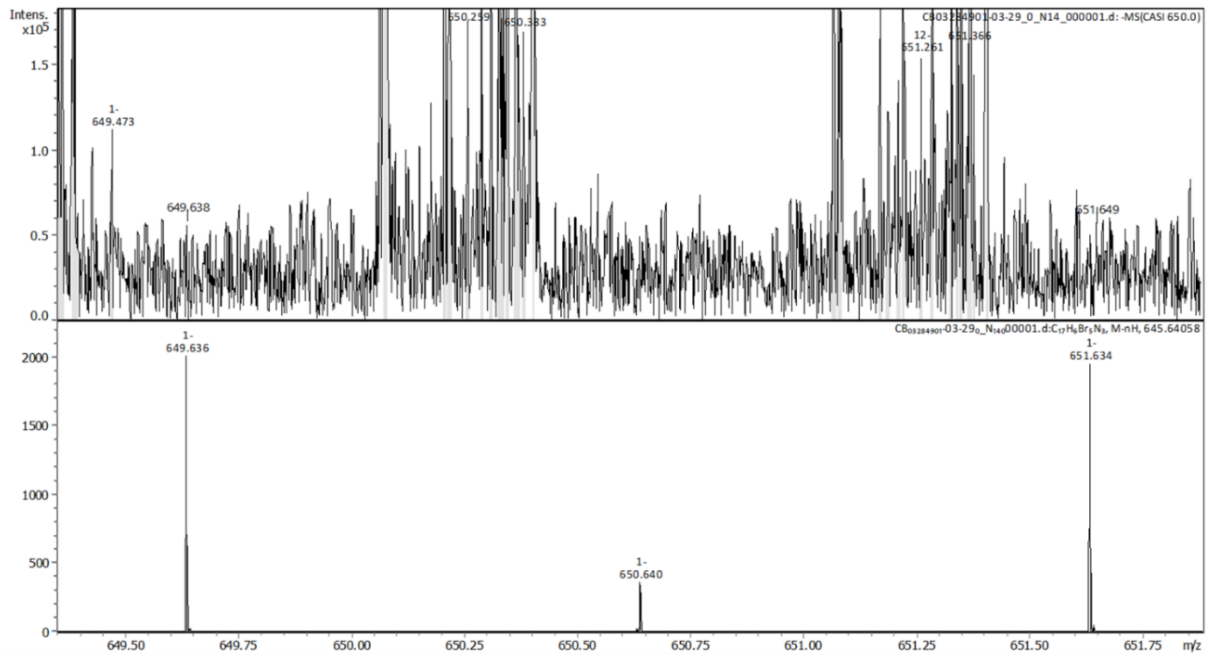

Figure 4.2: A wood duck egg from Long Branch Reservoir, Georgia, USA had only one peak identified using high-resolution MALDI (Matrix-assisted laser desorption/ionization), consistent with the presence of Aetokthonotoxin (AETX), which is considered an inconclusive result. The bottom portion of the graph is the AETX simulation for comparison with the egg yolk sample on the top portion of the graph.

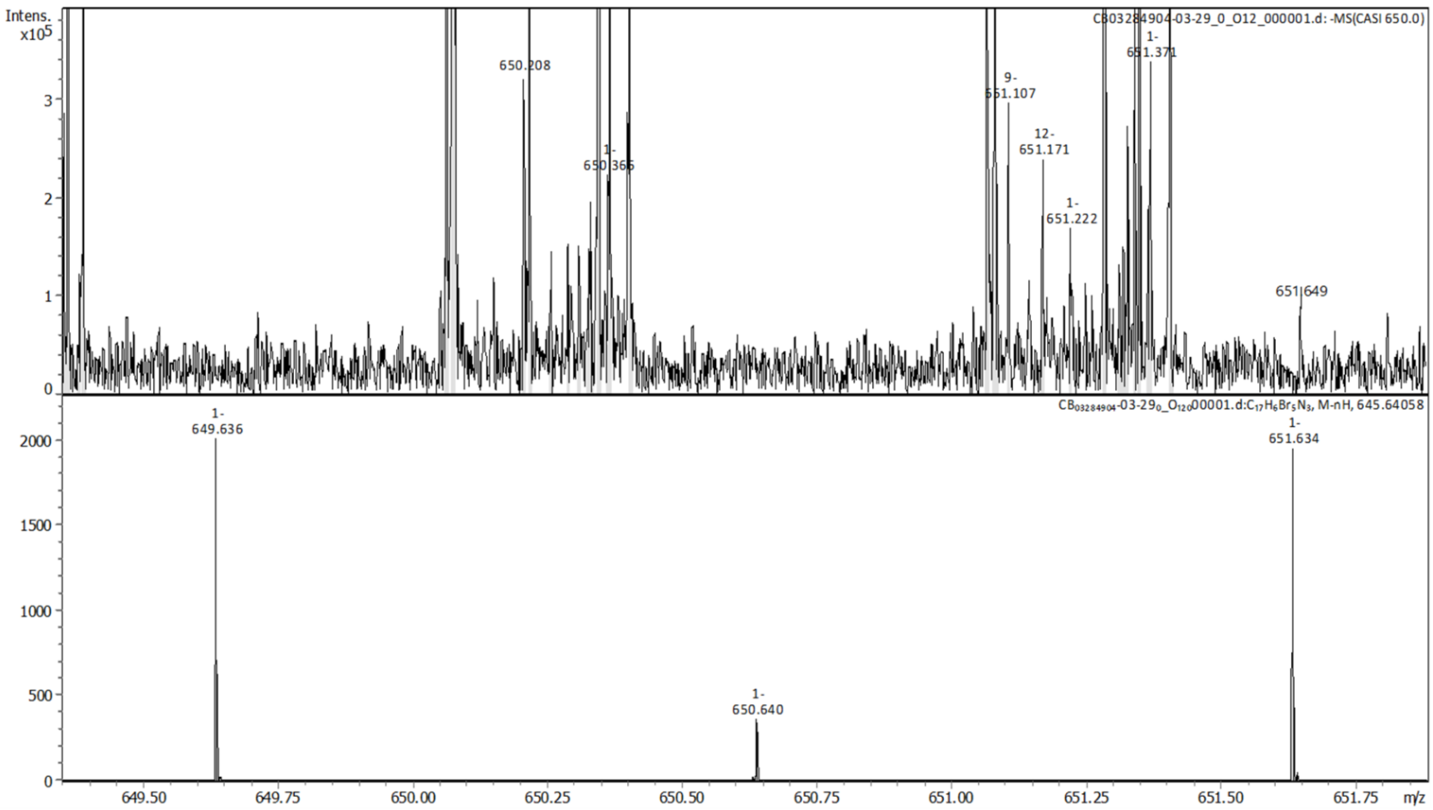

Figure 4.3: A wood duck egg from Long Branch Reservoir, Georgia, USA had no peaks within 0.001 of the simulation when analyzed using high-resolution MALDI (matrix-assisted laser desorption/ionization), indicating the absence of AETX.

