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Lipid Biochemistry of House Wren Egg Yolk and Possible Effects on Incubation Period

A Senior Research Honors Paper

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25 April 2001

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Lipid Biochemistry of House Wren Egg Yolk and Possible Effects on Incubation Period.

(Senior Honors Research Project)

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Abstract

House Wrens (*Troglodytes aedon*) are distributed throughout North and South America. As has been observed in many avian species, populations of House Wrens in the tropics have longer egg incubation periods than populations in temperate regions. The purpose of this study was to characterize incubation periods of a temperate population of House Wrens and to conduct a preliminary chemical analysis of the yellow and white yolk layers of House Wren eggs. The eggs were collected from a breeding population in Central Illinois from June-August 2000. Avian yolk is composed of yellow and white components, which are laid down in alternating layers around a core of white yolk called the latebra. White yolk is produced at night, while yellow yolk is produced during the day. Along with the temporal differences in formation, yellow and white yolk differ in their biochemical make-up in that yellow yolk is richer in high-energy lipids. The proportions of yellow and white yolk may differ in eggs from tropical and temperate populations of House Wrens, which may influence the rate of embryonic development and thus incubation periods.

There was a 90.0% hatching success from the artificial incubation of temperate House Wren eggs (n=30), which had a mean incubation period of 13.6 days ± 0.2SD, which is significantly different than the literature value, 12.2 days ± 0.2SD, of a natural population of House Wrens in a temperate region. The lipid components (triacylglycerol, phospholipid, free cholesterol, cholesterol ester and free fatty acid) of commercially sold unincubated chicken eggs were analyzed. Extraction of the lipids from the yolks was conducted using a modification of the Folch method, followed by the separation of lipid components using preparatory TLC. This method produced a 93.4-96.0% lipid recovery rate with little variability (<6.0%) in replicate portions of the same yolk. Future work will involve incubating eggs from tropical House Wrens and conducting a chemical analysis of their yolks. Yolk lipid analysis of temperate House Wren eggs will also be done.

Introduction

Factors Affecting Incubation Periods in Birds

The wide range of incubation periods (i.e. time from beginning of incubation by the parents until hatching) among bird species has gained the attention of many avian biologists (Geffen and Yom-Tov 2000). Factors that may affect incubation periods include parental health/behavior during incubation, local climate, egg size, and egg contents. The female's nutritional status during egg formation may influence the egg contents and therefore, the incubation period (Kendeigh et al. 1956, Schreiber and Lawrence 1976, Mills 1979, Murphy et al. 1984). The greater the assimilation of lipids by females, the more lipids that may be directed towards forming eggs. Food shortages during a breeding season may decrease the amount of lipids distributed to the yolks. Females are the sole incubators in some species, while in other species both parents incubate, or in rare instances, only the male will incubate the eggs (Johnson 1998, Grover 1984, Baicich and Harrison 1997). When both parents incubate the eggs, attentive periods (i.e. the time the parent is sitting on the eggs) may be more frequent, therefore, inattentive periods would be shorter. However, it would be incorrect to say for certain that this will increase the rate of embryonic development. Kendeigh (1963) showed that in House Wrens, the inside of the eggs remain at a fairly constant temperature (~35.0°C) throughout incubation even though there may an increase in the number of attentive periods. An increase in number and length of attentive periods should not increase the temperature of the inside of the egg and, therefore, there would not be a decrease in incubation period.

Ambient temperature (Patten 1964) and humidity (Rahn and Ar 1974) also affect egg incubation periods. In general, incubation temperatures are slightly below that of the body temperature of the incubating parent and within a certain range, the rate of development is proportional to temperature (Patten 1964). At temperatures below the adult body temperature, embryonic development will occur, but at a slower rate than if the eggs were incubated near the adult body temperature. Cooling of the egg during brief inattentive

periods by the parents does not result in the death of the embryo, but may slow development. Ambient temperature increases when the number of daylight hours in a day increases, which may decrease cooling of the egg during inattentive periods. Humidity may also affect incubation period. Water loss via the shell pores by evaporation is inevitable in an egg during incubation. In a more humid climate, water vapor gradients across the shell would be less than in dry climates and therefore, rate of water loss would be less (Rahn and Ar 1974). A high rate of water loss from the egg would soon cause dessication at a fatal level and this would restrict the time the embryo had to develop in the egg. Although not tested, an increase in water retention may allow the embryo to develop in the egg for a longer period of time.

Size of parents and eggs apparently affect the length of incubation (Geffen and Yom-Tov 2000). Larger birds tend to have a larger nutritional intake than smaller birds, and therefore, have the ability to supply more raw materials to form their eggs. This allows the larger birds to produce larger eggs. The increased nutritional components in the larger eggs allow the embryo to develop within the egg for a longer period of time. Birds are often separated into altricial and precocial species. When altricial birds hatch, they are blind, mostly featherless and helpless for a period of time. In contrast, precocial birds hatch with eyes open, down feathers, and are mostly self-sufficient. In precocial species, egg size is an accurate predictor of lipid content (Alisauskas 1986, Romanoff and Romanoff 1949, Parsons 1970, Ricklefs et al. 1978). In contrast, egg size has been found to be a poor predictor for lipid content in altricial species (Ricklefs 1984, Jones 1979, Schifferli 1973, Rickleffs 1977, Bryant 1978). However, contradictions have been found for both precocial (Arnold 1989) and altricial species (Muma and Ankney 1987, Ankney and Johnson 1985). Altricial birds produce eggs that have a smaller yolk to albumen ratio (Rohwer 1986, Ricklefs 1977, Romanoff and Romanoff 1949) and shorter incubation periods than precocial birds (Ricklefs 1979). The greater percentages of yolk and nutrients in precocial birds aid in their more advanced stage at hatching and influence their longer

incubation periods. The more nutrients available to an embryo, the greater the amount of energy and structural building blocks it has to develop. It is plausible that birds with longer incubation periods have larger percentages of yolk in their eggs.

Albumen, which is the major source of protein available to the embryo, is also the major water reservoir in eggs (Lillie 1940, Romanoff and Romanoff 1949). Some researchers have suggested that egg size is directly related to water content (Ricklefs and Montevecchi 1979, Meathrel and Ryder 1987, Arnold 1989, Warham 1983), and therefore, proportional to amount of albumen (Boersma 1982). Birds draw the yolk sac and remaining yolk into their gut at hatching (Lillie 1940, Alisauskas 1986), which suggests that yolk content is not a limiting factor for incubation period. A decrease in the percentage of yolk in larger eggs would, therefore, not limit incubation time, but may allow increased proportions of albumen in the egg. As a result, more water would be present in larger eggs. This increase in water availability may dampen the effect of water loss via shell pores and therefore, allow for longer periods of incubation (Warham 1983).

Factors affecting incubation period differences in temperate and tropical birds

Differences in incubation periods of birds from temperate and tropical regions have been the focus of many interspecific studies. Several studies have concluded that incubation periods are longer in tropical birds than temperate birds (Skutch 1949, Lack 1968, Ricklefs 1968, Woinarski 1985). However, Geffen and Yom-Tov (2000) claim previous studies on incubation periods in tropical and temperate environments were based on insufficient sample sizes, and therefore, have low statistical significance. They conclude that incubation periods tend to be similar in tropical and northern temperate birds. Most of these studies were interspecific and their results may or may not apply to intraspecific comparisons. Factors affecting differences in the length of incubation in

temperate and tropical birds include water loss from the egg, adult size, egg mass, clutch size, predation, immune system development, and yolk content.

Rahn and Ar (1974) concluded that incubation time is inversely proportional to water vapor conductance and water loss in eggs. Therefore, the greater the weight loss due to water evaporation through the shell, the shorter the incubation period for the developing bird. The geometry and abundance of pores in the shell and the water vapor gradient across the shell affect water loss during incubation. In humid climates, such as the tropics, water vapor gradients across the shell would be less than in temperate zones. As a result, water loss via pores in the shell would be less in the tropics than in temperate eggs and therefore, eggs would have a longer incubation period in the tropics. The surface area to volume ratio decreases as the egg size increases, therefore, the evaporative surface area is proportionally less in a larger egg than in a smaller egg. Thus, a larger egg would have a slower rate of water loss in relation to its size compared to a smaller egg (Martin and Arnold 1991).

Incubation period has been found to have a direct relationship to parental body mass in several avian species (Geffen and Yom-Tov 2000). In addition, length of incubation has been found to be proportional to egg weight raised to the 0.22 power for nearly 500 bird species (Rahn and Ar 1974). Therefore, larger birds would produce larger eggs that have longer incubation periods. Styrsky (pers. com.) observed that adult tropical House Wrens are larger and produce larger eggs with longer incubation periods than temperate House Wrens. However, this does not follow Bergmann's Law that sub-species in warmer climates are smaller than sub-species in cooler climates (Smith 1980).

Smaller clutch sizes are also associated with longer developmental periods in the egg (Geffen and Yom-Tov 2000). Assuming a constant total reproductive output, a female that lays a smaller clutch would be able to expend more energy per egg than if she were to lay many eggs. Even when interspecific studies have controlled for body size, clutch sizes in the tropics are smaller than in temperate regions (Yom-Tov 1994, Geffen and Yom-Tov

2000). Hence, an egg that has been supplied with more nutrients should have a longer incubation period; therefore, tropical birds should have longer incubation periods than temperate birds of the same species. Most of the studies have been interspecific comparisons, and it may be incorrect to predict intraspecific effects from these data. Although not addressed in the literature, smaller clutches may have a local environment with a lower relative humidity than larger clutches located in the same region. This is because fewer eggs would collectively lose less water to the environment than would a larger number of eggs. Therefore, eggs in small clutches may have a higher rate of water loss due to the lower local relative humidity.

