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Factors Affecting Tree Swallow (*Tachycineta bicolor*) Nestling Resting Metabolic Rate

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FACTORS AFFECTING TREE SWALLOW (*Tachycineta bicolor*) NESTLING RESTING METABOLIC RATE

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ABSTRACT

Metabolism is the major force that maintains the most rudimentary of functions, and, therefore, maintains life in every organism. Because of the immense effect metabolism can have on an individual's life history, it is key that the factors influencing metabolic rate are investigated. This study was designed to investigate the influential factors affecting Tree swallow, *Tachycineta bicolor*, nestling resting metabolic rates by observing maternal effects, early developmental conditions, age, and body mass in Tree swallow nestlings within collection sites at the Saint John's Abbey Arboretum and Kraemer Lake-Wildwood County Park in Stearns County, Minnesota. Due to the fact that there is little research on the resting metabolic rate of Tree swallows, this study contributes to the fields of ecology and organismal biology in allowing for greater knowledge on the resting metabolic rates of Tree swallows and, potentially, other small, short-lived animals. For this study, two predictions were made: the first being that the male Tree Swallow nestlings would have a higher resting metabolic rate than their female nestling counterparts. In the second prediction, it was anticipated that the resting metabolic rates of Tree Swallow nestlings from one nest box would differ significantly from the rates of nestlings within another nest box, as it was expected that their environmental influences would differ. After data analysis was completed, it was concluded that our first hypothesis, which states that males would have a higher resting metabolic rate than their female counterpart, was not supported, and that our second hypothesis that each nest box would have an average nestling resting metabolic rate different than the other nest boxes was supported.

INTRODUCTION

Metabolism, the process by which food resources containing high-quality chemical energy are degraded and oxidized to harness the useable, low-quality energy necessary for life's necessary chemical processes and physiological work, is the major force that maintains the most rudimentary of functions, and, therefore, maintains life in every organism. This metabolism places a toll on the surrounding environment near a habitat and the organism itself. In endotherms, specifically, resting metabolic rate accounts for 60 to 65 percent of all the energy expenditure in a day for some endotherms (Tisch 2005). Because of the immense effect metabolism can have on an individual's environment, in terms of resource demand and habitat drain, and life history, it is key that the factors influencing metabolic rate are investigated – especially when one's life is short-lived.

Short-lived organisms, such as Tree Swallows (*Tachycineta bicolor*), utilize their metabolism to increase their fitness through rapid growth rates, early maturation, and the production of many eggs and offspring. In order for these processes to occur, a swallow's useable energy stores must be continuously replenished, as it is these energy stores that allow for the required synthesis and deposition of amino acids and lipids required these processes. One such energy store utilized in these processes is adenosine triphosphate (ATP), which is replenished through aerobic metabolism – a combination of glycolysis, the citric acid cycle, electron transport chains and oxidative phosphorylation. The steps of aerobic metabolism involve the consumption of oxygen and the generation of carbon dioxide.

While necessary, the rapid production of usable energy through aerobic metabolism does come at a cost to the organism. This cost is observed in the form of reactive oxygen species (ROS), which have the ability to denature molecules such as nucleic acids and other proteins.

Although cells require a certain level of ROS to maintain standard functions, the production of ROS may result in the harm of the organism through the production of harmful byproducts, including hydrogen peroxide. For example, in a study conducted with snakes, it was found that the fast-living snakes, when placed under stress, produced more ROS, had less efficient DNA repair, and had a stronger immune response – all of which negatively impacted the snake's health (Flatt et al. 2011). In other small, short-lived organisms, such as Tree Swallows, it is likely that ROS levels in the body will be higher as a result of their higher aerobic metabolism and resting metabolic rates. These higher ROS levels will have long-term effects on the longevity of swallows, as swallows tend to have high metabolic rates and, therefore, high rates of aerobic metabolism and resting metabolic rate relative to their size (Flatt et al. 2011).

According to T. Burton et al. (2011), there are numerous influences that can affect the resting metabolic rate of individuals even within a species. Such factors include genotype, maternal effects, early developmental conditions, age, dietary history, reproductive state, personality differences, and body mass (T. Burton et al. 2011). Body mass, which is one trait in particular known to have an effect on the resting metabolic rate of an individual though the specific correlation between the two in organisms, remains somewhat unclear in its relation to resting metabolic rate. Numerous equations and methods have been derived in attempts to correlate organismal mass and metabolic rate (Gillooly et al. 2001), including a debatable mass-specific metabolic scaling component value (–0.33, –0.3, or –0.25), but it is fairly safe to conclude that smaller organisms will tend to have higher mass specific resting metabolic rates. However, it is still uncertain as to what specific factors affect resting metabolic rate. With this knowledge, multiple factors were observed and recorded in the pursuit of determining which factors have the most influence on the resting metabolic rate in tree swallow nestlings. Such

factors included body mass, environmental temperature, early developmental conditions, and age.

