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HATCHING ASYNCHRONY IN EUROPEAN STARLINGS
(*STURNUS VULGARIS*)

Jason T. Hanser

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Across a wide range of avian taxa, eggs within clutches hatch asynchronously, placing later hatched nestlings at a disadvantage. Here, we explore the proximate and ultimate causes of hatching asynchrony within European starlings, *Sturnus vulgaris*. Specifically, we investigate the effect of ambient temperature on egg viability and incubation behavior prior to clutch completion. Additionally, we examine the potential for storage time and maternally-deposited yolk testosterone to influence rates of embryonic development and hatching patterns within European starlings.

HATCHING ASYNCHRONY IN EUROPEAN STARLINGS
(*STURNUS VULGARIS*)

JASON T. HANSER

A Thesis Submitted in Partial
Fulfillment of the Requirements
for the Degree of

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2015

HATCHING ASYNCHRONY IN EUROPEAN STARLINGS
(*STURNUS VULGARIS*)

JASON T. HANSER

COMMITTEE MEMBERS:

Joseph M. Casto, Chair

Steven A. Juliano

Rachel M. Bowden

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J. T. H.

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CHAPTER I

THE USE OF GROWTH CURVES TO DESCRIBE INCUBATION
PATTERNS PRIOR TO CLUTCH COMPLETION IN
EUROPEAN STARLINGS (*STURNUS VULGARIS*)

Abstract

In many species of birds, the onset of incubation occurs prior to the completion of the clutch, causing eggs within a clutch to hatch asynchronously. Because nestlings that hatch later within a clutch are smaller and less able to compete with their siblings, they often suffer greater mortality than earlier hatched nestlings. While incubation prior to clutch completion has significant fitness consequences for both parents and offspring, little is known about the patterns of incubation during the laying stage that contribute to hatching asynchrony. Here, utilizing data collected by automated data loggers, we used a nonlinear mixed-model to construct growth curves that depict the application of heat to nests by parents. The use of growth curves allows us to examine variation between individuals with respect to changes in incubation behavior across the laying period and compare these curves to observed hatching patterns within starlings. While the use of growth curves is not a novel technique, their use here to model incubation behavior represents a unique application. Accordingly, we discuss their potential use in future studies, either as a conceptual tool when considering support for various explanations of

hatching asynchrony or an empirical tool to test predictions about the relationship between incubation during the laying stage and hatching patterns within clutches.

Introduction

Because embryonic development within most species of birds is dependent on parental incubation (Drent 1975), parents can influence hatching patterns by varying incubation onset (Wiebe et al. 1997; Ardia et al. 2006; Wang and Beissinger 2009; Lord et al. 2011). Within many avian species, parents often begin incubation prior to the last egg being laid, such that earlier laid eggs within a clutch may be incubated for several hours by the time the last egg is laid (Wang and Beissinger 2009; Podlas and Richner 2013). Consequently, eggs within a clutch frequently hatch asynchronously, with eggs typically hatching in the order in which they were laid (Clotfelter et al. 2000). Since later hatched nestlings are generally less able to compete with their older siblings (Skagen 1987) and less likely to fledge (Clotfelter et al. 2000), the degree of hatching asynchrony within a clutch (i.e. the time elapsed between the first and last hatched nestling) has important consequences for nestlings within a brood. A large body of research has explored the evolutionary and possible adaptive significance of hatching asynchrony (reviewed in Stenning 1996). However, despite a focus on the ultimate causes of hatching asynchrony within the scientific literature and its profound effect on offspring, our understanding of incubation patterns during the laying stage remains surprisingly limited. Prior studies have typically focused only on the total amount of incubation (i.e. time or heat transferred) prior to clutch completion, demonstrating that the amount of incubation during the laying stage correlates with the degree of hatching asynchrony within a nest (e.g. Ardia et al. 2006,

Lord et al. 2011). Unfortunately, these and most other studies have not typically considered variation in incubation behavior across the laying period within individual nests.