Predation could also be a factor affecting incubation periods. Skutch (1949) suggested that greater predation rates in the tropics may explain the smaller clutch size in the tropics, which would also explain longer incubation periods. However, Geffen and Yom-Tov (2000) believe that incubation periods would be shorter in the tropics in response to high rates of predation. Shorter incubation and fledging periods may have evolved for species with high nest predation rates so that the nestlings leave the nest earlier. Independent of predation, Ricklefs (1993) hypothesized that longer incubation periods would allow the immune system to develop more fully, which may lower mortality rates and increase survivability.

Another factor that may affect incubation periods is the content of the yolk. High lipid content makes egg yolk the most significant source of energy and raw materials for the developing bird embryo (Patten 1964). Lipid components of the yolk (cholesterol ester, triacylglycerol, fatty acid, free cholesterol, and phospholipid) are necessary for the development of the embryo. Lipoproteins, which transport lipids, vitamins, ions, and carbohydrates from the yolk to the developing embryo are derived from cholesterol esters (Royle et al. 1999). Although, fatty acids are oxidized to provide energy for development., triacylglycerol (glycerol with three fatty acid side-chains), the greatest lipid constituent of the yolk, provides most of the energy. Phospholipids are used as structural components of

cell membranes. If there is a large lipid reservoir in the yolk, the embryo will have sufficient energy and lipid building blocks to develop within the egg for a longer period of time than if there were less lipid in the yolk. Decreased amounts of yolk lipid would not allow for a long embryonic developmental period; the bird must hatch and begin receiving food from its parents (altricial) or finding food on its own (precocial). Differences in percentages of lipid classes, as well as total lipid content, may influence differences in incuabtion periods in tropical and temperature species.

Breeding Biology of House Wrens

In House Wrens, only the females have a brood patch and incubate the eggs (Johnson 1998). In temperate populations some egg-warming occurs during the first night after the first egg is laid. Nocturnal incubation by females increase in duration throughout egg-laying. Females lay one egg every successive day in the morning hours (Johnson 1998), and it is not until the night of the laying of the last egg that "full" incubation throughout the night occurs in temperate House Wrens. Kendeigh (1963) indicates that although the female may be on the nest, she is not always incubating. The lack of incubation may be achieved by pulling some of her feathers over her brood patch, which may prevent overheating of the eggs. Diurnal incubation is sometimes erratic during the first few days of egg-laying and regularly increases beginning the third to fifth day. Some females have been known to increase diurnal incubation regularly throughout egg-laying (Johnson 1998). After the last egg is laid, the female spends roughly 50-55% of the daylight hours and nearly the entire night on the nest. The duration of daylight incubation may increase slightly during the first days of incubation, but does not fluctuate much beyond that slight increase. Nearly half of the broods hatch synchronously, generally in the order they are laid (Johnson 1998). Eggs hatch during the night or early morning hours (Harper, pers. com.), although Johnson (1998) reported hatching only during daylight hours. The female is thought to control hatching patterns by the time she begins

incubating. If egg incubation begins before the last egg is laid, the first eggs incubated may hatch several days before the last egg laid. Unfortunately, few data have been collected on the incubation of tropical House Wrens. John Styrsky, a doctoral student at Auburn University, has observed that natural incubation periods in tropical House Wrens in Gamboa, Panama are longer than temperate House Wrens in Illinois (Styrsky, pers. com.). Styrsky artificially incubated one tropical House Wren egg and found the incubation period to be 14-15 days.

Number and size of clutches are different in temperate and tropical House Wrens. Temperate House Wrens produce two clutches per year with clutch sizes of 6-8 eggs in the early (first) season and 4-6 eggs in the late (second) season (Johnson 1998). In comparison, tropical House Wrens have 3 clutches per year, each with a clutch size of 3-4 eggs (Young 1996). This is in accordance with Jorden's Rule stating that races of birds in warmer climates lay fewer eggs per clutch than those races in the cooler climates (Smith 1980).

Anatomy of the Avian Egg and Yolk

The avian egg is a complex structure composed of a calcareous shell, albumen, yolk, and if fertilized, a developing embryo. The shell is composed of three layers: the innermost mammillary layer, the middle spongy layer, and the outer cuticle (Lillie 1940). The mammillary layer is composed of loosely fused calcareous particles. The spongy layer consists of thick calcareous strands, and the cuticle of the shell is calcareous and very porous (Lillie 1940) (Fig. 1A and 1B). The pores from the cuticle connect with openings in the lower two layers, and therefore, gas exchange between the inside and outside of the egg is possible. There are two organic shell membrane layers underneath the shell that are in contact with one another everywhere except at the blunt end of the egg. Here they are separate and an air sac forms between them at the time of laying (Carlson 1996, Lillie 1940). Albumen is the major source of water and proteins in the egg (Lillie 1940).

Rotation in the oviduct during formation of the egg twists some of the albumen on either end of the yolk, forming thick, spiral strands are called chalazae (Fig. 2), which are important in suspending the yolk in the albumen (Carlson 1996). The function of many of the albumen proteins is protection from infection (Schoenwolf 1995). Later in development, the embryo gains nutrition by ingesting the albumen proteins.

Avian eggs are macrolecithal with the yolk initially occupying a large portion of the entire egg. The yolk is a very important nutrient source for the embryo during development (Carey et al. 1980). Yolk is composed of yellow and white yolk (named for their color), which are laid down in alternating rings around a core of white yolk called the latebra (Spohn and Riddle 1916, Carlson 1996) (Fig.2). The two types of yolk are laid down at different times; white is laid down at night, while the yellow yolk is laid down during the day (Carlson 1996). In addition to differences in the time of formation, yellow and white yolk differ in their biochemical make-up, specifically in the percentage of different lipids that they contain (Spohn and Riddle 1916, Carlson 1996). Yellow yolk contains more carotenoid-rich lipids, which gives the daytime yolk its characteristic yellow color (Carlson 1996). The white layer is relatively low in lipid content, but higher in protein content than the yellow layer. Therefore, the chemical composition of the two yolks accounts for the stratified appearance of the whole yolk.

There are five classes of lipids found in yolk: triacylglycerol, phospholipid, free cholesterol, cholesterol esters, and fatty acids. Triacylglycerides are the major lipid component in yolk (Royle et al. 1999), followed by phospholipids. Minor fractions, in descending quantities, include free cholesterol, cholesterol ester, and free fatty acid. All five lipid fractions are important in the development of the embryo. A decrease in a lipid component may decrease the chances of survival for the embryo. An increase in these components provides the necessary nutrients for a longer period of development inside the egg. There is no need for the embryo to hatch earlier for the purpose of eating.

Purpose of this Study

The purpose of this study was to compare incubation periods and yolk lipid content of eggs in two populations of House Wrens (*Troglodytes aedon*); one from the tropics (Panama) and one from a temperate zone (Illinois). The first hypothesis I tested was that differences in egg incubation period between tropical and temperate House Wrens is due to differences in total yolk content. If incubation period is influenced by yolk content, then there are two opposing explanations to explain either a direct or inverse relationship between the two variables. If incubation period is related to total yolk content, the larger Panamanian House Wren eggs should have a smaller yolk to whole egg ratio. Therefore, assuming that the size of the air sacs in both types of eggs are proportionally the same, the larger Panamanian eggs will have a greater percentage of albumen than the Illinois House Wren eggs. The larger water source from the increased percentage of albumen would allow a longer incubation period because it will decrease the effects of water loss via shell pores (Warham 1983). Excess yolk is taken up by the embryo at hatching (Lillie 1940), and therefore, yolk may not be a limiting factor of incubation, because it is in excess during embryonic development.

The second prediction is that the percentage of yolk in the eggs of the tropical House Wrens should be larger than in the temperate wrens and, therefore, the tropical wren eggs will have a greater percentage of lipids. This assumes that the quality (i.e. classes of lipids per volume of yolk) of yolk is the same in both the tropical and temperate eggs. A larger proportion of lipids in tropical House Wren eggs would allow more nutrients for the developing embryo to mature longer before hatching.

If yolk to whole egg mass ratios in tropical and temperate eggs are the same, the third hypothesis is that yellow to white yolk ratios may differ in between tropical and temperate eggs. If lipid nutrition is a determining factor of incubation, then a greater percentage of yellow yolk should be found in eggs with longer incubation periods, because yellow yolk has a greater percentage of lipids than does white yolk.

Methods

Field Work

All temperate zone field work was done in an established study area at the Merwin Nature Preserve south of the Mackinaw River in Central Illinois (Fig.3, 40°31'N, 85°59'W, elevation 220m) during the late breeding season (June-August) 2000. Twice-weekly during morning hours, all nest boxes (n = 244) were checked for nest-building. Once a nest box was found to have a lined or nearly-lined nest, it was monitored every morning thereafter for the first laid egg. Using a thin-tip permanent marker, the first egg was labeled directly on the shell and returned to the nest. Labels denoted the nest box number, nest number for that box that year, and the egg number (e.g. the second egg laid in the second nest at box YA4 is labeled YA4-2#2). The second and third eggs of the clutch were collected so differences between egg number laid could be analyzed. To prevent any potential change in parental behavior, plaster eggs of the same size and similar color were used to replace each wren egg removed. Within a few hours of collection, the eggs were transported back to the lab in egg cartons lined with cotton.