Using this knowledge, two predictions were made: the first being that male Tree Swallow nestlings would have a higher resting metabolic rate than females due to our assumption that male and female swallow nestlings would differ in body mass and hormone levels. This prediction stems from our thought that male nestlings would have a different body composition and growth rate when compared to their female nest mates. The second prediction was that the resting metabolic rates of Tree Swallow nestlings would vary significantly from one nest box to another due to the differences in the quality of the environment surrounding each individual nest based on resource availability and land coverage box and/or maternal influences, including maternal hormone levels, temperament, and overall condition.

METHODS

Study Sites

Data collection took place from May 13th through July 14th, 2015, between the hours of 5:00 AM and 1:00 PM. Collection sites included locations within Saint John's Abbey Arboretum and Kraemer Lake-Wildwood County Park in Stearns County, Minnesota, in which populations of Tree Swallows resided. The dates of collection corresponded with Tree Swallow egg laying, incubation, nestling care, and nestling fledging. Testing was completed prior to 1:00 PM during data collection to avoid exerting the Tree Swallows during the afternoon heat. At each collection, the date, time, and temperature were recorded. In total, 23 nest boxes produced fledglings; within these 23 nest boxes, 23 adult females and 1 adult male were captured and banded during incubation in addition to the 86 nestlings captured and banded during nestling care.

Adult Female Capture and Sampling

Once a pair of Tree Swallows established a nest, the nest box was observed at least every other day in order to monitor the nest height, the number of feathers present within the nest, when the first egg was laid, the total number of eggs laid, the date of first egg hatch, and any predation or abandonment of the nest box. When the pair was incubating the eggs, the female occupying the nest box was captured. Blood (≤ 100 μL) was drawn from the female's brachial artery; this blood sample was later centrifuged down to separate out plasma from red blood cells for plasma testosterone measurements. After blood was drawn, the female was massed and her wing chord, tarsus length, and number of wing mites were recorded. Female swallows were aged based on their general coloration of plumage; females with a dull brown color were classified as second year (SY) females, or those in their first mating season, and those with the blue-green color of their male counterparts were classified as after second year (ASY) females. If the Tree Swallow was previously banded, her USGS band number was recorded and compared to data of previous years. If she was not banded, a new band was placed around her tarsus, and the band number was recorded. Prior to release, the female's white chest and/or tail feathers were colored with a Crayola® washable marker in order to identify her in flight during later temperament tests.

Maternal Temperament Testing

Over the duration of incubation and nestling care, the female of the nest box underwent a series of temperament tests including nest defense, 2-D neophobia, and 3-D neophobia; the order of these tests were assigned by a random number generator based on the female's capture order number. The nest defense test was conducted once during incubation and once during nestling care. For nest defense, one researcher stood at the nest box for 5 minutes and recorded the

female's nest flush as she approached the nest box, her first vocalization, number of dives made by the female towards the researcher, number of these dives within 1 meter of the researcher, and percentage of fly time during the 5-minute period. 2-D neophobia involved taping a piece of paper with red bull's-eye image over the front of the nest box, and 3-D neophobia involved placing a tennis ball on the front of the nest box. The female's nest flush was recorded for each test as well. Neophobia tests lasted 15 minutes, or until the female re-entered the nest box. Again, the female's first vocalization was recorded, as well as the number of times she flew by the nest box, the duration of time she hovered in front of the nest box, and the percentage of time she flew during the 15-minute period.

Resting Metabolic Rate Measurements of Nestlings

During nestling care, resting metabolic rates of nestlings were recorded while the nestlings were between 4- and 12-days-old with the use of a laptop equipped with Logger Pro 3.8.6.2 software, a Vernier Software and Technology CO_2 Gas Sensor apparatus, and a Fluke 52II Thermometer. Nestlings were taken from the nest box and placed into a lined holding container that had ventilation holes in the lid. Nestlings were kept in the holding container for 5 minutes to relax and acclimate to the new environment prior to the measurement of resting metabolic rate. After 5 minutes had passed, a nestling was selected randomly from the holding container, was gently wrapped in a collection chamber liner, and was placed into the collection chamber. A tight seal was made between the collection chamber and its lid and between the lid and the CO_2 gas sensor prior to beginning data collection; this ensured that there were no air leaks from the collection chamber. A dark cloth was wrapped around the collection chamber to create a dark environment similar to that of the holding container. Logger Pro was set to collect the production of CO_2 over time by measuring the percent of CO_2 produced in a two-minute