Variation in incubation behavior over the course of the laying period should affect hatching patterns and, consequently, the degree of hatching asynchrony within a clutch. For example, even in cases in which the total amount of incubation is similar among nests, differences in the pattern of incubation behavior during the laying stage may still exist – affecting the timing of hatching events and the degree of asynchrony within a nest. In order to visualize how incubation behavior during egg laying may vary, we can construct hypothetical curves that reflect different incubation patterns (Figure 1) by graphing the cumulative amount of heat transferred to eggs throughout the course of the laying period. These curves depict the total amount of heat applied to eggs up until a point in time, such that the slope of curve at a given point indicates the rate at which heat is transferred (i.e. the intensity of incubation) at that time. For all curves, the amount of heat transferred to eggs at the start of the laying period is equal to zero, but increases with time at a varying rate as it approaches some maximum value. In some cases (Figure 1, curve a), the curve may reach the maximum value well before clutch completion, indicating parents began incubation prior to clutch completion but later stopped applying heat before the laying sequence finished. However, the specific shape of the curve may vary and will be dictated by parameters that control the maximum value (i.e. asymptotic value), the steepness of the curve, and the location of the inflection point. Changes in the maximum or asymptotic values reflect variation in the total amount of heat transferred to eggs. However, the steepness of the curve may also vary between individuals, with a less

steep curve (Figure 1, curve b) indicating that parents apply heat at a relatively constant rate during the laying stage. Alternatively, the inflection point of curves may differ. By occurring later, the curve – if considered only for the duration of the laying period – can resemble an exponential function (Figure 1, curve c) – suggesting parents are increasingly engaging in incubation behavior as the laying sequence progresses.

Since it is generally assumed that hatching patterns within clutches are reflective of incubation prior to clutch completion (Wiebe et al. 1997), these curves may be used to make predictions related to hatching asynchrony and the degree of competition among nestlings. For example, assuming a clutch size of at least three eggs, if the amount of heat applied to eggs increases exponentially during the laying stage (Figure 1, curve c), the amount of time that elapses between successive hatchings should increase with subsequent hatchings. That is, the amount of time that elapses between the first two eggs that hatch should be significantly less the amount of time that elapses between the last two eggs to hatch. As a result, nestlings of earlier laid eggs – which should hatch over a narrow time frame – should be relatively evenly matched in terms of size and ability to compete for food, while the last hatched nestling should be substantially smaller. However, if heat is transferred at a fairly constant rate across the laying period (Figure 1, curve b), the amount of time that elapses between successive hatchings should be relatively equal. In such cases, we might expect the mass of nestlings within a clutch to decline linearly with hatching order, such that that difference in mass between the first two nestlings to hatch should be similar to the difference in mass of the last two nestlings to hatch. While this pattern of incubation will still produce a hierarchy with respect to the mass of nestlings within a clutch, the differences in the mass of nestlings within a brood

should be different than if all but one of the nestlings hatched at approximately the same time.

Consequently, by altering the pattern of incubation during the laying stage, parents may be able to promote nestling size hierarchies that are beneficial under certain environmental conditions. For example, blood-feeding ectoparasites are common within the nests of many cavity nesting species and impose significant costs on adults and offspring. By feeding on incubating adults and developing nestlings, ectoparasites deprive individuals of nutrients and energy (Christie et al. 1996, Richner and Tripet 1999, O'Brien et al. 2011) – limiting their ability to invest in growth, maintenance, and reproduction. In several species, including starlings, adults often attempt to minimize their exposure to ectoparasites, either by avoiding nest boxes that contain old nest material (Oppliger et al. 1994) or by incorporating green aromatic plant matter that limits ectoparasite numbers in their nest (Clark and Mason 1985, Shutler and Campbell 2007, Mennerat et al. 2009). Still, ectoparasites are present within most starling nests (Pryor and Casto 2015) and nestlings from nests with high ectoparasite levels are less likely to fledge (Gwinner & Berger 2005, Cantarero et al. 2013). In such cases where ectoparasites are present and the survival of all offspring is not reasonably assured, parents may benefit from the production of nestling size hierarchy. Similar to the brood-reduction hypothesis (Lack 1954), by engaging in incubation during the early portion the laying stage and staggering the hatching times of nestlings, parents could focus their efforts and resources on the first hatched nestlings – ensuring the survival of at least a portion of the brood.