Incubation

Immediately upon arrival at the lab, eggs marked for the incubation experiment were weighed to the nearest 0.001g on an Acculab PocketPro 2060D portable electronic balance and placed on the top shelf of a Grumbach incubator (Fig. 4) with two shelves, one over the other. The date, time, temperature, humidity, and egg-rotation speed were noted at the beginning of incubation. Temperature and humidity were maintained at 37.8-38.0°C and 85-90%, respectively. These settings have previously been successful in incubating House Wrens to hatching (Criley pers. com.) The eggs were automatically rotated approximately 3/4 of their circumference over a 10 minute period every hour to prevent adhesion of the embryo to the shell membrane. To monitor water loss, everyday the eggs

were weighed to the nearest 0.001g at 0500 and 1700. For the first 10 days of incubation, the eggs were rotated automatically on the top shelf. Aluminum foil was placed on the upper half of the incubator window to prevent illumination of these eggs. On day 10 of incubation, the eggs were placed, individually, into small petri dishes lined with cotton on a stationary bottom shelf to record the hatching of each chick on video. A Panasonic video camera programmed with an interval timer that recorded in 1 second "on"/ 50 second "off" intervals was used to monitor hatching. Florescent lights (cool-white) were placed outside the incubator window to provide sufficient light for videotaping the eggs. The hatchlings were weighed to the nearest 0.001g (0-5h) after hatching and then sacrificed and preserved for an ongoing embryonic stage series project.

Lipid Extraction

Yolks from commercially purchased chicken eggs were used for all lipid analysis. Royle (1999) demonstrated that the Folch method (Folch 1956) as modified by Ways and Hanahan (1964) was an exhaustive extraction procedure for all lipids from yolk. The extraction was further modified in my procedure to obtain higher percent recovery. The eggs were frozen and during thawing the shell and albumen were removed in order to isolate the yolk. A half gram of yolk was homogenized in 60ml chloroform/methanol (2:1 v/v) using a hand-held blender. After gravity filtration through Whatman 4 filter paper, the solid residue was scraped from the filter paper and was rehomogenized in 60ml chloroform/methanol (2:1 v/v). The homogenate was filtered, the two filtrates were combined, and they were washed with 30ml aq. 0.88% KCl. The mixture was shaken, allowed to settle, and the upper aqueous layer was removed by pipetting and discarded. The organic layer was dried over anhydrous $NaSO_4$ and dried by rotary evaporation. The extracted lipid was quantified gravimetrically.

Lipid Class Separation - Preparative TLC with Silica Gel 60

Preparative TLC with a hexane/diethyl ether/formic acid (80:20:1 v/v) eluent confirmed the presence of 5 classes of lipids (cholesterol ester, triacylglycerol, fatty acid, free cholesterol, and phospholipid) in the extracted yolk lipids. Standards obtained from Sigma (cholesteryl oleate, palmitic acid, cholesterol, triolein, and phosphatidylcholine) were used to identify the different lipid classes. Glass preparative TLC plates (20 x 20 cm; EM reagents) precoated with Silica Gel 60 (layer thickness 0.25mm) were spotted using a glass pipette with the standards and 0.10-0.20g of extracted lipids dissolved in a minimal amount of chloroform. The silica gel did not contain a fluorescence indicator. Before developing the TLC plate, the chamber was allowed to sit 30 min. to give time for the hexane/diethyl ether/formic acid (80:20:1 v/v) eluent to saturate the air in the chamber. This was aided by 2 large filter papers in the chamber that acted as solvent wicks. The preparative TLC plate was placed in the chamber to develop it, and the solvent front was allowed to run to nearly the top of the plate. The plate was removed and allowed to dry for 5 min. A minimal amount of 0.1% (w/v in methanol) 2,7-dichlorofluorescein was sprayed onto the plate and allowed to dry for 10 min. With the aid of both short (254nm) and long (365nm) wave ultraviolet light, the bands of lipids were identified and outlined in pencil.

Lipid Class Separation – Column Chromatography

Column chromatography was also examined as a method to separate the classes of lipids. Originally, silica gel for flash chromatography was used in the column. Next, silica gel for wet-column chromatography was used. A slurry of silica gel in hexane/diethyl ether/formic acid (80:20:1 v/v) was made. For every gram of sample analyzed, 20g of silica gel was added to the column. The sample was loaded by a pipette and the eluent, hexane/diethyl ether/formic acid (80:20:1 v/v), was added. Samples were collected in 10ml test tubes. Samples from the test tubes were spotted next to the standards on aluminum backed TLC plates and developed in hexane/diethyl ether/formic acid (80:20:1 v/v).

Lipid Class Separation - Preparative TLC with Silica Gel G

Glass preparative TLC plates (20 x 20 cm) precoated with Silica Gel G (layer thickness of 1000μm) were spotted using a 1cc syringe fitted with a 26 gauge needle with the standards and 0.10-0.20g of extracted lipids dissolved in a minimal amount of chloroform. The silica gel did not contain a fluorescence indicator. Before developing the TLC plate, the chamber was allowed to sit 30 min. to give time for the hexane/diethyl ether/formic acid (80:20:1 v/v) eluent to saturate the air in the chamber. This was aided by 2 large filter papers in the chamber that acted as wicks. The preparative TLC plate was placed in the chamber to develop it. After the solvent front was allowed to run to nearly the top of the plate, the plate was removed and allowed to dry for 15 min. The plate was returned to the chamber, and the new solvent front was allowed to run to the point where the first solvent front was stopped. The plate was removed and allowed to dry for 10 min. A minimal amount of 0.1% (w/v in methanol) 2,7-dichlorofluorescein was sprayed onto the plate and allowed to dry for 15 min. With the aid of both short (254nm) and long (365nm) wave ultraviolet light, the bands of lipids were identified and outlined in pencil.

Eluting the Lipids from the Silica Gel

The bands of silica that contained the lipid fractions were scraped off into separate round bottom flasks and labeled by fraction. The lipids were eluted from the silica by vortex mixing with either 3 x 5ml of chloroform/methanol/distilled water (5:5:1 v/v; phospholipid) or 3 x 5ml diethyl ether (triacylglycerol, cholesterol ester, free cholesterol, free fatty acid). The fractions were weighed gravimetrically to the nearest 0.01g.

Staining of Yolk Layers with Potassium Dichromate

To visualize the layers of yellow and white yolk, a 6% $K_2Cr_2O_7$ stain was used to stain yolks previously halved and fixed in 2 changes of 4% formalin for 20 minutes each. The yolk was stained with $K_2Cr_2O_7$ until the rings were distinguishable from one another. The stained yolk half was used as a map to collect samples of both yellow and white yolk from the non-stained yolk half.

Statistical Analysis

The mean incubation period observed in this study was compared to the published value of natural incubation periods of House Wrens in Illinois during the second season using a t-test (Heath 2000). Linear regression analysis (Heath 2000) using the computer program SPSS (Norusis/SPSS) was used to determine the significance of weight loss vs. time of incubation and the rate of weight loss. The mean mass of hatchlings in this study and the published value of mass of recent hatchlings in nature were compared using a t-test (Heath 2000).

Results

The hatching success of collected House Wren eggs in the Summer 2000 was 90.0% (27/30). Of the three eggs that did not hatch, one contained no embryonic development, while the other two went through significant development (>8 days) before the embryos died. The mean period from the beginning of incubation to hatching was 13.6 days ± 0.2SD (range 0.8d, n=27), which is significantly greater than the literature value of natural incubation period for second season clutches in Illinois (Johnson 1998), 12.2 days ± 0.9SD (Fig. 5, $t = 11.9$, $df = 93$, $p<0.001$).

The rate of weight loss in House Wren eggs was different at the beginning and end of incubation. Linear regression was used to analyze the rate of weight loss of the eggs during the period of incubation. The rate of weight loss increased significantly at the end of the incubation compared to the beginning (Fig. 6, $F = 15.45$, $df = 26$, $P < 0.001$). There was no significant difference in the variation in rate of weight loss among eggs (Fig. 7, $F = 0.094$, $df = 26$, $p = 0.762$).

Mean initial mass of the incubated eggs was 1.432 g ± 0.14SD (n=27). The mean net mass change of the lab incubated eggs was -0.162 g ± 0.06SD (n=27). The mean final mass of the eggs (i.e. last weight of egg measured before hatching) was 1.270 g ± 0.14SD (n=27), which was significantly less than the mean initial mass, 1.432 g ± 0.14SD (Fig. 8, $t = 5.983$, $df = 52$, $p < 0.001$). The mean weight of the incubated hatchlings (Fig. 9) was 1.060 g ± 0.13SD (n=27), which was not significantly different than natural incubation results, 1.07 g ± 0.15SD, reported by Johnson (1998) (Fig. 10, $t = 0.166$, $df = 31$, $p > 0.05$).