period. At the conclusion of the resting metabolic rate data collection, the temperature within the collection chamber and the linear slope of the collected data were recorded. The nestling was then removed from the collection chamber and was banded with a USGS band, massed, and measured for tarsus and wing chord lengths. If the nestlings in a nest box were younger than 11-days-old, their claws were colored and/or clipped to identify one from another during resting metabolic rate testing. Blood (≤ 30 μL) was drawn from the nestling's brachial artery for molecular sexing. If blood was not easily drawn, a small amount of feathers were plucked instead. The nestling was then returned to the nest box or kept in a holding location while its siblings were tested.

Molecular Sexing of Nestlings

In the lab, nestling blood was used to molecularly sex each nestling by DNA isolation, polymerase chain reaction PCR of the CHD gene, a HAE III Restriction digest, and electrophoresis. Nestling DNA was isolated from the blood or feather sample taken during nestling banding. The Bench Protocol: Animal Blood (Spin-Column Protocol by Qiagen DNeasy® Blood & Tissue Kit was utilized for DNA isolation from blood. For DNA isolation from feather samples, the User Developed Protocol from the DNeasy® Blood & Tissue Kit was used. For detailed methodology of nestling molecular sexing, see Appendix 1.

Statistical Analysis

All collected data was entered into a Microsoft Excel workbook and Minitab 17 Software. A preliminary data analysis was completed to determine which day post-nestling hatch would yield the greatest number and best quality results; a one-way analysis of variables (ANOVA) by nestling age was completed for nestlings that had undergone metabolic rate testing

each day from day 6 to day 12 to determine if there was a specific day of age that stood out as being more unique than other days of testing; the resulting graph can be viewed in Figure 1. This ANOVA was followed by a Tukey Simultaneous 95% Confidence Interval test to determine if the data from one day of nestling age was statistically different from another. The Tukey showed that days 9, 10, 11, and 12 were not significantly different from one another. However, within the graph produced by the one-way ANOVA, a peak-dip-peak trend was observed over days 10, 11, and 12. Based on these findings, it was determined that, in order to decrease variability and potential error in resulting findings, only 11 day old nestlings would be used in subsequent tests due to the fact that 11-day-old nestlings had the least variation between individuals and that we had the most replicates of data from that nestling age. Further analysis involved one-way ANOVA of all 11-day-old nestlings sexes by mass, a one-way ANOVA of mean resting metabolic rates for male and female nestlings after correcting for environmental temperature, a one-way ANOVA of all 11-day-old nestlings by nest box number, and a tukey post-hoc test of mean resting metabolic rate for each nest box on day 11 corrected for nestling mass and temperature. In addition, a multiple regression of 11-day-old nestlings average per box against a list of factors found in Table 3 that were thought to have a potential influence on nestling resting metabolic rates. For this multiple regression, "dummy variables" were used to indicate whether data was available for each variable tested, such as maternal testosterone levels and complete broods, as the procedures for maternal plasma testosterone measurements and nestling molecular sexing did not always produce successful results. Because three different tests were conducted using the same dataset, a Bonferroni Correction was done to the p-value ($p < 0.0167$).

RESULTS

Male and female nestling mean masses were compared with the use of a one-way ANOVA of nesting sex by mass. As seen in Figure 2, the male and female nestling masses are significantly different from one another ($F_{1,1} = 7.72$, $p = 0.007$). While the masses of the sexes differ significantly, the mean resting metabolic rate residuals between the sexes were not significantly different. The mean resting metabolic rate residual of male nestlings was 0.00113 CO_2 produced per gram per minute, which is greater than the females' at 0.00044 CO_2 produced per gram per minute. However, this difference was not statistically significant ($F_{1,1} = 0.06$, $p = 0.809$), as illustrated in Figure 3.

A Tukey post-hoc pairwise comparison was completed of each nest box with 11-day-old mean nestling resting metabolic rate for each nest box testing data against the residuals of the mean nestling resting metabolic rate corrected for mass and temperature. As illustrated in Table 3, many boxes are not significantly different from one another, but there are a few exceptions, such as box 1, 16, and 17. Because nest boxes 1, 16, and 17 do not correspond to multiple groups, they are significantly different from the rest.