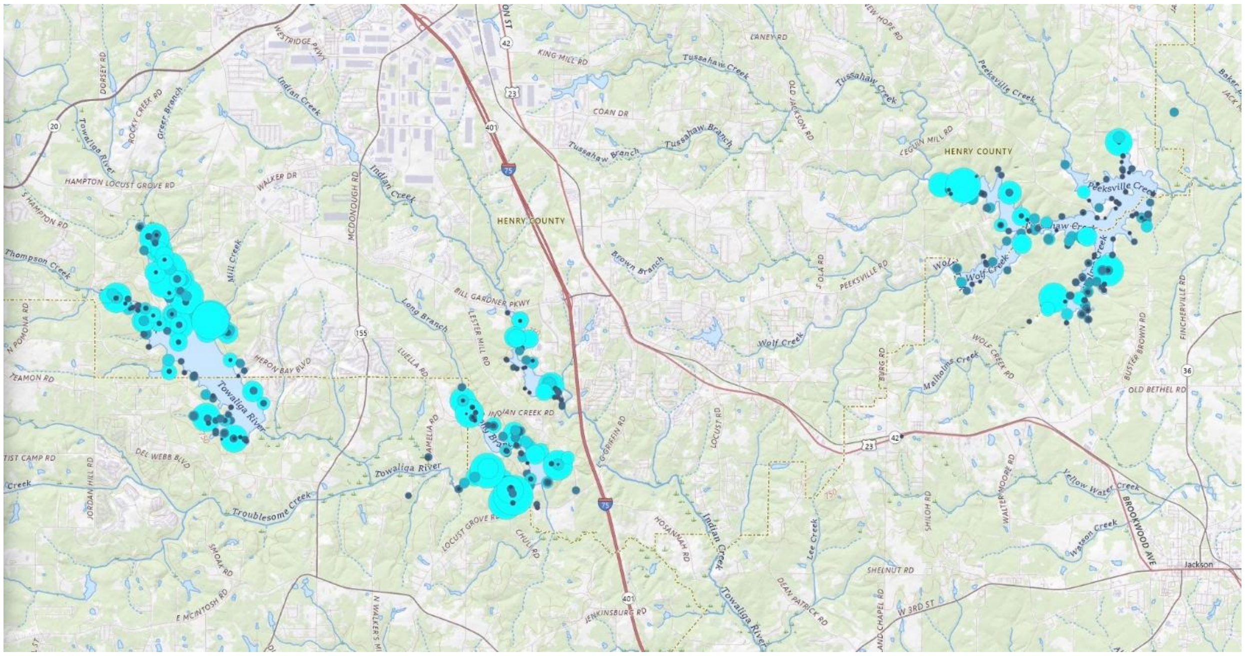

Figure 4.4: A map of unhatched egg densities within nest boxes in Henry County Reservoirs. From left to right: Upper Towaliga, Lower Towaliga (near the words "Towaliga River"), Long Branch, Gardner (slightly above Long Branch), and Tussahaw Reservoirs. The lighter the point (nest box location), the more the unhatched egg density; darker points indicate fewer unhatched eggs.

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CHAPTER 5

CONCLUSIONS

Considerations and Limitations

It is important to consider that human interpretation of hatched eggs (membrane counts) may not be consistent. Eggshell pieces could be incorrectly identified as successful hatchlings. The membrane counts at each box are determined by technicians, which can vary, as the same technician may not be present during each post-nesting season. Additionally, membrane counts are not the most accurate way to judge a specific nest. In other words, some nest boxes may have two or more incubations by different females. While the membrane count may be high, it could be lower than expected, if more than one hen incubated during the nesting season.

Similarly, in one of the photos from the study, a hen was observed facing out of the box with an egg in her bill. Nest tidiness by females is not considered in this study. It can be hypothesized that females clean nest boxes (of debris and eggs) to ensure a successful and healthy hatching. Without visual confirmation of a hen cleaning out older membranes or eggs, these instances are missed during nest checks. Another consideration, as was displayed in chapter 2, was the inconsistencies in site nest checks. During those years, technicians may have been limited by help, time, or even lack of access to boxes due to flooding or drought. Thus, while a site may have shown a lower success, this is dependent on how many boxes were checked and therefore does not provide an accurate assessment of nest usage and productivity. In 2024, B.F. Grant WMA boxes were not cleaned out by technicians; therefore, any failed nests with rotting eggs or other nest debris were left and nesting material (cedar shavings) was lacking and needed to be replaced in many boxes.

Box placement was another area of concern at most of the sites in this study. Most nest boxes were in areas of open space/water. Nesting out in the open can easily attract the attention of predators. Placement of nest boxes in areas with thick emergent vegetation is essential for the survival of ducklings. Without vegetation, there is no cover for hatchlings to hide from predators. Additionally, box maintenance and condition should be further considered. The nest boxes and predator guards at B.F. Grant WMA were in poor condition and needed to be replaced. Specifically, a door (where technicians can open and clean out boxes) was missing from one of the B.F. Grant WMA boxes. A broad opening to nest boxes makes it easier for predators to enter.

As is usually the case with field cameras, there are malfunctions. While trigger time can be manipulated to capture images quickly, camera wake-up time is not always accurate. In many photo instances, the camera was not able to accurately interpret movement and therefore missed the duck's activity. Setting the trail cameras to video mode provided more insight on duck behavior/activity. However, many videos also had a delay and only showed a short time frame of the box moving or a splash of water near the entrance. It can be hypothesized that it captured the time after or before the hen left or came into the box. However, the behavior of the hen or other cavity-nesting species can only be predicted. Images and videos that were blank (but showed movement or trace) were eliminated from the study to exclude inconsistencies.

Future Research

Emerging technology and additional data collection will improve our assessments of the costs and benefits of wood duck nesting within artificial nest boxes. Females and males could be tracked via GPS-enabled radio transmitters. By implementing these devices, researchers can obtain accurate tracking as well as migration routes for wood ducks. As areas/bodies of high activity are determined, local managers may want to adjust by removing older boxes or adding more boxes to a certain area to reduce dump nesting. Recognizing highly populated bodies of water and roost sites can allow researchers to study the potential for disease. As highlighted in the fourth chapter, wood duck eggs can be used to detect environmental contaminants such as Aetokthonotoxin and provide an indicator for potential disease events like Vacuolar Myelinopathy. For example, if wood ducks are migrating to or frequenting a body of water with dense hydrilla mats, this area can be isolated as "high risk". Even when eggs were identified as showing trace amounts of VM, there was no way to identify where or what body of water held hydrilla. Additionally, examining harvested wood ducks (tissue samples, eggs, feathers) could provide more insight as to which part of the duck VM can be found. By examining these areas, researchers can hypothesize whether VM is a concern for wood duck populations.

Another area for research is the effect the ambient temperature and percentage of precipitation have on nesting and incubating hens. In the Southeastern United States, due to warmer spring and summer temperatures, females are known to have multiple nests during one nesting season. Consistent nest incubation can be observed in seasons of abnormally high or low temperatures. Additionally, as seen in this study, high amounts of precipitation can cause box flooding. Areas of box decline can be attributed to these abnormal temperatures and precipitation amounts. As such, this is an important area for managers to consider. While technicians cannot change boxes based on temperature, boxes can be better maintained for high precipitation. In the Carrollton sites, boxes were extended on posts. This increased height lowered the chances for flooding of the nests as well as the potential for nest predation.

APPENDICES

APPENDIX A

Results: Site-specific total hatched and unhatched wood duck eggs by year

This appendix file includes the site-specific counts of membranes and unhatched eggs by year. It is important to note that not every site was checked each year, and some sites were only checked once.

B.F. Grant had usage in 2007, 2013, 2014, 2015, 2016, 2017, 2018, and 2019. In 2007, 18 membranes and 16 unhatched eggs were counted. There was a slight decrease in membranes between 2013 (68) and 2014 (48) before a spike in 2015 (79). From 2016 to 2018 there was a downward trend in membranes, with 2018 being the lowest (12 membranes). B.F. Grant was checked once more in 2019, and the membrane count had positively increased to 84.

Big Lazer Creek had recorded usage from 2001 to 2020. The highest membrane count was 163 in 2004. Except for a small spike in 2006 (123 membranes), from 2004 to 2009 there was a decline in membranes. In 2011, the lowest number of unhatched eggs was recorded (2), compared with 41 successful membranes found. Another notable peak in membrane counts was observed between 2013 (103) and 2014 (104).