To determine how incubation effort changes over the course of the laying period, as well as the effect of ectoparasite abundance on patterns of incubation prior to clutch

completion, we examined incubation behavior in European starlings (*Sturnus vulgaris*), a cavity nesting species. Utilizing automated temperature data loggers, we monitored ambient and nest cup temperature during the laying stage and determined the amount of heat transferred to eggs via incubation behavior. Using a non-linear mixed model, we constructed growth curves that depict the application of heat to eggs during the laying period and compared these curves to observed hatching patterns. Then, by scoring the ectoparasite abundance within each nest, we investigated the effect of ectoparasite abundance on incubation behavior prior to clutch completion, and in doing so, we demonstrate the utility of growth curves when studying incubation.

Methods

Study System and General Methods

This research was conducted in a European starling nest box colony in Normal, IL (40.5221° N, 89.0127° W) between April and July of 2013. European starlings are a cavity nestling species that exhibit a moderate and variable degree of hatching asynchrony (Stouffer and Power 1990), making them a suitable model species to investigate variation in incubation behavior during the egg laying stage. During the breeding season, nest boxes were visited daily to check for the initiation of new nests. Upon discovery, a subset of nests was assigned to an experimental treatment group as part of another study. Each egg from these nests was removed shortly after laying and replaced with a painted wooden replacement egg. The number of nests included within the entire study and each subset of nests is summarized within Table 1.

To monitor incubation prior to clutch completion, we used automated temperature data loggers (iButtons, Maxim Integrated Products; Sunnyvale, CA). Data loggers were installed inside the cup of each nest and on the underside of each nest box to monitor nest and ambient temperature, respectively. All data loggers were installed in nests shortly after the first egg was laid and programmed to record temperature every 15 minutes. A third data logger was installed inside each nest box to monitor ambient temperature within the nest box. However, a preliminary analysis of these data suggests that ambient temperature inside the nest box did not differ from ambient temperature outside the nest box. As a result, within our analyses, we did not consider data from data loggers that recorded ambient temperatures within the nest box. Instead, we assumed that ambient temperatures within the nests were the same as ambient temperatures outside the nests. Finally, to prevent females from ejecting or repositioning data loggers within the nest cup, data loggers were placed in cloth pouches and tied in place to fix their position within the nest cup. Data loggers installed on the underside of nest boxes were held in place by hard plastic fobs (Embedded Data Systems; Lawrenceburg, KY.).

By comparing ambient and nest temperatures and assuming that deviations from ambient temperatures reflected parental incubation, we determined the amount of heat that could be attributed to parental incubation. Additionally, since not all heat transferred by parents to eggs results in embryonic development, we only considered heat that was also capable of inducing embryonic development (i.e. temperatures greater than physiological zero). Thus, we calculated the number of degree hours above 24° C that could be attributed to parental incubation, since the minimum temperature required for embryonic development falls between 24-27° C for most species of birds (Webb 1987).

To determine total amount of heat that had been applied to a nest at any given time, we summed the number of degree hours above 24° C prior to the specific time that were attributable to parental incubation.

In order to examine hatching patterns, in the nests that retained their original eggs throughout the laying period, we numbered eggs shortly after they were laid to indicate their position within the laying sequence and later monitored hatching within clutches. Starting on the projected hatch day, nests were checked every two hours from 0600 – 2000 until either all eggs hatched or no additional eggs hatched for 36 hours. Based on the contents of the nests, we were able to infer from which egg each nestling had hatched. To minimize disturbance at the nest during this period, nest checks were brief and usually lasted less than one minute.