All data taken was then quantified and formatted to fit a general linear model, which was then reduced to the final general linear model via removal of factors with high p-values ($p > 0.11$) with a multiple regression, as observed in Table 4. For this model, provisioning conditions over a nestlings' time in the nest box, approximately a period of 14 days, were added as a potential factor affecting nestling resting metabolic rates.

DISCUSSION

After data analysis was completed, it was concluded that neither of the two predictions of this study were supported. While there was a statistically significant difference between male and

female body masses, these differences in body mass did not lead to statistically significant differences in resting metabolic rates between the two sexes as predicted. In addition, the majority of nest boxes did not exhibit significantly different resting metabolic rates – with the exception of three nest boxes observed in this study.

The general linear model indicates that the most important factors influencing resting metabolic rate are brood sex ratio and maternal average flush distance, though the associated probability was greater than 0.05. This suggests the need for future research to increase sample size, as it is possible that an insufficient sample size marginalized the impact of some factors that may truly be significant. It could also be that, as Burton et al. (2011) suggests, the factors included in the analysis are context-dependent upon other factors, such as environmental or physiological conditions. By furthering this study using a larger sample size, determining what these factors are and what influences them could be possible.

The low p-value of the average maternal nest flush signifies this factor may have an impact on increasing the metabolic rate of the nestlings. In a study conducted on zebra finches by Criscuolo, Monaghan and Metcalfe (2008), the idea that metabolic rate is strongly impacted by the feeding patterns of the parents during early nestling development was supported. Those nestlings that were fed a lower protein diet had higher metabolic rates as they grew. With this idea in mind, it's possible that mothers who are more likely to flush the nest in the presence of a stressor may also be reluctant to return to the nest if any danger is sensed. If mothers are returning to the nest less frequently, it's likely that the nestlings could be being fed less frequently, which would potentially lower the protein intake of the nestling and, as a result, raise the nestling's resting metabolic rate. Like the zebra finches in the study, the tree swallow

nestlings receiving less protein-rich food could have resultant higher metabolic rate as supported in Criscuolo, Monaghan and Metcalfe's study.

In another study conducted by Bentz (2012), it was observed that females enduring more frequent aggressive situations inducing high stress had more sympathetic input and therefore higher breathing rates and corresponding metabolic rates. If resting metabolic rates in tree swallows are in fact heritable and not controlled by early nutrition and development, it's possible that females more easily stressed by human presence, or another stressful encounter, would flush from the nest box more readily due to being more cautious of her environment. If this behavior resulted in a greater rate of nestling fledging, the genetic component coding for intensified sympathetic input, and metabolic rates, would be passed on to the female's offspring though genetic inheritance.

The other factor approaching significance was the sex ratio within each brood. According to our results, the resting metabolic rate, when corrected for nestling mass and environmental temperature, was lower when there were more males within the nest. Few studies have investigated or even mentioned this phenomenon in avian species giving little insight as to what the difference between having male or female predominant nests is. In one study conducted by Magrath (2007), it was found that, in populations of brown songlarks (*Cinclorhamphus cruralis*), nests with higher male to female nestling ratios required more food resource delivery by the parents of the nestlings, as male nestlings were larger than their female counterparts and required more resources. If the parents were unable to provide enough resources to their nestlings and had a male-dominate set of nestlings, the resting metabolic rate of the nestlings would decrease due to a limited nutrient availability.

As illustrated in Table 4, the nest box itself was an influential factor in nestling resting metabolic rate, although the nest box was not a statistically significant factor for all nest boxes. Nest boxes 1, 16 and 17 were significantly different from others in terms of nestling resting metabolic rate. This difference could be due to a multitude of reasons ranging from nest box location relative to other landmarks or relative to Tree Swallow density. Being located in a more desirable location or in an area of high swallow density would imply that there was a higher amount of aggressive interactions in the area during nest box acquisition. According to Bentz (2012) and Whittingham et al. (2001), maternal aggressive interactions can attribute to nestlings with increased yolk testosterone levels, which can lead to an increase in nestling resting metabolic rates.

In furthering this study, one could look further into the effects of body composition on resting metabolic rate; this is suggested for the continuation of this research due to the reporting of Burton et al. (2011) that showed that the relative size of an organism's intestines, liver, kidneys, and heart had a positive relationship with resting metabolic rate due to the highly metabolically active tissues that reside in the walls of these organs.