Blanton Creek had recorded usage from 2001 to 2020. This site had the highest membrane count (207) in 2003. From 2003 to 2010 there was a steady decline in the number of membranes. After 2010, there was some slight variability of spikes and declines each year.

Notably, only 1 unhatched egg was recorded in 2013, compared with 47 membranes found that year.

Cedar Creek had usage in 2001, 2002, 2014, 2015, 2016, 2017, 2018, and 2019. The most notable were 2001 and 2002 where 0 unhatched eggs were found. The year 2002 yielded the most membranes (81). After a drop in membranes in 2014, the number began to increase until another drop between 2018 and 2019.

Chattahoochee Fall Line had recorded usage in 2017 to 2020. From 2018 to 2020 there was an increase in membranes and a decrease in unhatched eggs. The highest membrane count (21) was recorded in 2020, with only one unhatched egg that same year.

Clarks Hill had usage in 2001, 2002, 2003, 2004, 2005, 2006, 2008, 2013, 2014, 2016, 2017, 2019, and 2020. From 2004 to 2005 there was no recorded box usage. From 2006 to 2016 there were no membranes counted. In 2017, there were 19 membranes with this site hitting its highest membrane count (30) in 2019.

Clybel had usage in 2001, 2004, 2005, 2006, 2007, 2008, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, and 2020. From 2001 to 2006, nest data showed a notably high unhatched eggs. Nest success shifted to a higher number of membranes compared to unhatched eggs from 2012 to 2020, with the highest number of membranes (100) in 2017.

Dawson Forest had usage in 2001, 2002, 2003, 2004, 2005, 2006, and 2012. Overall, nests showed a higher number of membranes compared to unhatched. However, in 2005 sums were closely related, with the total membranes (15) and total unhatched (76). There was a notable change in 2012, the last year of recorded usage when there was a total of 20 unhatched eggs and 0 membranes.

Fishing Creek had wood duck nest usage in 2001, 2002, 2003, and 2004. Membrane counts were higher than unhatched until 2019. In 2019 the highest number of membranes were counted (27) as well as the highest number of unhatched eggs (37). In 2020, there were 6 membranes and 31 unhatched eggs.

Hart County had usage from 2001 to 2005. There was a notable increase in membranes (138) in 2003. The highest number of unhatched eggs (100) were counted in 2005. In this year, the number of membranes (83) were fewer than the previous year.

J.L. Lester had usage from 2006 to 2020. The highest number of membranes (34) were counted in 2019. This same year also exhibited the highest count of unhatched eggs (29).

Joe Kurz had usage in 2001, 2002, 2003, 2004, 2006, 2007, 2008, 2009, 2011, 2012, 2013, 2014, 2016, 2017, 2018, 2019, and 2020. Unhatched eggs were higher (86) in 2004. The highest number of membranes was recorded as 75 in 2018. In 2019 and 2020 the membrane count remained the same (71), while 2020 had more unhatched eggs (65).

Lake Lanier had usage in 2001, 2002, 2003, 2004, 2005, 2006, 2011, and 2012. There was a high sum of unhatched eggs (143) compared to membranes (54) in 2005. Another high success year was 2011, with 134 membranes and 26 unhatched eggs.

McDuffie PFA had usage in 2020. There were 50 membranes and 25 unhatched eggs within this year. Since there were no other usage years recorded no trends could be identified.

McGraw Ford had usage from 2014 to 2019. The highest number of membranes (8) was recorded in 2016. The year 2018 was the only year that had unhatched eggs (4).

Oconee had usage in 2002, 2003, 2004, 2005, 2006, 2008, 2010, 2017, 2018, 2019, and 2020. Both 2019 and 2020 showed an upward trend in unhatched eggs (83 and 87). The highest number of membranes was identified in 2019 as a total of 53.

Paulding Forest had usage in 2002, 2006, 2007, 2008, 2009, 2011, 2013, 2014, 2015, 2016, 2017, 2018, 2019, and 2020. After 2009, except 2014 and 2018, unhatched egg counts were higher than the number of membranes counted. The highest number of membranes (15) and unhatched eggs (20) were recorded in 2011.

Phinizy Swamp only had wood duck usage in 2003. There were no recorded membranes within this site. Only 3 unhatched eggs were identified. Since there were no other usage years recorded no trends could be identified.

Pine Log Mountain had usage in 2006, 2009, 2011, 2012, 2013, 2014, 2016, 2017, 2018, and 2019. Unhatched eggs (8) were only recorded in 2009. The highest number of membranes (8) were identified in 2018. The years 2011, 2013, and 2014 did not show any membrane or unhatched egg counts.

Redlands had usage from 2015 to 2019. The two highest membrane counts were in 2015 (213) and 2019 (250). There were a large number of unhatched eggs in all years except 2019. In 2016, the highest number of unhatched eggs (244) were recorded.

Rum Creek had usage in 2002, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2013, 2014, 2015, 2016, 2017, 2018, 2019, and 2020. Higher membrane counts were exhibited in 2016 (113), 2017 (121), 2019 (177), 2020(156). The highest number of unhatched eggs were identified in 2019 (113) and 2020 (100).

Vaughter only had usage in 2020 when there were 12 membranes and 9 unhatched eggs recorded. Since there were no other usage years recorded, no trends could be identified.

West Point had usage in 2001, 2003, 2004, 2005, 2006, 2008, 2010, 2011, 2012, 2014, 2015, 2016, 2017, 2018, 2019, and 2020. The highest number of membranes were recorded in

2001 (126), 2003 (116), 2008 (109), 2010 (143), 2011 (123), and 2012 (168). The highest unhatched counts were recorded in 2012 (203), 2014 (133), and 2015 (100).

APPENDIX B

AETX Toxin Extraction Protocols for Wood Duck Egg Tissue

Cullen, Brown

7/25/2023

Overview:

1. Duck Egg Tissue Extraction Protocol
2. N-Vap
3. Solid Phase Extraction
4. LC-MS/MS

Duck Egg Tissue Extraction Protocol

Materials:

- Small Weigh boats
- MeOH (Methanol) in a squirt bottle
- CHCl3 (Chloroform)
- (CH3)2CO (Acetone)
- Dichloromethane (DCM)
- 50 ml graduated cylinder
- Mortars and pestles

- Scoopulas
- 50 ml centrifuge tubes
- Funnels
- Tin foil
- Filter paper (Whatman #42 ashless)
- Sharpies
- Sonicator
- Vortex

** Each extraction batch extracted on the same day will need to include the following Quality Controls:

1. Solvent blank
2. Solvent spike
3. Blank control tissue (Chicken thigh)
4. Control tissue spike (Chicken thigh - 10uL from stock solution of 1 ppm AETX in 100% DMSO)

Preparation:

1. Clean and condition enough mortars, pestles, spoonulas, and funnels for blank solvent, chicken negative control, chicken spiked control, and unknown felid tissues with methanol, acetone, then chloroform
2. Label small weight boats, mortars, pestles, and funnels with extraction id
3. Label two sets of 50 mL centrifuge tubes with matching extraction id.