The procedure for lipid extraction was nearly exhaustive. A third extraction of the solid residue with 60ml chloroform/methanol (2:1 v/v) did not recover additional lipids (detection limit 0.01g). There was low variation in the total lipids extracted in replicate proportions (variation < 6.0%) of the same yolk. When 0.50g of peanut oil (Sigma) and

0.50 g of palmitic acid were extracted using the above procedure, 93.4% and 96.0% were recovered, respectively.

Rings of yellow and white yolk were often distinguishable from one another when stained with the $K_2Cr_2O_7$. Unfortunately, the yolk often fell apart during the staining with $K_2Cr_2O_7$.

Discussion

Any of several factors might explain the difference in the natural and laboratory incubation results. In the field, the initiation of incubation and the time of hatching is difficult to accurately determine. The female begins incubation during the early night of the laying of the last egg and the eggs start to hatch either in early morning or late at night (Johnson 1998). Another factor that may explain differences in incubation period results are the habits of the female House Wrens. In the lab, the eggs are held at a constant temperature and humidity, but in the wild, female House Wrens occasionally leave the nest to feed (Johnson 1998). At this time, the air temperature and relative humidity around the eggs begins to drop Thus, in the wild, there are fluctuations in the air temperature and relative humidity, whereas in the lab these variables are held nearly constant. It is expected that without the drops in temperature and humidity in lab that may slow incubation, the eggs would hatch earlier and the incubation period would be less than in nature. However, laboratory incubation times were longer. It is possible that the air temperature in the incubator, 37.8-38.0^{o}C was lower than the air temperature of the nests in nature. However, Kendeigh (1963) used thermocouples to record the temperature of the inside of the egg throughout incubation, and he reported that the inside of the egg was held at a near constant 35^{o}C, which is lower than the air temperature that was used in our incubation study. Although this was not investigated, developing systems in the embryo may depend on the fluctuations in temperature that occur when the female incubates then leaves the nest for brief periods of time, and a relatively constant temperature in the incubator may have slowed the developing systems. The 90.0% hatching success is evidence that the temperature, humidity and rotation rate was adequate for incubating House Wrens in the lab.

The increased rate of weight loss at the end of incubation may be due to the beginning of the hatching process when the embryo begins to use its bill to chip away the

inside of the shell. This would increase the evaporative surface, and therefore, the rate of evaporation from the egg would increase.

Although there was a significant difference between the incubation periods found in this study and by Johnson (1998), there was no significant difference in the mean weights of the hatchlings. This demonstrates that there was no significant increase in weight of the embryo during the longer incubation period in the laboratory. So it is likely that development took longer in the laboratory embryos, and thus the embryos incubated in the laboratory hatched later.

There were problems differentiating the phospholipid and free cholesterol, as well as the triacylglycerol and fatty acid components, when preparative TLC with Silica Gel 60 was used to separate the lipid classes. Spotting with a 1cc syringe fitted with a 26 gauge needle worked better, because it resulted in a decrease in the width of the spotting layer. The spotting was more controlled, and when the plate was developed, there was better separation of the lipid classes. However, the classes of lipids still did not sufficiently separate to allow for accurate removal. A different, and more polar, solvent [hexane/diethyl ether/formic acid (70:30:1 v/v)] was used in hopes of sufficiently separating the lipid classes. Increasing the polarity of the TLC solvent separated the classes of lipids better than the less polar solvent. Increasing the polarity of the solvent did separate the triacylglycerol and fatty acid classes, but the cholesterol and phospholipid classes could not be separated.

The advantages of column chromatography over preparative TLC are that the lipids do not need to be eluted from the silica after column chromatography and also, larger samples may be loaded onto a column (Zubrick 1984). However, column chromatography may not be as accurate for smaller samples. Silica gel for flash chromatography was too reactive with the lipids and the less polar lipids (phospholipid and cholesterol) did not pass through the column in an efficient time. When silica gel for gravity column chromatography was used, the phospholipid and the cholesterol classes still did not pass

through the column. Column chromatography of yolk samples was discontinued. Flash chromatography could be studied for as a more efficient method in separating the lipids into the five classes. Switching to a more polar eluent after the cholesterol ester, triacylglycerol, and fatty acid fraction are collected may move the phospholipid and cholesterol fractions through the column faster. Once the cholesterol fraction has been collected, an even more polar eluent may be used to pass the least polar fraction, the phospholipid, through the column.

The properties of the binding agents in Silica Gel 60 did not allow the triacylglycerol and fatty acid classes, as well as cholesterol and phospholipid classes to separate from one another. Switching to Silica Gel G, which has a different binding agent, separated all five classes from one another. When Silica Gel G on the preparative TLC plates was used and the plate was developed twice, the 5 classes of lipids were separated without overlap so that scraping of the silica from the plate allowed the classes to be scraped off separately.

When trying to stain the yellow and white yolk with $K_2Cr_2O_7$, the yolk often fell apart. Fixing the yolk longer in the 4% formalin or in a stronger concentration of formalin may be beneficial. The procedure for staining the two layers needs to be adjusted and improved.

Future Studies

In the summer of 2001, more eggs will be collected from populations of House Wrens breeding in Illinois and Panama. In Illinois, second and third eggs of clutches will be collected from both brood seasons. The protocols for incubation and yolk lipid analysis reported in this paper will be repeated. These data will be used for a comparison between the second seasons of summer 2000 and summer 2001, as well as between early and late season eggs. The eggs collected from Panama in summer 2001 will be used to make comparisons between tropical and temperate House Wren yolk lipids.

Although there was a 90.0% success in hatching, the laboratory incubated House Wren eggs hatched about a day later than those in the field did. This may be due to different criteria for calculating incubation times in the field. More accurate determinations of incubation periods in the field should be determined.

A study designed to look at the differences in yolk lipids between the first 2 eggs laid in a clutch to the last 2 laid eggs will also be performed in summer 2001. There is now evidence that the first two eggs laid are usually females and the last two eggs laid are males (Thompson pers.com.). In Illinois, each successively laid egg is larger than the previous (Johnson 1998). The female parent may allot each successively laid egg more nutrients for development. Variations in nutrients in eggs may affect incubation periods.

Female parent size and yolk content comparisons will be made in summer 2001. It is possible that the size of the female may affect yolk content of eggs. The female condition will be determined using a formula that takes into consideration female mass, tarsus length, flattened wing-chord length, and tail length (Eckerle pers. com.). The volume of the airspace in the eggs will be measured, which will allow more accurate conclusions in studies comparing components of the egg to the total egg size. The sizes of the pores in the House Wren egg shells will also be measured using scanning electron microscopy. Since pore size is one of the factors determining rate of evaporation from eggs, a comparison of

pore size in tropical and temperate egg shells should help determine which House Wren eggs have a greater rate of water loss. Environmental data from both regions of egg collection will be taken to determine effects of humidity, altitude, and temperature on incubation periods.

Comparisons of lipids between the yellow and white yolk will be made. An efficient method for analysis of the yolk layers is needed. To determine the relative amounts of yellow and white yolk the potassium dichromate procedure will be used. Digital photographs of the stained yolk on a grid background will be taken and the relative amounts of yellow and white yolk will then be calculated. Energy content studies using a bomb calorimeter should be performed to compare both total yolk and also yellow and white layers of yolk in tropical and temperate House Wrens.