If one does not wish to sacrifice nestlings, there are other areas to be explored as well. Maternal effects on resting metabolic rate has not had much attention in the past, but they could very well be an important influential factor to be studied in conjunction with the factors already included in this study. Maternal deposition of androgens, like testosterone, during oocyte and yolk development has been found to lead to increases in resting metabolic rate, nestling competitive behaviors, and growth rate (Bentz 2012). Future research could utilize Bentz work with that of Schmaltz et al. (2007) to observe the effects of yolk testosterone levels on egg laying order. With this, it would be key in this study to see if nestling growth rates differ between nest

mates and to observe how that potential differences in testosterone levels and in growth rate affect the resting metabolic rate of the nestlings. Tobler et al. (2007) and Whittingham et al. (2001) have found a positive relationship with nestling resting metabolic rates. It is possible that growth rate could yield more significant data, as demonstrated through the work Klassen and Bech (1992); this would require data collections to involve an increased number of series of data where nestling resting metabolic rate and other measurements are completed daily from the time the nestling is 6-days-old until the nestling is 12-days-old.

The collection of a series, as described above, could also aid in investigating the peak-dip-peak pattern observed over days 10, 11, and 12, as seen in Figure 1. It is uncertain as to what exactly caused the reduction in resting metabolic rate when the nestlings are 11-days-old, which is why it would be interesting to further investigate this repeatable observation with a larger sample size to discover what causes the pattern. A larger sample size could also aid in determining if this pattern is significant, as it is not significant with the sample size used in this study.

A final set of factors that should be monitored in future studies should include the impact of handling nestlings, as handling may result in a stress response in the nestlings and elevation of metabolism, which would skew or mask other potential resting metabolic rate influential factors. Having the knowledge of the time of the nestling's last meal prior to resting metabolic rate sampling would also be beneficial due to the influence of digestive processes on metabolism. It would also be beneficial to record whether or not the nestling was actively moving within the collection chamber, as a nestling's motion may affect data collection. Measuring the oxygen consumed during nestling resting metabolic rate testing in addition to the carbon dioxide produce would also provide more accurate data and results.