To assess parasite abundance, we estimated the spottiness of eggs on the day before the projected hatch day. When first laid, starling eggs are immaculately blue, but – as the laying and incubation periods progress – they often accumulate dark reddish-brown blood spots due to bites to the parent by blood-feeding ectoparasites (Lopez-Rull et al. 2007, Hornsby et al. 2013). Prior work in this nest box colony has demonstrated that the number of blood spots on eggs within a clutch correlates positively with the abundance of Northern fowl mites (*Ornithonyssus sylviarum*) found in nests during the nestling stage (Pryor 2012). We used a method similar to Pryor (2012) to categorize nests as having either a low- or high-parasite burden. Nests in which individual eggs had on average more than fifty spots were considered to have a high parasite burden. Similarly, those nests in which individual eggs averaged less than 50 spots had a low parasite burden.

Model and Statistical Analyses

To model the application of heat prior to clutch completion, we constructed growth curves using a non-linear mixed model (Aggrey 2009; Wang and Zuidhof 2004). Based on a visual inspection of the raw data as well as its versatility and ubiquity in analyzing growth data (Robertson 1923, France et al. 1996), we fit our data to a logistic function:

$$H_{ij} = \frac{\beta_1 + u_{i1}}{1 + e^{\frac{-Time - (\beta_2 + u_{i2})}{\beta_3 + u_{i3}}}}$$

where H_{ij} is the cumulative amount of heat (degree hours above 24° C) transferred by parents to eggs within nest i at time j . Parameters β_1 , β_2 , and β_3 are fixed-effects that describe the asymptotic amount of heat transferred, the inflection point of the curve, and the rate at which heat is transferred, respectively. To account for differences between nests, the full model (shown above) also includes three between-subject random-effects: u_{i1} , u_{i2} , u_{i3} . Inclusion of u_{i1} , u_{i2} , u_{i3} , allows for individual variation in each of the fixed-effect parameters: β_1 , β_2 , and β_3 , respectively. Since not all between-subject effects may have been needed to adequately fit the data, between-subject effects were systematically added and removed to construct several competing models. Because the inclusion of multiple between-subject effects results in a substantial increase in the number of parameter estimates due to the addition of covariance parameters, we were unable to include additional parameters in our model – such as egg type (wooden eggs versus real eggs) or parasite burden. Consequently, to examine possible effects of egg type and

parasite burden, we compared the between-subject parameter estimates of our final models using a factorial MANOVA.

In addition to the logistic growth function, we also considered an alternative and simplified solution by fitting our data to an exponential function:

$$H_{ij} = Time^{(\beta_1 + u_{i1})}$$

where H_{ij} is the cumulative amount of heat (degree hours above 24° C) transferred by parents to eggs within nest i at time j . Parameters β_1 is a fixed-effect and u_{i1} is a between-subject effect.

Because clutch size is variable within European starlings, we restricted our analyses to four-egg (n=17) and five-egg clutches (n=23), which includes more than 60% of all nests. Additionally, because the length of the laying period differs by clutch size, we constructed separate models for four and five egg clutches.

All models were later evaluated by comparing corrected AIC (AICc) values, where a lower AICc value indicates greater support for a model (Burnham et al. 2011). All fixed and random effect parameters were entered into the model as normally distributed variables. All statistical analyses were performed using SAS (version 9.3).

Results

Fit statistics, including AICc, of the various models for four and five eggs clutches are summarized in Table 2. Comparison of AICc values indicate that all logistic growth models outperformed the exponential model and that, among the logistic growth models

considered, the full models for both four and five egg clutches, containing all three between-subject random effects (i.e. u_{i1} , u_{i2} , and u_{i3}), provided the best fit.