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Figure 1A

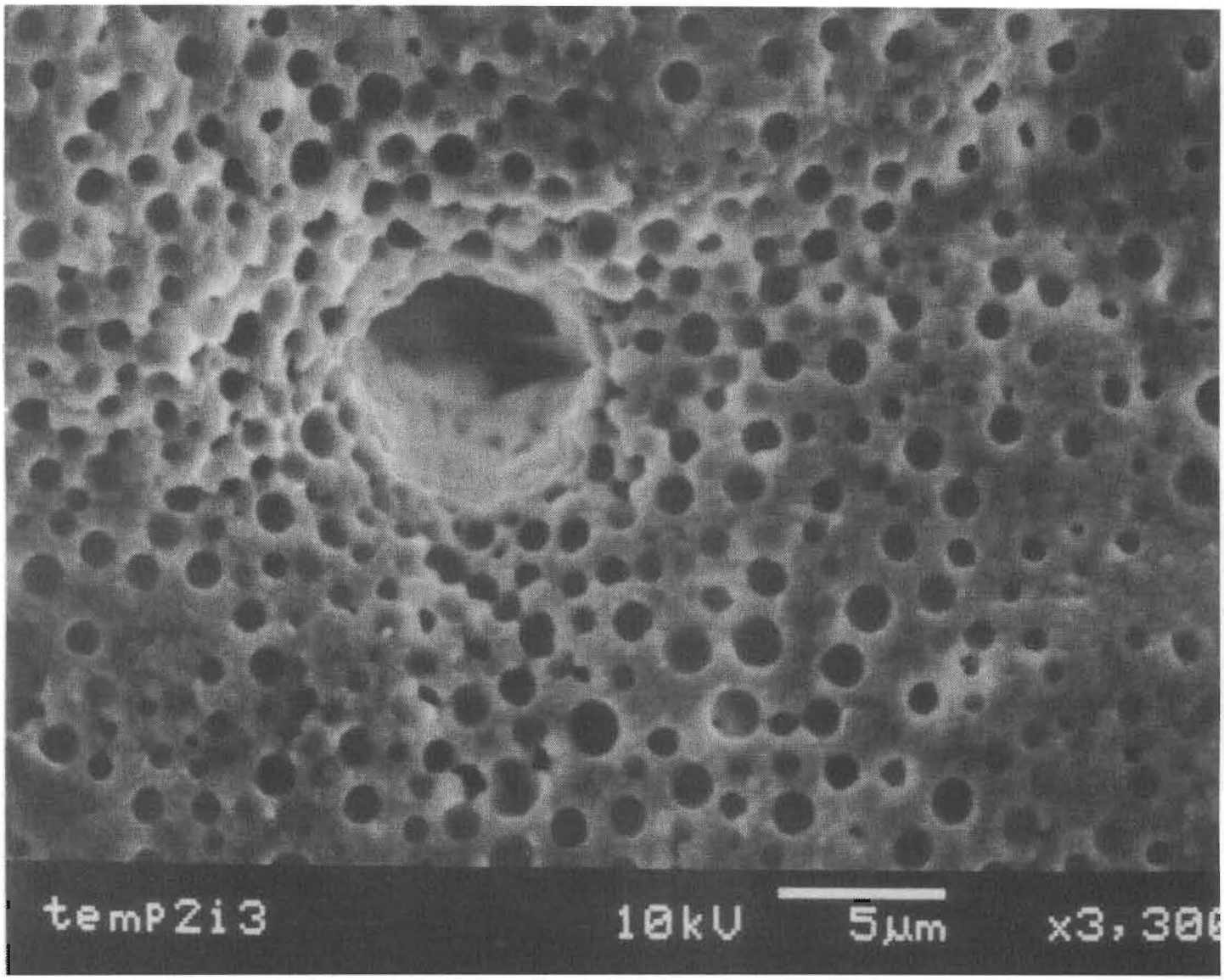

Figure 1A: SEM photograph of the inside layer of the egg shell showing the pores. The pore is the large opening near the center of the photograph. The opening is about 5.725µm wide. The pores extend all the way through the shell. The smaller openings do not appear to extend through the entire shell and are not considered pores. Pores allow passive diffusion of gases to be exchanged between the microenvironment surrounding the eggs and the inside of the egg itself.

Figure 1B

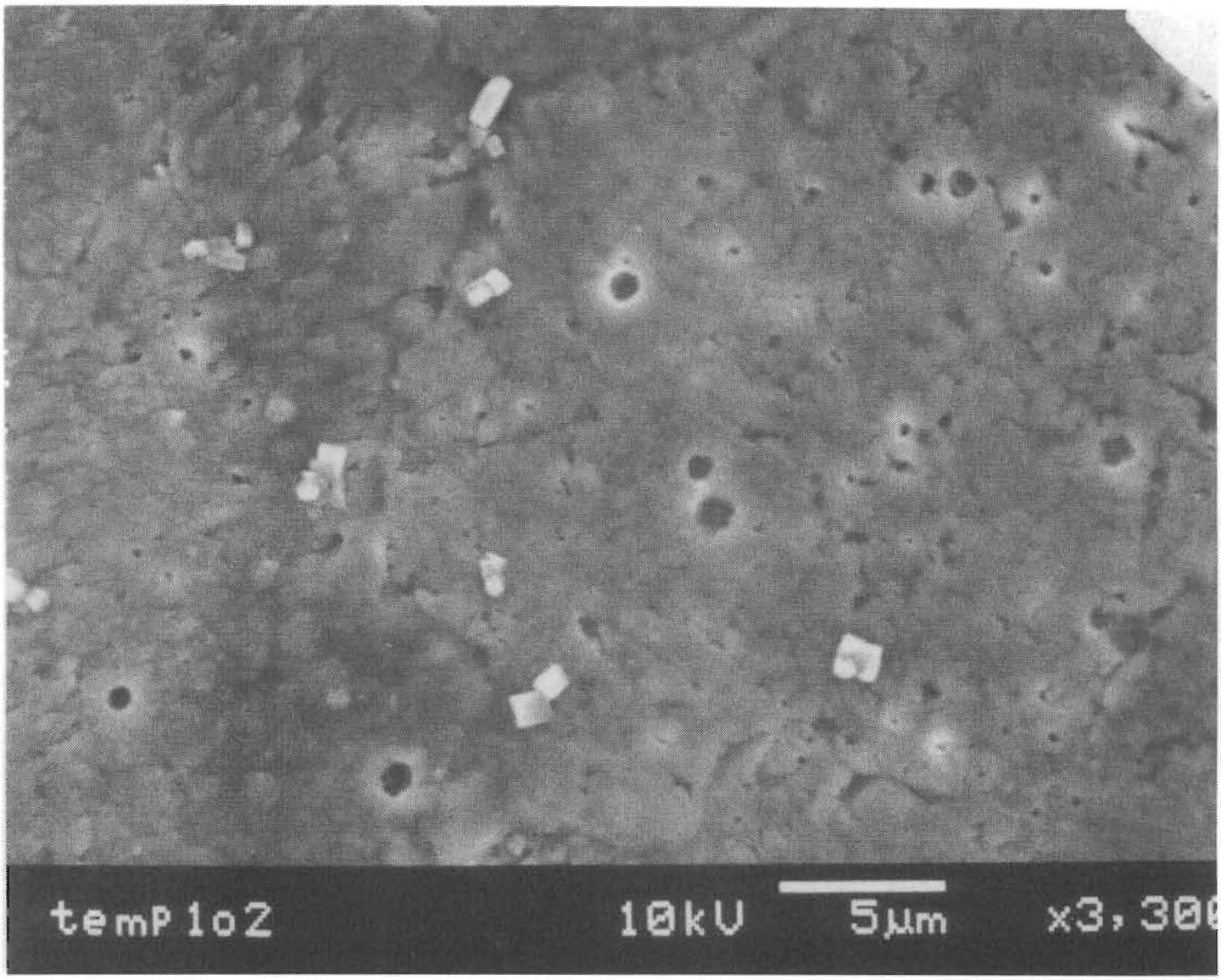

Figure 1A: SEM photograph of the outside layer of the egg shell showing the pores. The pores extend all the way through the shell. Pores are conical; on the inside openings are large, and they decrease in circumference as they extend outward. Here on the outside shell, the pores are less than 1 µm wide.

Figure 2

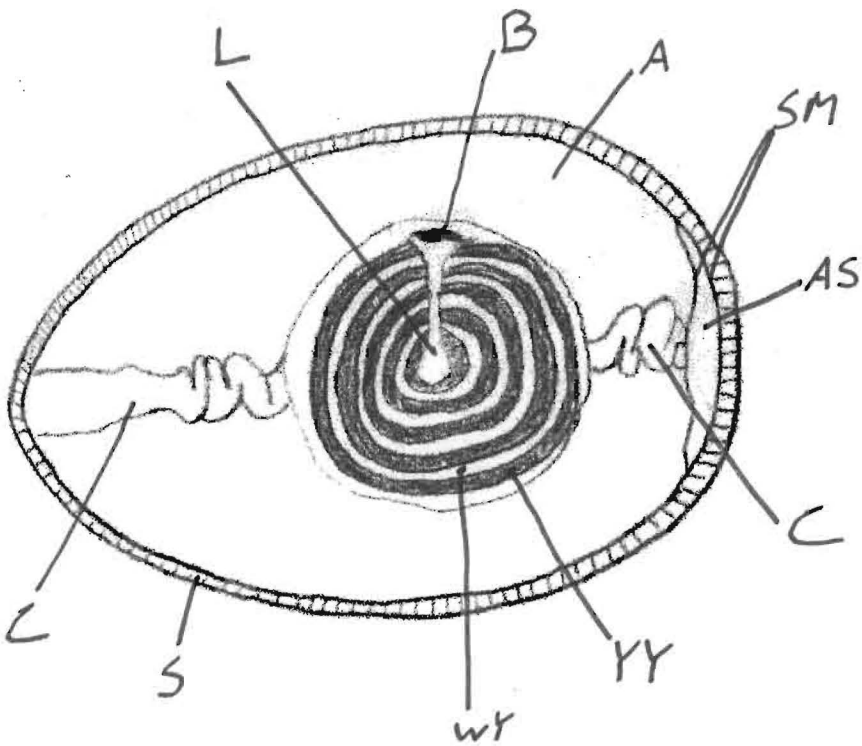

Figure 2: A generalized view of a mid-saggital section through a whole egg immediately after laying. Major components are labeled: A=albumen, AS=airspace, B=blastoderm, C=chalazae, L=latebra, S=shell, SM=shell membranes, WY=white yolk, YY=yellow yolk.