4. Add 15.0 g of anhydrous sodium sulfate to mortar
5. Use weight boat and measure 1.0 g of wet tissue (separate samples of yolk, white, and egg) to the nearest 0.1 g and record weight in extraction log.
6. Add 1.0 g wet weight of tissue to a mortar
7. Grind sample to an even grain with mortar and pestle.
8. Transfer sample to 50 mL centrifuge tube using spoonula.
9. Add 10 mL methanol to each 50mL centrifuge tube. Cap and shake well.

a. Create positive controls with chicken thigh and solvent

i. Vortex AETX stock for 20 seconds

ii. After adding 10 mL methanol, spike control tissue in centrifuge tube with 10uL AETX stock of 1ppm AETX in 100% DMSO

iii. Repeat procedure for spiking solvent

iv. Cap and parafilm AETX stock

v. Allow the spiked tissue and solvent tubes to sit for at least 1 minute before continuing to next step

i. Reduce the matrix suppression for LCMS-MS analysis by adding in Triclosan Vortex Triclosan for 20 seconds

ii. Add in 5uL of Triclosan to every sample (including those spiked with AETX)

iii. Cap and parafilm Triclosan

iv. Return to the refrigerator

10. Vortex for 30 seconds, then shake well 5-10 seconds.

11. Uncap samples and add 20 mL chloroform, cap and shake well.
12. Vortex 30 seconds, and then shake for 5-10 seconds.
13. Sonicate for a total of 30 min.
14. Remove samples from sonicator and dry outside of tubes.
15. Shake tubes well.
16. Gravity filter through funnel with Whatman #42 ashless filter paper into new, clean, labeled 50 mL conical centrifuge tubes.
17. Rinse initial conical centrifuge tubes and remaining tissue in the filter paper with 5mL chloroform and 5 mL methanol into clean centrifuge tube. Two separate rinses: once with methanol, then once again with chloroform.
18. Cap and store in fridge until ready for N-Vap steps.
19. Copy extraction log info into digital extraction log on Wilde MS Teams Page

N-Vap Duck Egg Tissue Protocol

Use N-evaporator in ABEL Toxicology Laboratory Rm. 205 or forced air from the fume hood in Fish Lab Rm. 116

1. Slightly open necessary valves on N-evaporator
2. Wipe nozzles with methanol and kimwipe
3. Turn on fume hood fan and light
4. Place samples on N-evaporator
5. Turn on N-Vap leaving heat off

6. Open both nitrogen tank valves with steer bar horizontal
7. Slowly open LPM meter and adjust to 2-3
8. Lower nozzles to appropriate height to cause agitation without bubbling
9. Continue running until totally dry [a]. Transfer more solution when necessary

a. To conserve nitrogen, if samples dry to 2-3 mL mark, transfer to forced air until totally dry.

Duck Egg Tissue Solid Phase Extraction

Materials:

- 6cc 500mg 30µm Oasis brand HLB SPE columns
- MeOH (Methanol)
- Deionized water (DI water)
- C2H6OS (DMSO) 10%
- Dichloromethane (DCM)
- MeCN (Acetonitrile)
- 15 mL glass tubes
- Sonicator
- Vortex
- Manifold
- Vacuum pump generator

Procedure:

1. Label SPE columns
2. Label 15 mL glass tubes
3. Reconstitute dry tissue extracts in 6 mL of 10% DMSO 90% DI water mix
4. Vortex for 30 seconds
5. Sonicate for 10 minutes, set aside
6. Set up manifold and vacuum

a. Clean and condition manifold with methanol

b. Load 6cc 200mg 30µm Oasis brand HLB SPE columns onto vacuum filter

7. Condition 6cc SPE columns into waste bin

a. 6 mL methanol

b. 6 mL deionized water

c. Adjust vacuum pressure to 1 drip/second

8. Vacuum filter 6 ml of sample through SPE columns on wet column bed into waste container
9. Rinse sample tubes with 6 mL of 100% DI water through SPE columns into waste container
10. Allow vacuum filter to run ~30 minutes after drips stopped (or until sorbent bed is dry)
11. Rinse 50 mL conical tube with 6 mL methanol and transfer to SPE column to elute sample into 15 mL glass tubes
12. Repeat with 6 mL of dichloromethane
13. Cap samples and fridge until they can be transported to the EPA.

14. Dry samples in N-Vap using N-Vap Protocol below
15. Reconstitute in the glass tube with 1 mL of methanol
16. Vortex for 30 seconds, sonicate for 10 minutes, then transfer to HPLC vial
17. Dry sample in vial completely
18. Reconstitute in the glass tube with 1 mL of dichloromethane
19. Vortex for 30 seconds, sonicate for 10 minutes, then transfer to HPLC vial
20. Dry sample in vial completely
21. Reconstitute HPLC vial samples in 1.0 mL of 60:40 acetonitrile:DI water
22. Vortex for 30 seconds
23. Store at 4° C until LC-MS/MS analysis can be conducted

N-Vap Duck Egg Tissue Protocol

Close all valves

1. slightly open necessary valves and LPM air gauge
2. Wipe nozzles with methanol and kimwipe
3. Turn on fan and light
4. Turn on N-Vap leaving heat off
5. Open both nitrogen tank valves with steer bar horizontal
6. Adjust LPM meter to 2-3
7. Lower nozzles to appropriate height to cause agitation without bubbling
8. Continue running until totally dry

LC-MS/MS

Materials:

1. Method blank vial
2. Unknown sample vials
3. Control vials

a. Solvent blank

b. Solvent spike

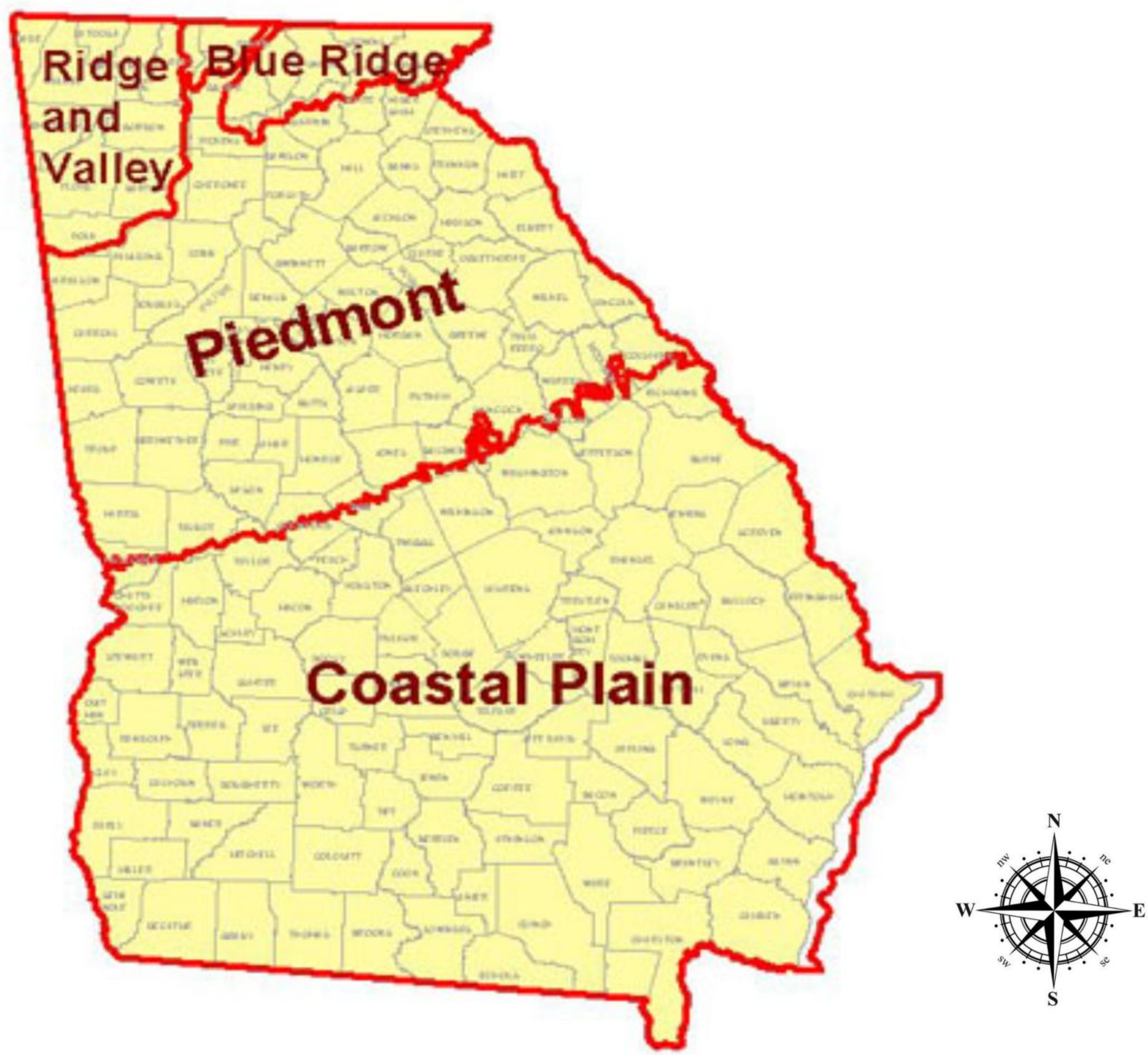
c. Tissue blank

d. Tissue spike

4. HPLC machine (Thermo Accela HPLC coupled to TSQ Quantum Ultra Mass Spectrometer at U.S. EPA lab in Athens Ga, USA)

APPENDIX C

Geographic Region and Site Maps



Map 1. A state geographical area map used to delineate Piedmont sites.

Source: http://coastgis.marsci.uga.edu/summit/provincesmap.htm

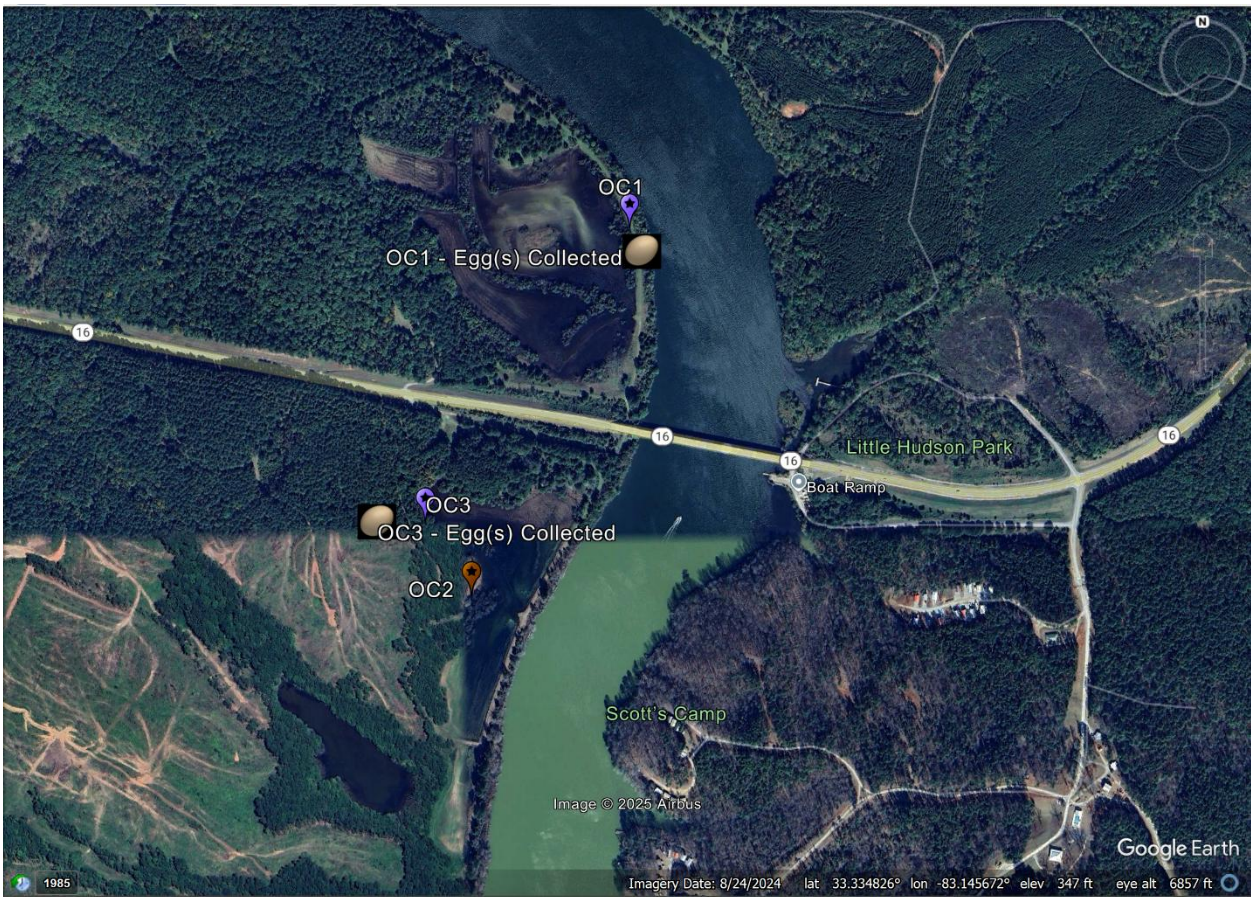


Figure 1.1: A complete aerial view of observed nest boxes (3) at Oconee WMA in 2023 and 2024; purple points indicate the presence of an iButton and camera, orange points indicate only an iButton (no camera), and the egg symbol indicates a box in which eggs were collected.