FIGURES AND TABLES

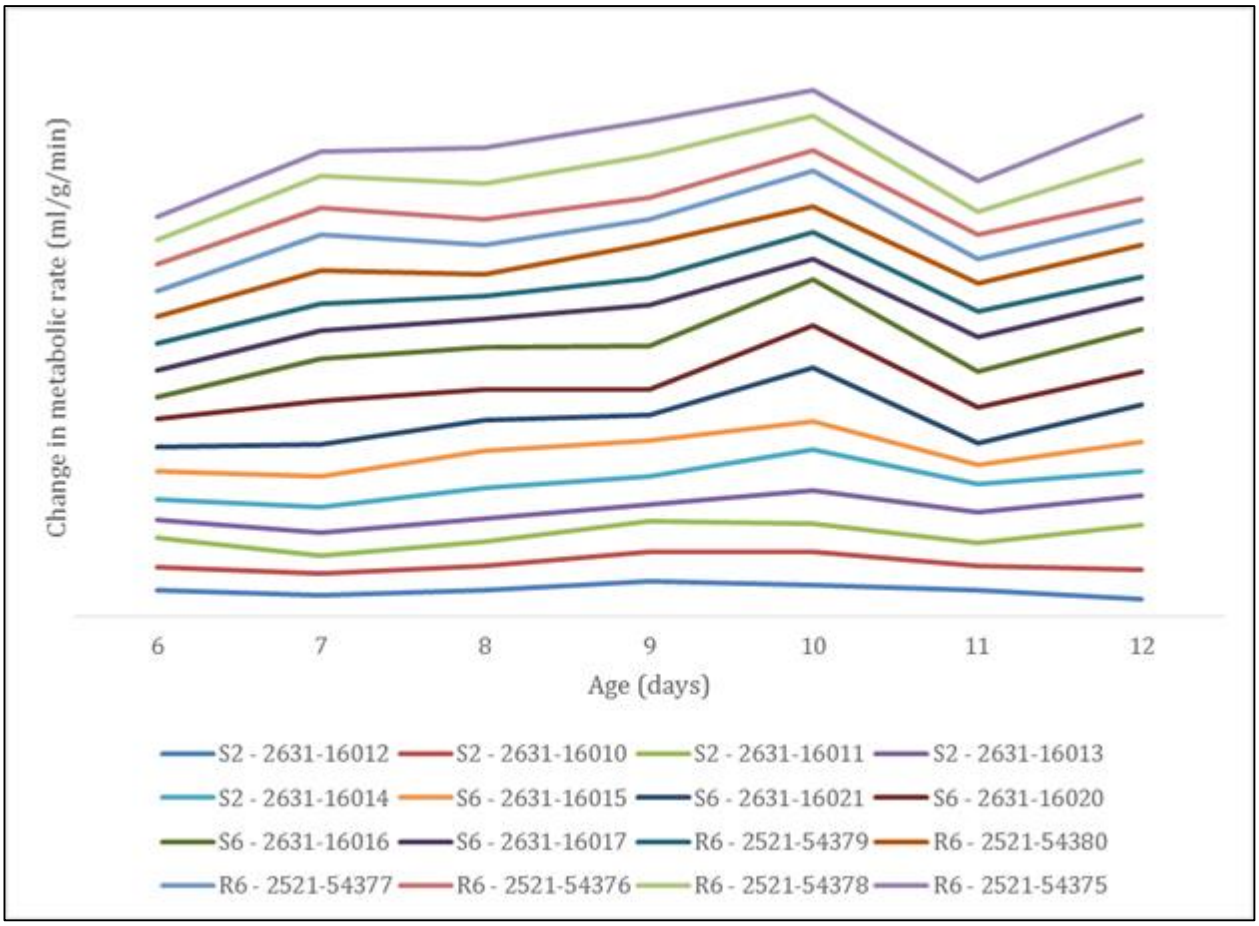

Figure 1. Stacked column graph showing resting metabolic rate over time for 16 individual nestlings that underwent resting metabolic rate testing one time per day beginning when the individuals were 6-days-old and ending when the individuals were 12-days-old.

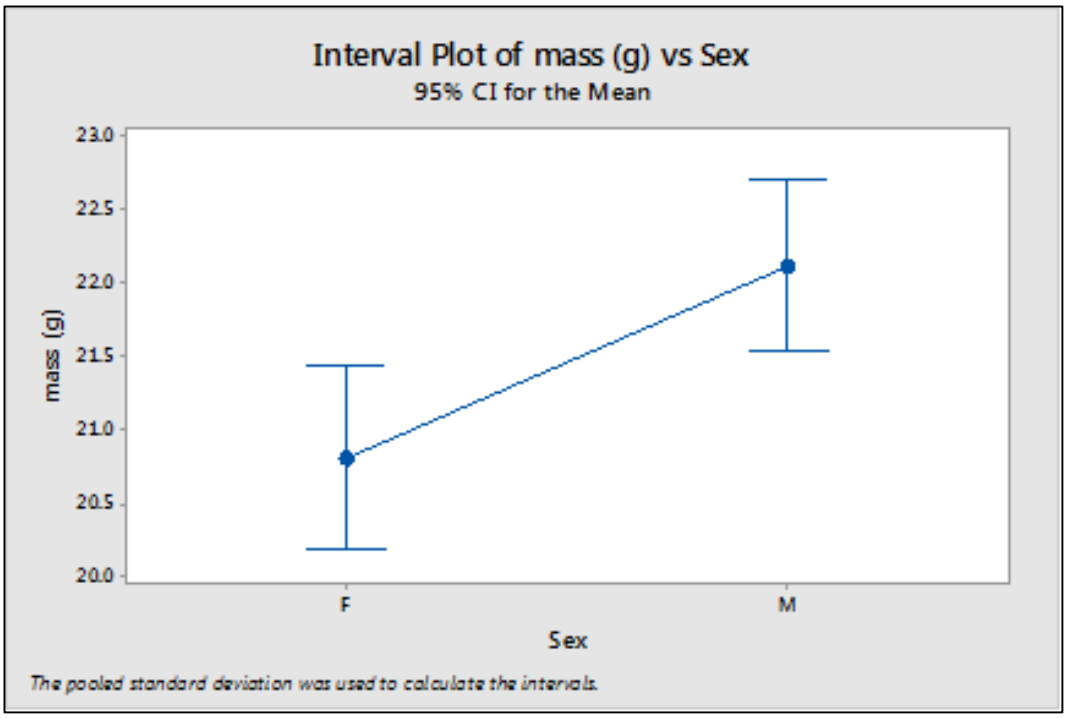

Figure 2. One-way ANOVA of nestling sex by mass with standard deviation intervals ($F_{1,1}$ = 7.72, p = 0.007).