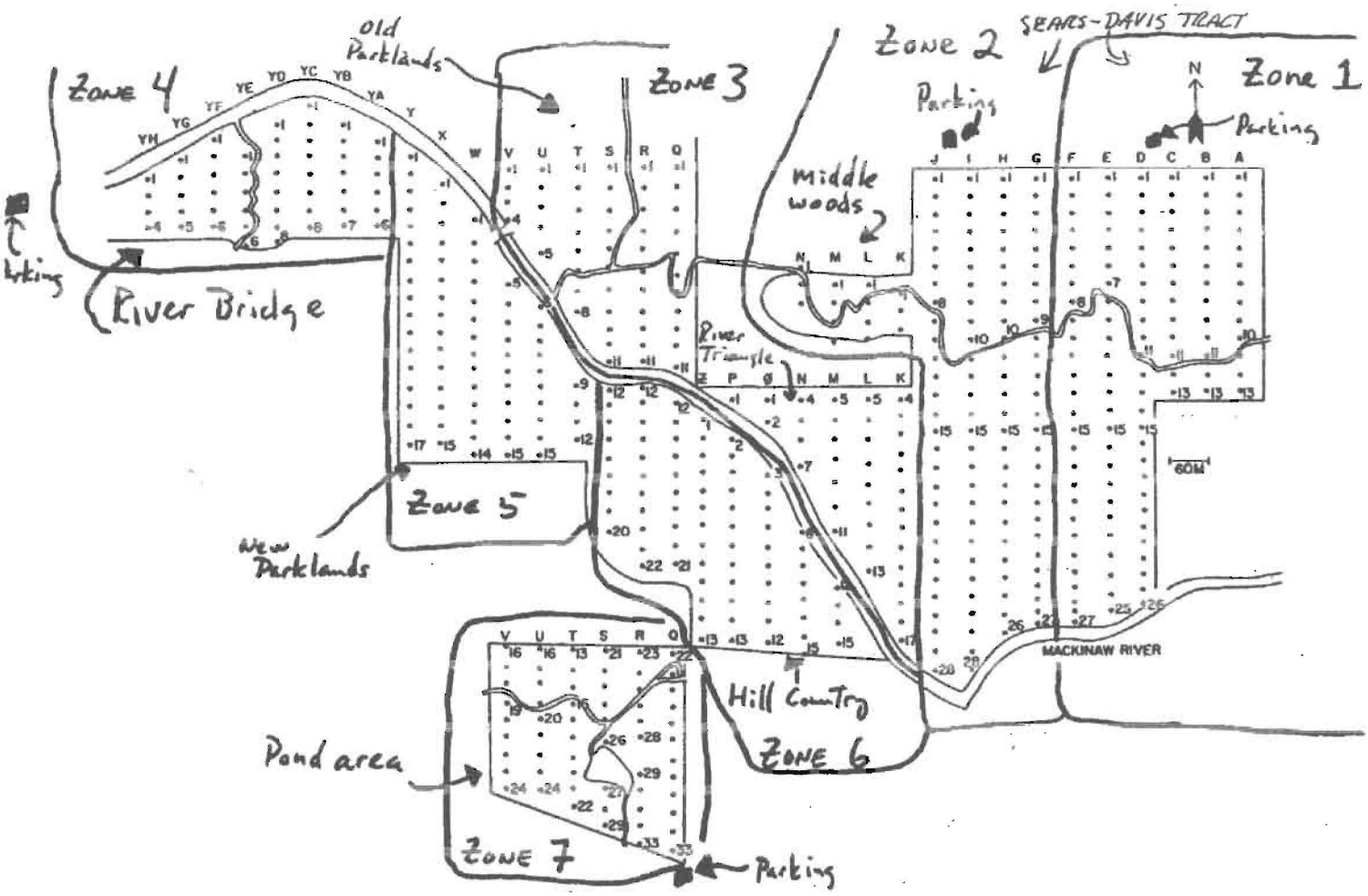

Figure 3

Figure 3: Map of nest boxes present at the Merwin Nature Preserve in Central Illinois (40°31'N, 85°59'W, elevation 220m). Eggs collected for this study were from zones 4, 5, 6, and 7. Nest boxes are located at each dot.

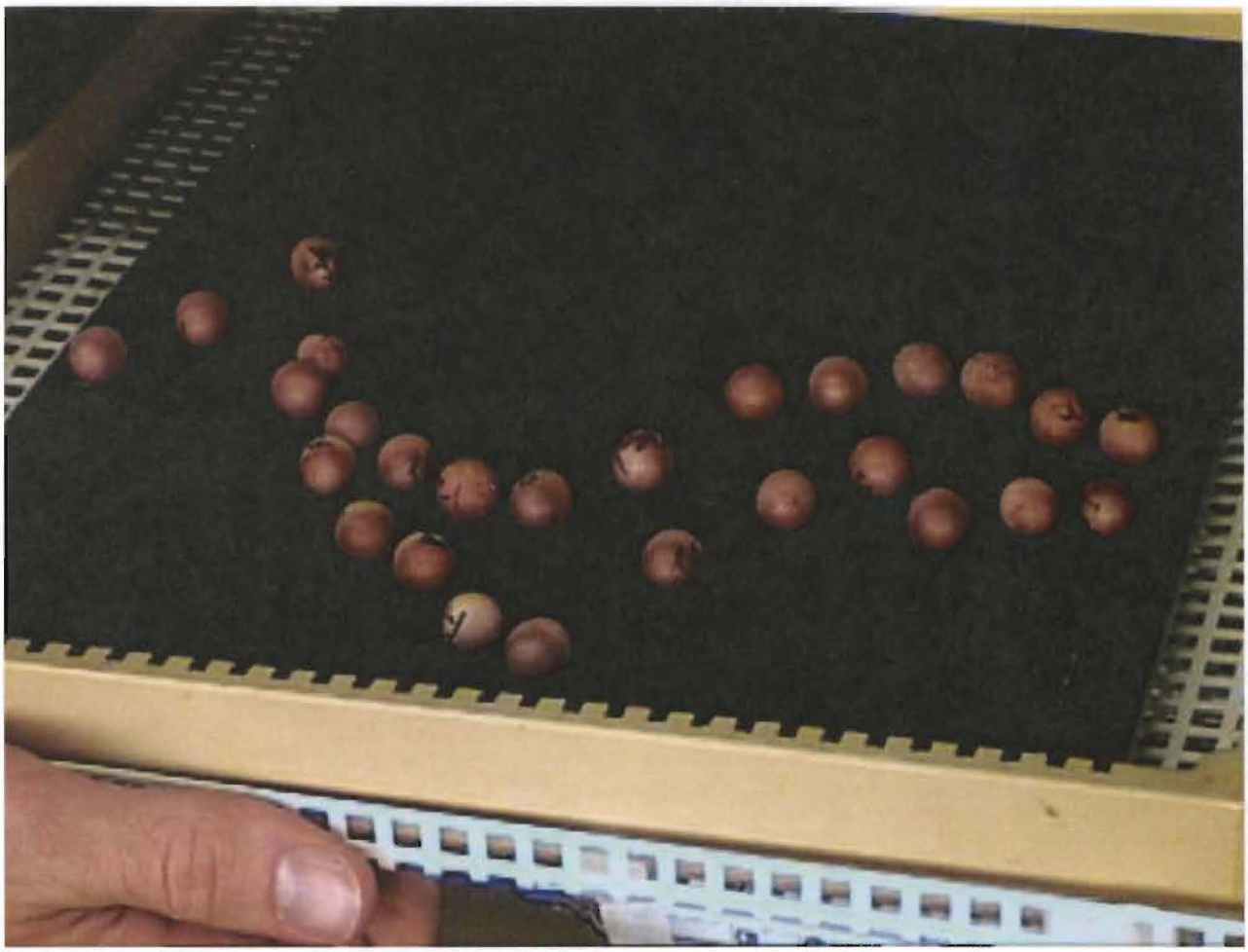

Figure 4

Figure 4: House Wren eggs in Grumbach incubator. Temperature and humidity were maintained at 37.8-38.0°C and 85-90%, respectively. For the first ten days, they were rotated automatically 3/4 of their circumference over a ten minute period every hour.