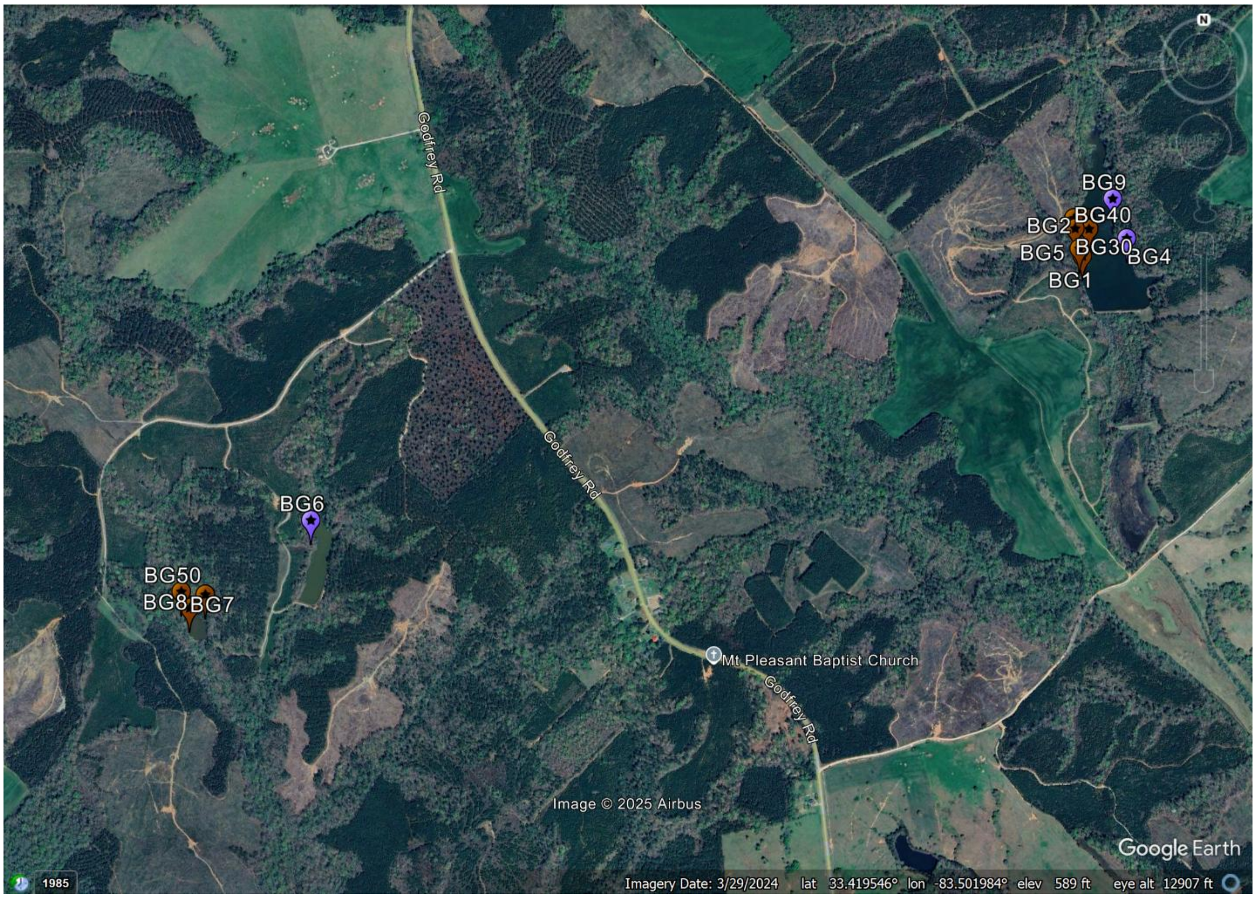


Figure 1.2: A complete aerial view of observed nest boxes (12) at B.F. Grant WMA in 2023 and 2024; purple points indicate the presence of an iButton and camera, orange points indicate only an iButton (no camera). From left to right: Little Steinbeck Pond boxes, Big Steinbeck Pond box, Marsh Pond boxes.

Figure 1.3: An enlarged aerial image of observed boxes (8) in the Marsh Pond at B.F. Grant WMA in 2023 and 2024; purple points indicate the presence of an iButton and camera, orange points indicate only an iButton (no camera).

Figure 1.4: An enlarged aerial image of Little Steinbeck Pond observed nest boxes (3 boxes on the left) and Big Steinbeck Pond (1 box on the right) at B.F Grant WMA in 2023 and 2024; purple points indicate presence of an iButton and camera, orange points indicate only an iButton (no camera).

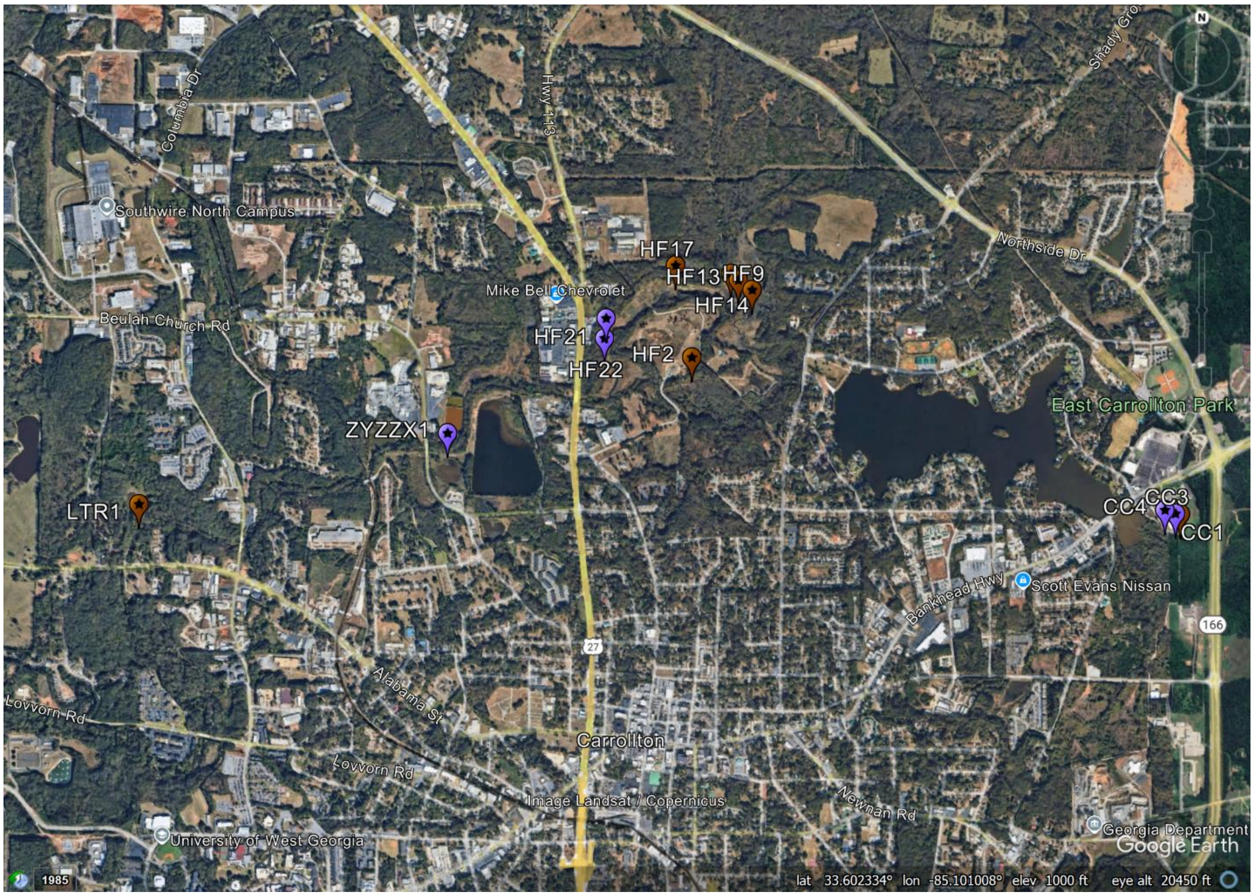


Figure 1.5: A complete aerial view of observed nest boxes within Carroll County (12 boxes in Carrollton, GA) in 2023 and 2024; purple points indicate the presence of an iButton and camera, orange points indicate only an iButton (no camera). From left to right: Little Tallapoosa box, Zyzzx Street boxes, Hobbs Farm boxes, and Curtis Creek boxes.