Parameter estimates for the final model are detailed in Table 3. Within both four- and five-egg clutches, all three between-subject random effects were significant, indicating significant variation among the curves of individual nests with respect to asymptotic value, steepness, and inflection point. Moreover, within four- and five- egg clutches, several covariance parameters were significant. For both four and five clutches, we observed significant parameter estimates for the covariance between the random effects u_{i1} and u_{i2} (σ_{u1u2}^2) as well as u_{i1} and u_{i3} (σ_{u1u3}^2). The positive estimates for σ_{u1u2}^2 and σ_{u1u3}^2 indicate that as the asymptotic amount of heat applied increases, the value for the inflection point of a curve increases and maximum rate at which heat is transferred decreases. Additionally, within four egg clutches, a significant and positive parameter estimate for the covariance between u_{i2} and u_{i3} (σ_{u2u3}^2) indicates that as the value for the inflection point of a curve increases the maximum rate at which heat is transferred decreases.

Between-subject parameter estimates for the final models did not differ by egg type for four ($F_{3,9}=0.11$, $p=0.9537$) or five egg clutches ($F_{3,16}=0.40$, $p=0.7560$), indicating that females did not alter incubation effort based whether the nest contained real eggs or wooden decoys. Similarly, between-subject parameter estimates did not differ as a result of parasite burden for four ($F_{3,9}=0.58$, $p=0.6420$) or five egg clutches ($F_{3,16}=0.30$, $p=0.8238$), nor was the interaction between parasite burden and egg type statistically significant for four ($F_{3,9}=0.36$, $p=0.7808$) or five egg clutches ($F_{3,16}=0.07$, $p=.9745$).

Hatching patterns for four and five egg clutches are depicted in Figure 3. Within four- and five- egg clutches, the amount of time that elapsed before an egg hatched, relative to the hatch time of the first hatched egg, increased with laying order in an exponential fashion.

Discussion

We examined variation in incubation behavior prior to clutch completion by using a nonlinear mixed model to construct growth curves that depict the transfer of heat to eggs by parents during the laying stage. In doing so, we were able to demonstrate significant variation between individuals with respect to incubation prior to clutch completion.

While the application of heat by parents generally increased exponentially across the laying period, our analysis revealed that a model based on logistic growth provided the best fit – indicating that variation in incubation behavior is not limited to the total amount of heat applied to eggs during the laying period, but also includes differences in the intensity of incubation over the course of the laying stage (Figure 2). Additionally, because of the significant covariance between the between-subject effects u_{i1} and u_{i3} (σ_{u1u3}^2), this pattern was more exaggerated when parents engaged in more incubation during the laying stage, such that parents that apply the most heat to eggs during laying are also more likely to apply heat in an exponential fashion. Thus, within nests that exhibit the greatest degree of hatching asynchrony, most of the asynchrony should be due to hatch time of the last laid egg relative to hatch times of earlier laid eggs. Altogether, these results suggest that parents employ different incubation strategies during the laying period and may select strategies based on environmental cues as a means of manipulating

hatching patterns and brood composition. While we found no support for the hypothesis that parents alter incubation behavior as a result of parasite burden, our analysis nonetheless illustrates how growth curves may be used to examine variation in incubation patterns prior to clutch completion. Future studies that utilize growth curves in their analyses could similarly compare between-subject parameter estimates to determine the effect of a wide variety of factors on incubation behavior.

While often overlooked, conceptual models of incubation behavior prior to clutch completion are important for understanding hatching patterns and hatching asynchrony in birds (Wiebe et al. 1998; Wang and Beissinger 2009). While most studies of hatching asynchrony have typically examined either the amount of time spent incubating prior to clutch completion (e.g. Haftorn 1981; Bortolotti and Wiebe 1993) or the total amount of heat applied to eggs during the laying period (e.g. Johnson et al. 2013), variation among individuals is not limited to differences in the total amount of incubation prior to clutch completion. Rather, even in situations in which the total amount of heat transferred to eggs does not differ between individuals, differences in incubation patterns (Figure 1) could produce different hatching patterns. These patterns are important to consider when investigating hatching asynchrony as some incubation patterns may be more applicable to specific hypotheses. For example, according to the peak load hypothesis, parents benefit from hatching asynchrony by staggering the age of nestlings, such that the energetic demands of nestlings within a brood are not equal and the energetic demand of the entire brood is less than if all the nestlings hatched at the same time (Mock and Schwagmeyer 1990). Since parents should experience the greatest benefit when the hatching times of nestlings are equally spaced, we might expect parents to apply heat at a constant rate

across the laying period. While past studies have generally not considered variation in incubation behavior during the laying period as it relates to hypotheses that explain hatching asynchrony, future studies should consider these patterns in accordance with various hypotheses when predicting how individuals should alter incubation behavior prior to clutch completion.