Figure 5
Laboratory vs. Natural Incubation Periods

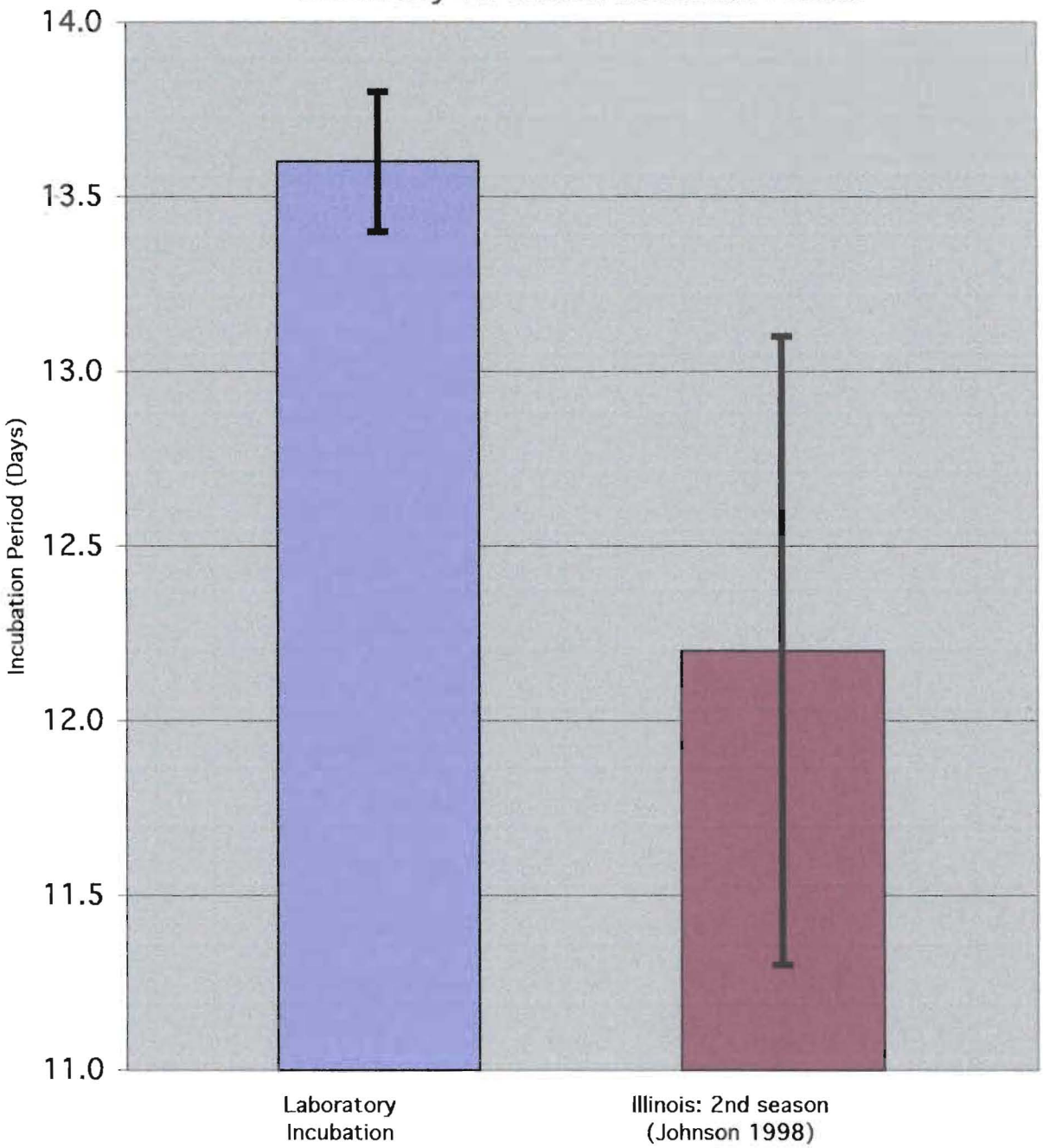

Figure 5: Bar graph showing the significant difference ($t = 11.9$, $df = 93$, $p < 0.001$) of the incubation periods between the laboratory incubated eggs and the naturally incubated eggs reported by Johnson (1998).

Figure 6
Weight Loss vs. Time of Incubation

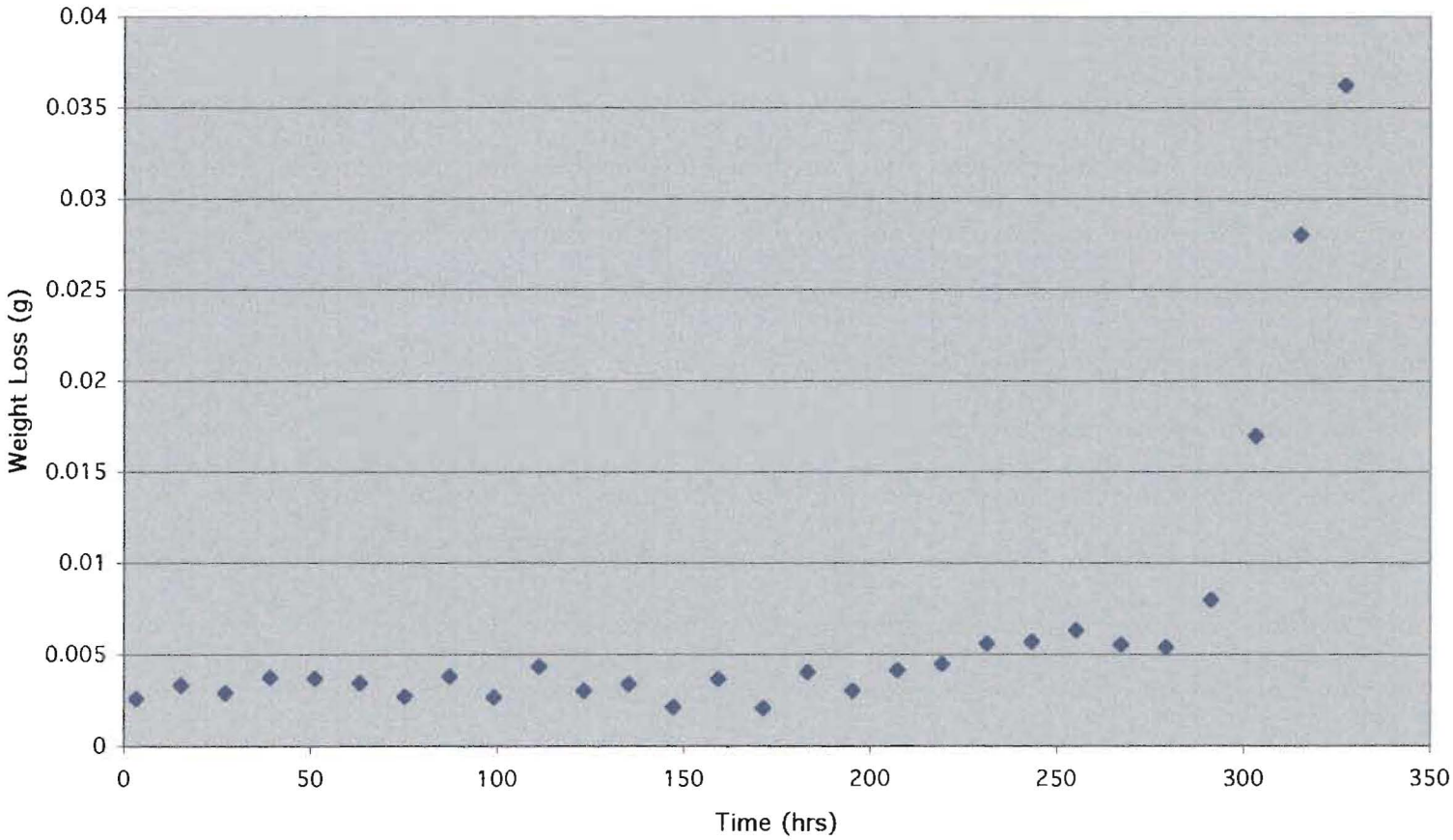

Figure 6: Scatter plot of mean weight loss of incubated eggs versus hours of incubation. Weight loss is due to the evaporation of water from the albumen via the pores in the shell. The loss of water is fairly constant during incubation until the period right before hatching. Here, the weight loss increases significantly ($F = 15.45$, $df = 27$, $p < 0.001$).

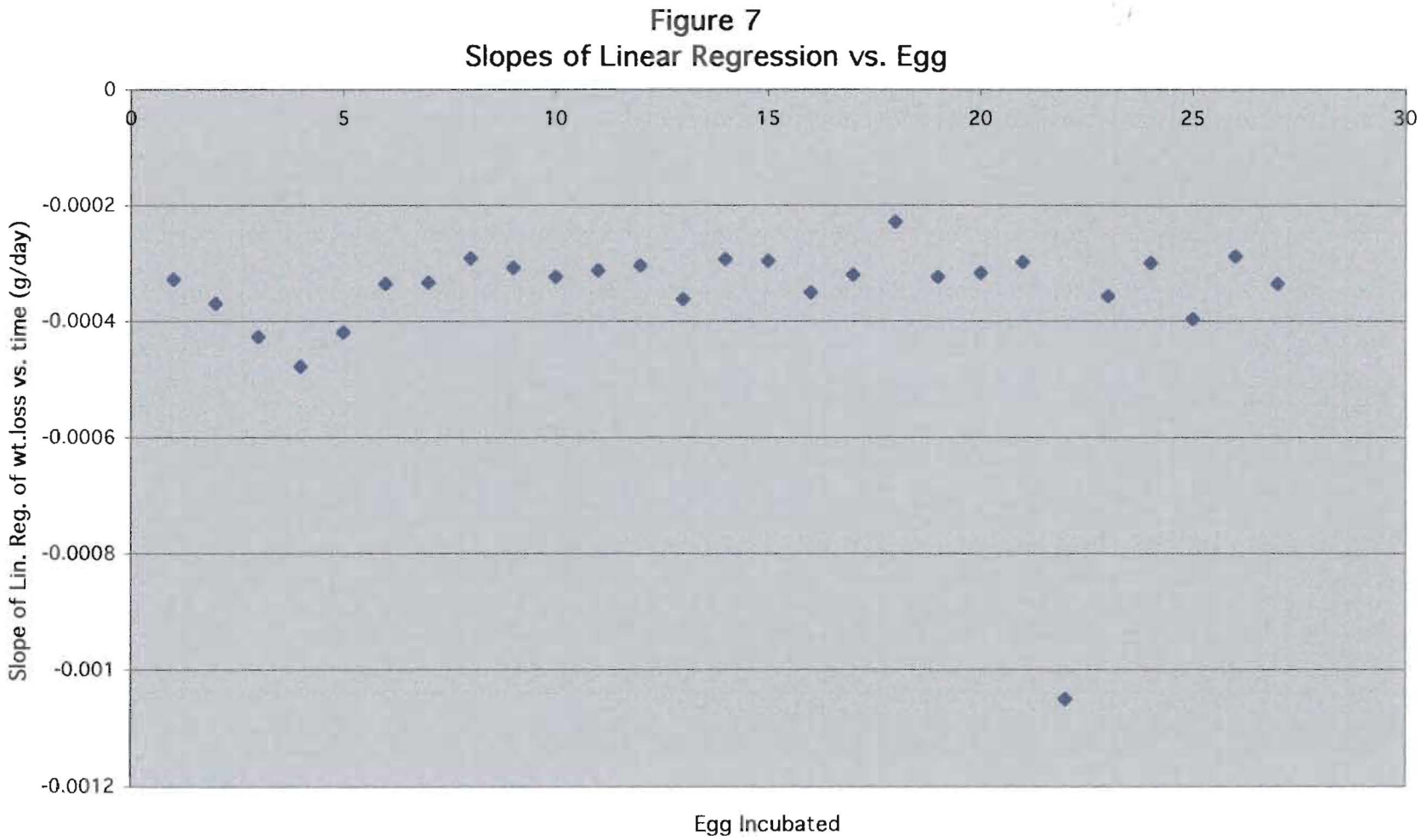

Figure 7: Scatter plot of the slopes of lines of best fit for linear regression of individual egg weight loss over time. There was no significant difference in the rate of weight loss between the eggs ($F = 0.094$, $df = 26$, $p = 0.762$)