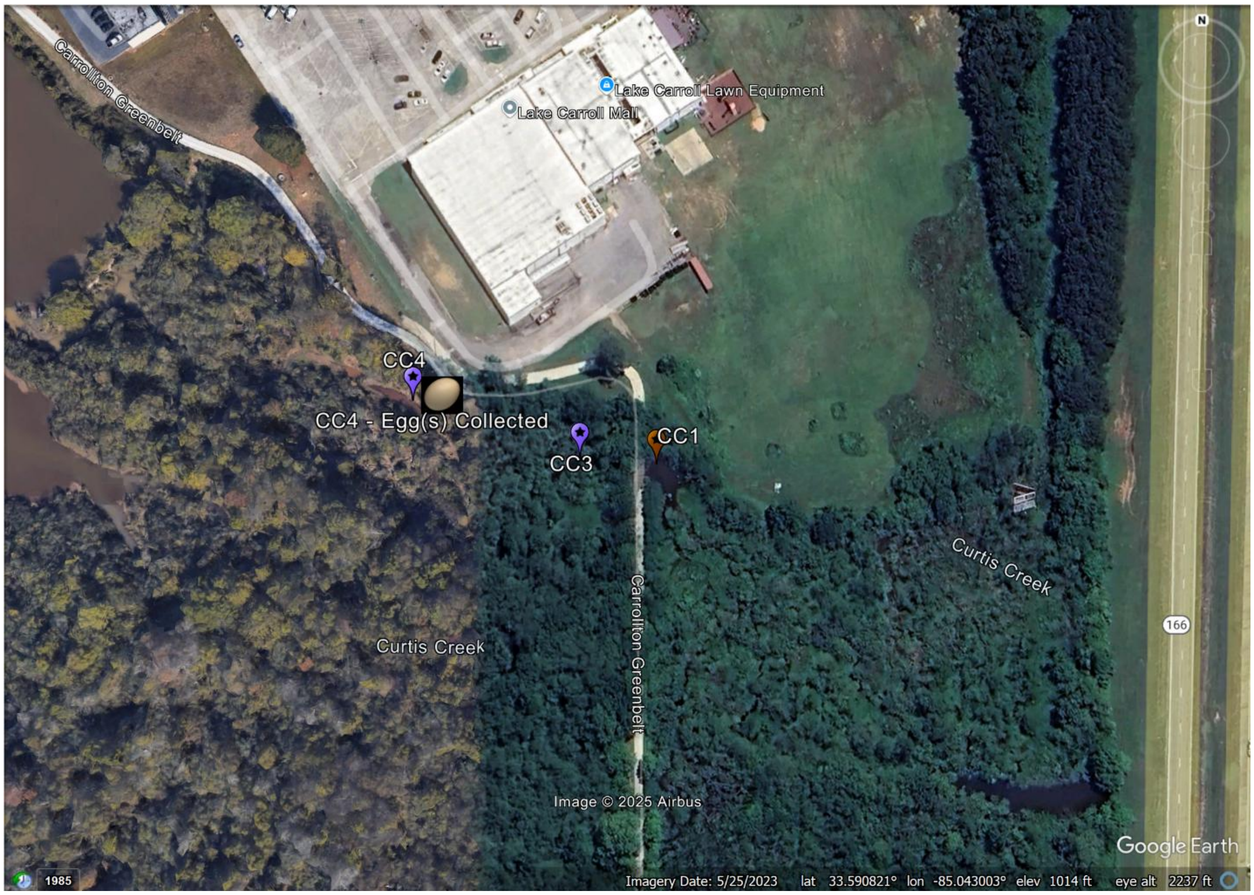


Figure 1.6: An enlarged aerial image of Curtis Creek boxes (3) in 2023 and 2024; purple points indicate the presence of an iButton and camera, orange points indicate only an iButton (no camera), and the egg symbol indicates a box in which eggs were collected.

Figure 1.7: An enlarged aerial image of Hobbs Farm boxes (7) in 2023 and 2024; purple points indicate presence of an iButton and camera, orange points indicate only an iButton (no camera), the warning symbol indicates an egg that tested positive for Aetokthonotoxin (AETX), and the egg symbol indicates a box in which eggs were collected.

Figure 1.8: An enlarged aerial image of the Little Tallapoosa box (2023 and 2024); the orange point indicates only an iButton (no camera), and the egg symbol indicates a box in which eggs were collected.

Figure 1.9: An enlarged aerial image of the Zyzzx Street box in 2023 and 2024; the purple point indicates the presence of an iButton and camera, and the egg symbol indicates a box in which eggs were collected.

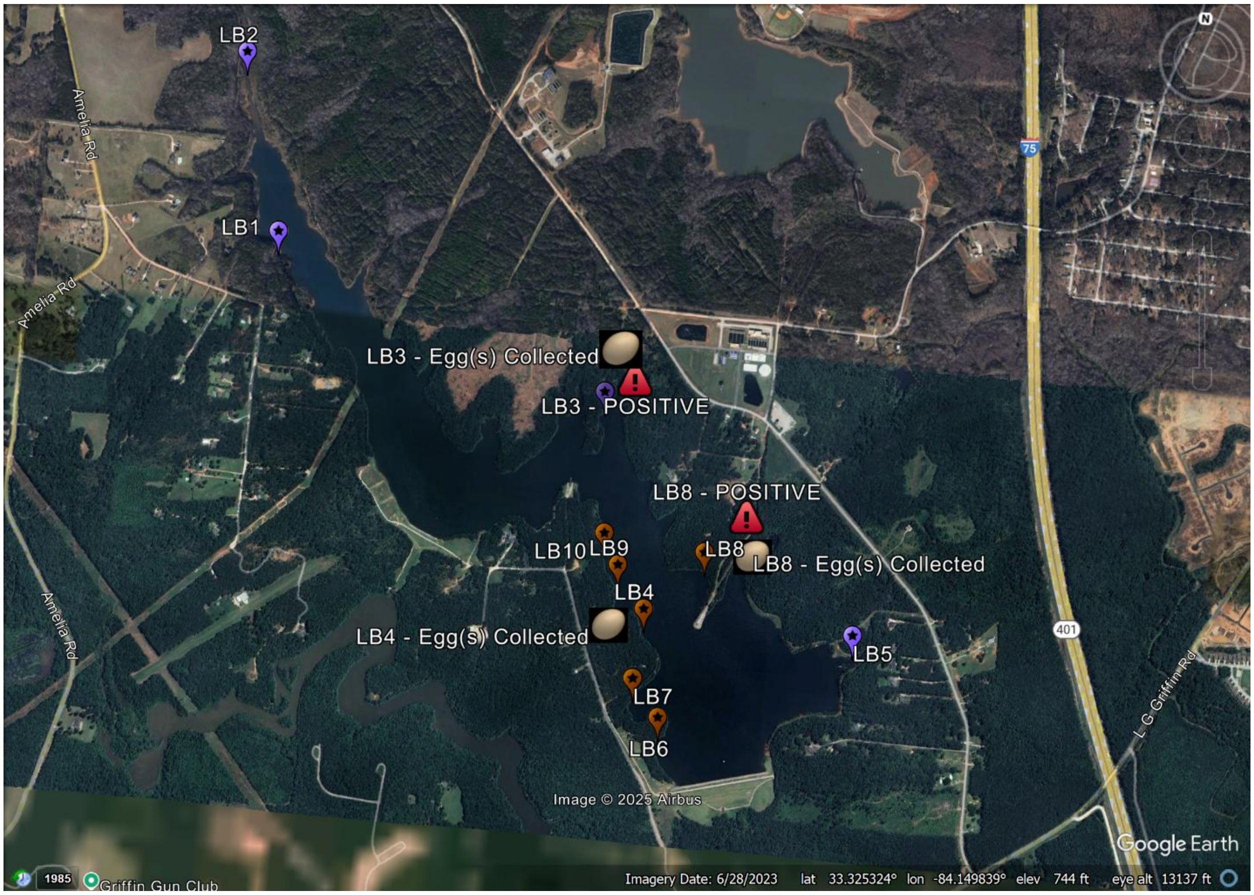


Figure 1.10: A complete aerial view of observed boxes (10) within Long Branch Reservoir in Henry County in 2023 and 2024; purple points indicate presence of an iButton and camera, orange points indicate only an iButton (no camera), the warning symbols indicates an egg that tested positive for Aetokthonotoxin (AETX), and the egg symbol indicates a box in which eggs were collected.