The use of growth curves to model incubation behavior may also prove useful when testing predictions about the relationship between incubation prior to clutch completion and hatching patterns within a clutch. Hatching asynchrony has generally been assumed to be caused by differences in the onset of development among eggs within a clutch, stemming from parents engaging in incubation before the last egg is laid (Wiebe et al. 1998; Wang and Beissinger 2009). However, differences in the rate of development among eggs within a clutch may also contribute to hatching patterns. A study of hatching asynchrony in American kestrels found that hatching patterns could not be fully explained by incubation prior to clutch completion (Bortolotti and Wiebe 1993). More recently, several studies have found that eggs within a clutch may differ in their rate of development, such that later laid eggs within a clutch require shorter periods of incubation before hatching compared to earlier laid eggs (Muck and Nager 2006; Boonstra et al. 2010; Hadfield et al. 2013). While observed hatching patterns were largely reflective of incubation patterns within this study (Figure 3), the use of growth curves to model incubation may enable researchers to investigate the possibility of other forces mediating hatching asynchrony when experimental methods are not feasible.

Because of their relatively small size and low cost, temperature data loggers – such as iButtons – have become increasingly popular in studies of incubation behavior (Joyce et

al. 2001; Hartman and Oring 2006; Schneider and McWillaims 2007; Ardia et al. 2009; Johnson et al. 2013). By monitoring temperature remotely and automatically, these devices have enabled researchers to collect large amounts of data with relative ease. However, owing to difficulties in managing and analyzing large data sets (Jennrich and Schluchter 1986, Lynch 2008, Hampton et al. 2013), the full potential of these data is rarely realized. Instead, studies that have employed automated data loggers to examine incubation behavior have frequently relied on data reduction methods to illustrate broad patterns within species (e.g. Ardia et al. 2009). While appropriate and sufficient in many circumstances, these techniques oversimplify incubation behavior and result in the loss of information that may obscure more nuanced patterns (See Chapter 2). Conversely, some have simply reported raw temperature data from individual nests to demonstrate the variation in incubation behavior within and among individuals (e.g. Podlas and Richner 2013; Johnson et al. 2013). While anecdotally informative, it is difficult to draw conclusions or test predictions using raw data. Ideally, in order to strike a balance between the loss of information and the ease of analysis, future studies of incubation should consider these issues as well as the scope and aim of their study when considering how to analyze and present data on incubation behavior. Here, we have demonstrated that non-linear mixed models can be used to construct growth curves that illustrate the application of heat to individual nests by parents. While not necessarily appropriate for all cases, this method reduces the loss of information and allows us examine variation in incubation behavior within and among individuals over the entire laying period.

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Table 1. *Summary of sample sizes according to treatment and analysis.* Because not all iButtons were retrieved, the total number of nests with iButton data is not equal to the number of nests that hatched.

Total Number of Nest	77
Number of Nests that Hatched	57
Four and Five Egg Clutches w/ iButton Data	
4 Egg Clutches	
Real Eggs	6
Wooden Eggs	11
5 Egg Clutches	
Real Eggs	9
Wooden Eggs	12
Nests with Hatching Data	
4 Egg Clutches	6
5 Egg Clutches	10

Table 2. Comparison of Corrected Akaike Information Criterion (AICc) values¹ for models containing various within subject effects. Models for four and five eggs clutches were constructed and evaluated independently.