Figure 8
Difference in Egg Mass

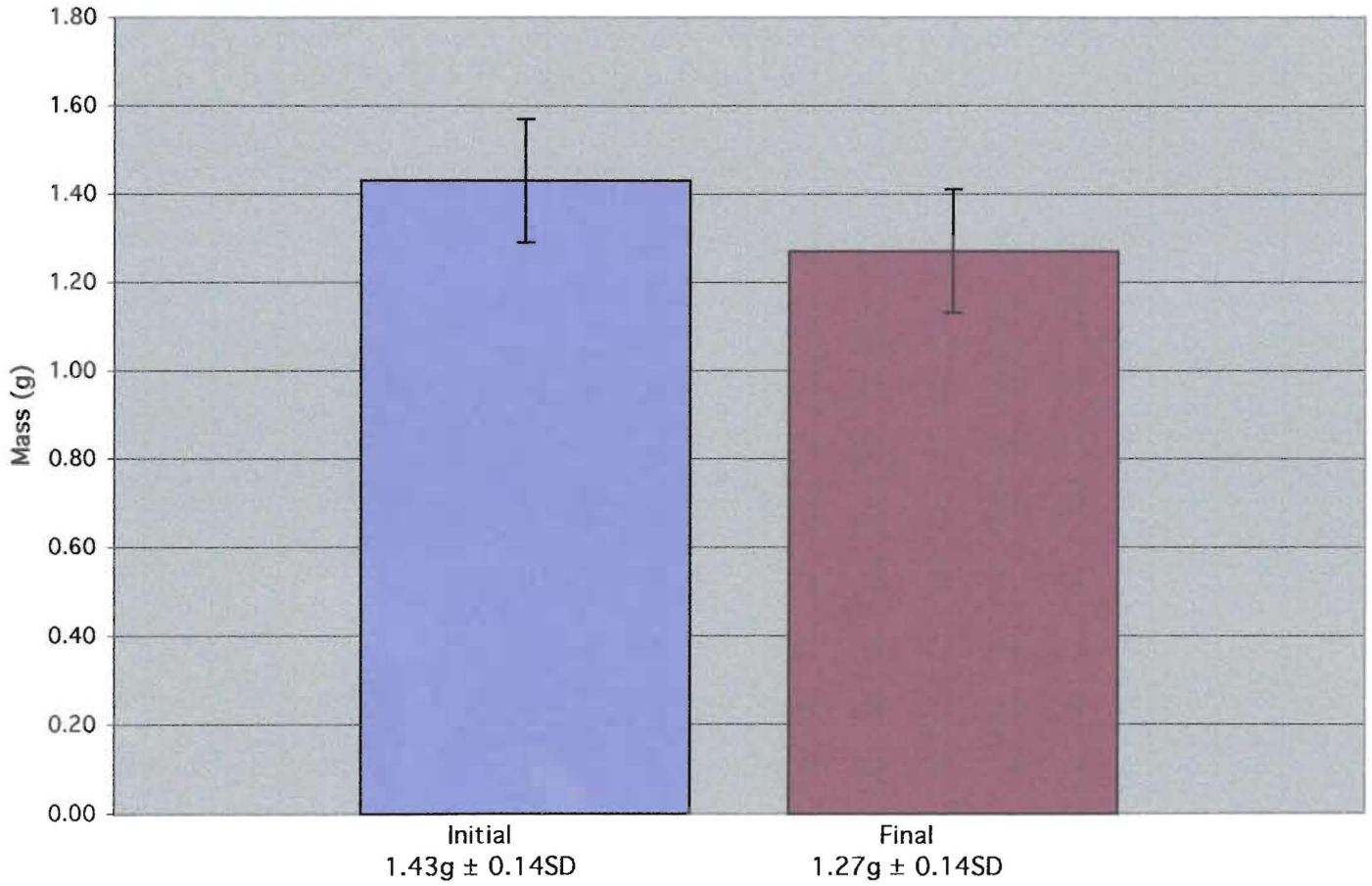

Figure 8: Bar Graph showing the significant difference ($t = 5.98$, $df = 52$, $p < 0.001$) between the egg masses weighed before incubation and the final egg masses right before hatching.

Figure 9

Figure 9: A newly hatched House Wren.

Figure 10
Mass of Hatchlings

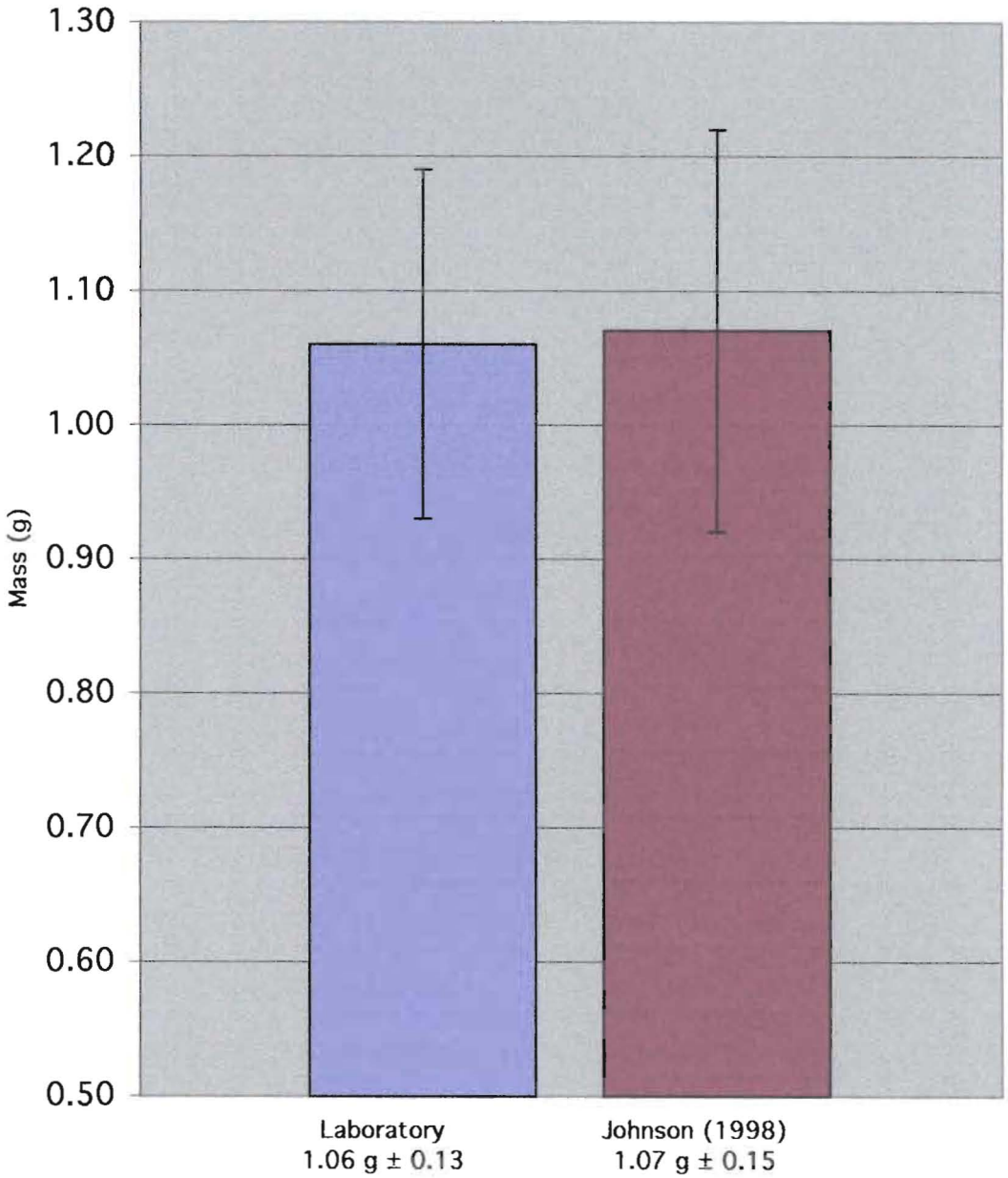

Figure 10: A bar graph showing no significant difference ($t = 0.16$, $df = 31$, $p > 0.05$) of mass of hatchlings between the laboratory and the field.