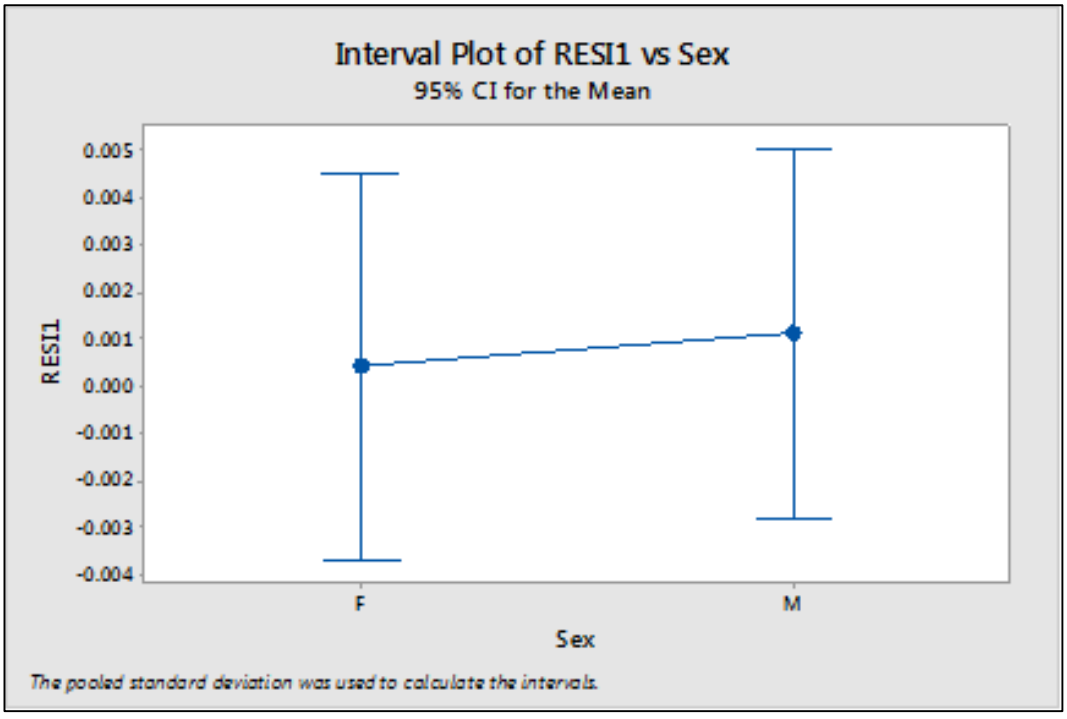

Figure 3. One-way ANOVA of male and female nestling mass of residuals by nestling resting metabolic rate corrected for environmental temperature with standard deviation intervals ($F_{1,1}$ = 0.06, p = 0.809).

Table 1. Female Tree Swallow average condition summary by age.

	Second Year Females (n=12)	After Second Year Females (n=20)
Body Mass (g)	21.45 ± 1.398	21.45 ± 1.212
Tarsus Length (mm)	11.425 ± 0.575	11.91 ± 0.995
Wing Chord (mm)	114.5 ± 3.555	115.85 ± 3.422
Wing Mite Count	13 ± 7.604	7.05 ± 4.261
Blood Plasma Testosterone Volume (μL)	2.583 ± 3.449	2.35 ± 2.681

Table 2. Average maternal behavior by age with standard deviations.

	Second Year Females (n=12)	After Second Year Females (n=20)
Mean Flush Distance (m)	6.773 ± 3.384	8.495 ± 5.952
Mean Percent Fly Time (%)	85.095 ± 14.548	77.925 ± 16.029
Nest Defense Dive Count	4.91 ± 6.732	5.858 ± 7.996
Nest Defense Dives within 1 Meter of Researcher Count	2.45 ± 2.681	3.875 ± 5.558
2-D Neophobia Fly By Count	14.35 ± 9.761	16.639 ± 13.212
2-D Neophobia Hover Time in Front of Nest Box (sec)	4.95 ± 5.058	2.898 ± 3.659
2-D Neophobia Nest Box Re-entry Time (min)	9.942 ± 5.431	13.82 ± 4.055
3-D Neophobia Fly By Count	20 ± 18.477	26.839 ± 19.937
3-D Neophobia Hover Time in Front of Nest Box (sec)	3.525 ± 2.556	4.5 ± 5.493
3-D Neophobia Nest Box Re-entry Time (min)	12.325 ±4.074	13.982 ± 3.732

Table 3. Tukey post-hoc pairwise comparison of nest box by the residual of nestling resting metabolic rate corrected for mass and temperature.