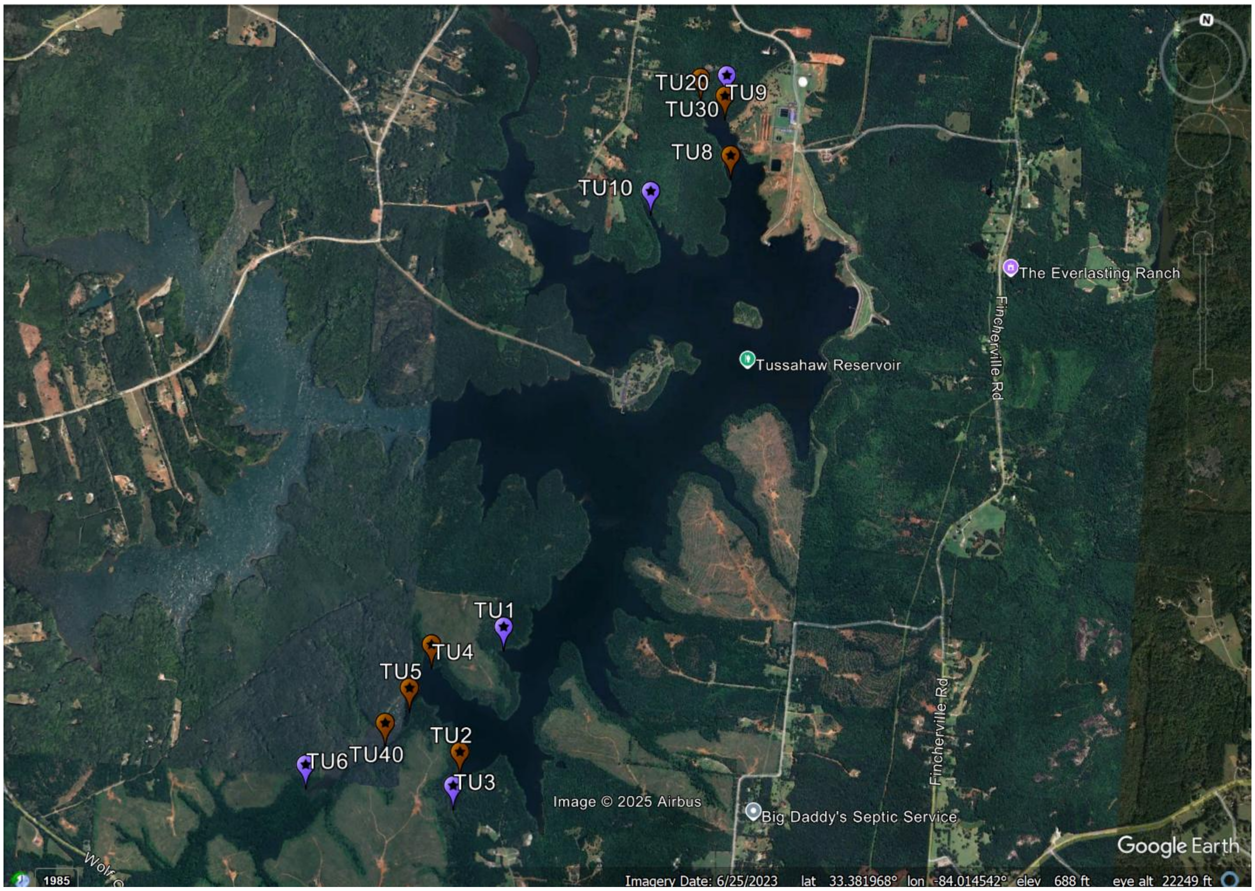


Figure 1.11: A complete aerial view of boxes (12) observed within Tussahaw Reservoir in Henry County in 2023 and 2024; purple points indicate the presence of an iButton and camera, and orange points indicate only an iButton (no camera).

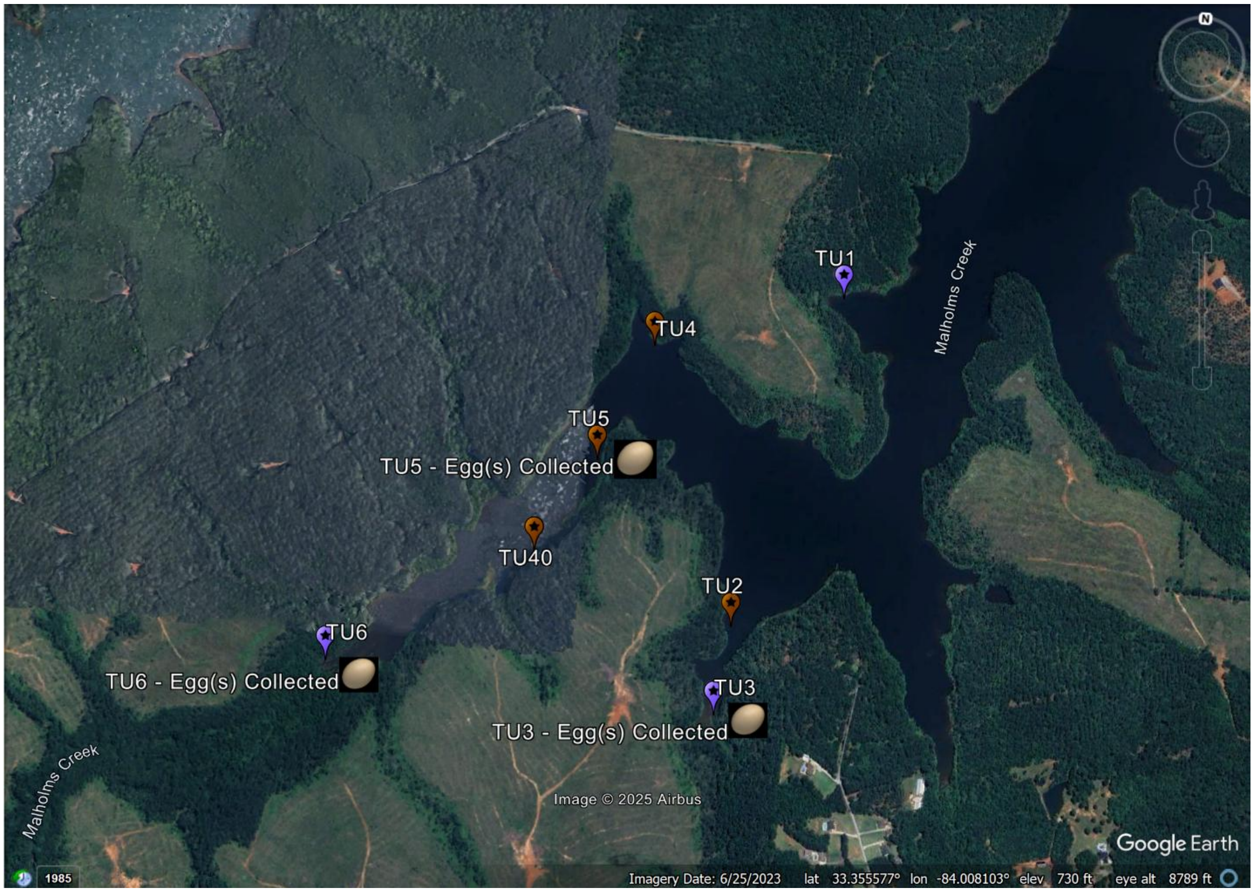


Figure 1.12: An enlarged aerial image of the nest boxes (7) in the Malholms Creek portion of Tussahaw Reservoir located in Henry County in 2023 and 2024; purple points indicate presence of an iButton and camera, orange points indicate only an iButton (no camera), and the egg symbol indicates a box in which eggs were collected.

Figure 1.13: An enlarged aerial image of the nest boxes (5) in the Peeksville Creek portion of Tussahaw Reservoir, located in Henry County in 2023 and 2024; purple points indicate the presence of an iButton and camera, and orange points indicate only an iButton (no camera).