Box Number	Mean	Group
1	0.01995	A
2	0.01859	A B
3	0.01094	A B C
4	0.00955	A B C
5	0.006	A B C
6	0.00597	A B C
7	0.00443	A B C
8	0.00131	A B C
9	0.00073	A B C
10	0.00067	A B C
11	0.00052	A B C
12	-0.00236	A B C
13	-0.00307	B C
14	-0.00467	B C
15	-0.00705	B C
16	-0.00742	C
17	-0.0085	C

Table 4. Multiple regression of factors affecting 11-day-old nestling resting metabolic rate data averages per box model prior to reduction and remaining factors after reduction.

	Coefficient	T-Value	P-Value	Coefficient	T-Value	P-Value
Constant	-0.363	-1.07				
Brood Sex Ratio	-0.0255	-1.81	0.167	-0.0199	-1.82	0.094
Maternal Average Flush Distance	0.00036	0.77	0.497	0.000545	1.76	0.104
Maternal Condition	0.302	1.5	0.231			
Maternal LOG-Testosterone	0.0088	0.86	0.452			
Maternal Activity Level	0.0129	0.97	0.405			
Maternal Number of Dives	4.5E-05	0.07	0.951			
Maternal Parasite Load	-0.0002	-0.35	0.751			
14-Day Provisioning Mean Temperature	0.00391	0.84	0.46			
14-Day Provisioning Rain Total	-0.0015	-0.26	0.815			
Maternal Age	0.00706	1.2	0.316			
DummyVariable: Maternal Testosterone	-0.0272	-1.02	0.383			
DummyVariable: Complete Brood	0.0041	0.35	0.748	0.104	0.33	0.751

APPENDIX 1

Molecular Sexing of Nestlings Detailed Methodology

In the lab, nestling blood was used to molecularly sex each nestling by DNA isolation, polymerase chain reaction PCR of the CHD gene, a HAE III Restriction digest, and electrophoresis. Nestling DNA was isolated from the blood or feather sample taken during nestling banding. The Bench Protocol: Animal Blood (Spin-Column Protocol by Qiagen DNeasy® Blood & Tissue Kit was utilized for DNA isolation from blood. For DNA isolation from feather samples, the User Developed Protocol from the DNeasy® Blood & Tissue Kit was used. Once Nestling DNA was isolated, NanoDrop 2000/2000c software and a NanoDrop 2000 Spectrophotometer by Thermo Fisher Scientific were used to calculate the amount of microliters

of isolated DNA and microliters of proteomics grade water needed to perform a PCR in order to amplify the CDH gene. These calculated amounts were placed into TAQ bead tubes labeled with DNA sample numbers. P2 and P8 primers (Griffiths et al. 1998) were added to each TAQ bead tube (1 μL 250 ng/mL of each), and the tubes were tapped to ensure that all constituents were mixed together prior to their placement in an Applied Biosystems 2720 Thermal Cycler. The thermal cycler was then set to run a series of five steps. In the first step, the thermal cycler heated the samples at 94 °C for 2 minutes. This was followed by step two that involved a repeated series; during the series, the temperature was held at 94 °C for 30 seconds, dropped to 47 °C for 45 seconds, and then brought up to 76 °C for another 45 seconds. This series was repeated 35 times to increase product yield. The third step brought the thermal cycler's temperature down to 48 °C for 1 minute and was then followed by a rise to 76 °C in step four that lasted for 5 minutes. Once step four was completed, the thermal cycler brought the temperature to 4 °C in step five to cool the samples until HAE III Restriction digest was ready to be performed. For the HAE III Restriction digest, 5 μL of PCR product, 5 μL 5 U *Hae*III, 1 μL Restriction enzyme 10X buffer, and 3.5 μL sterile water were pippetted into a thermal cycler tube. The Applied Biosystems 2720 Thermal Cycler was set to run for 2 hours at 37 °C followed by a drop in temperature to 4 °C until electrophoresis is ready to be completed. Electrophoresis was completed with a 3% Agarose gel held in 1X TAE solution within a Bio-Rad Mini-Sub® Cell GT chamber attached to a Bio-Rad PowerPac Basic power supply. In the middle gel well of each row, 11 μL of ladder was pipetted into the well. The HAE III Restriction digest product was then added to 1 μL of ficoll and Orange G, and the mixture (11 μL total) was then loaded into a gel well. For each sample, the gel well number was recorded. The PowerPac Basic was then set to run at 100 volts, and electrophoresis began. After 30 minutes of electrophoresis, the PowerPac was shut off and

disconnected. A GelDoc-It^2 Imager and Life Science software from UVP VisionWorksLS Image Acquisition and Analysis Software were used to observe the samples' banding on the gel; if there was a band at 300 BP, the nestling was a male, and, if a pair of bands appears with one at 300 BP and another at 350 BP, the nestling was a female.

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