	Within-subject Parameters Included	Four Egg Clutches				Five Egg Clutches			
		σ_e	AIC	AICc	$\Delta AICc$	σ_e	AIC	AICc	$\Delta AICc$
<i>Logistic Growth Function</i>	u_1	49.1119	28587	28587	3883	50.4756	48951	48951	8194
	u_2	49.0556	28734	28734	4030	52.1856	48927	48927	8170
	u_3	52.0548	28825	28825	4121	53.3794	49355	49355	8598
	u_1, u_2	25.7683	25896	25896	1192	22.1147	43436	43436	2679
	u_1, u_3	27.5264	26142	26142	1438	18.9906	42228	42228	1471
	u_2, u_3	29.1825	26292	26293	1589	19.9668	42587	42587	1830
	u_1, u_2, u_3	18.9608	24704	24704	0	15.2963	40457	40757	0
<i>Exponential Growth Function</i>	u_1	230.37	34950	34950	10246	515.63	65909	65909	25152

¹Lower AICc values indicate a better fit.

Table 3. *Parameter estimates and standard errors for the final model, including both fixed and mixed effects. Final model was selected by comparing AICc values.*

Parameter	Four Egg Clutches				Five Egg Clutches			
	Estimate	SE	t-value	p	Estimate	SE	t-value	p
Fixed Effects								
β_1	225.99	42.60	5.30	0.0001	267.44	48.21	5.55	<0.0001
β_2	277.79	18.56	14.96	<0.0001	402.39	21.75	18.50	<0.0001
β_3	48.11	4.94	9.73	<0.0001	57.84	6.24	9.26	<0.0001
Random Effects¹								
σ_{u1}^2	26651.00	5216.86	5.11	0.0002	37976.00	8533.42	4.45	0.0003
σ_{u2}^2	5455.78	1274.15	4.28	0.0008	9242.06	2626.48	3.52	0.0025
σ_{u3}^2	385.25	116.04	3.32	0.0051	735.28	248.90	2.95	0.0085
σ_{u1u2}^2	8452.19	1908.69	4.43	0.0006	11594.00	3716.59	3.12	0.0059
σ_{u1u3}^2	1466.60	488.81	3.00	0.0095	1819.75	969.62	1.88	0.0769
σ_{u2u3}^2	1036.63	321.02	3.23	0.0061	-434.14	573.52	-0.76	0.4589
σ_e^2	18.96	0.42	45.42	<0.0001	15.30	0.26	59.89	<0.0001

¹ σ_{u1}^2 , σ_{u2}^2 , σ_{u3}^2 = individual variance in β_1 , β_1 , β_1 , respectively; σ_{u1u2}^2 , σ_{u1u3}^2 , and σ_{u2u3}^2 = covariance between various random effects; σ_e^2 = residual variance in amount of heat transferred

Figure 1. *Three hypothetical patterns of incubation prior to clutch completion based on a logistic growth function.* Each line represents a different incubation pattern and depicts the application of heat across the laying period.

Figure 2. *Individual and general growth curves depicting the transfer of heat to eggs by parents over the course of laying period for (a) four egg clutches (n=17) and (b) five egg clutches (n=21). Dotted lines represent growth curves for individual nests. General growth curves for four and five egg clutches (dark lines) were graphed using only the fixed effect parameter estimates for four and five egg clutches.*

Figure 3. *Hatching patterns for (a) four ($n=6$) and (b) five egg clutches ($n=10$).* Hatching lag refers to the elapsed time between the first egg to hatch within a clutch and the focal egg. Within most nests, the first laid egg was the first to hatch and, thus, had a hatch lag equal to zero. However, within one of the four egg clutches, the first laid egg was not the first egg to hatch. Consequently, the average hatch lag of first laid eggs within four egg clutches was not equal to zero.

CHAPTER II

DOES THE EGG VIABILITY HYPOTHESIS EXPLAIN HATCHING
ASYNCHRONY IN EUROPEAN STARLINGS?

Abstract

In many species of birds, the onset of incubation occurs prior to clutch completion, causing eggs within a clutch to hatch asynchronously. Nestlings that hatch later within clutches are often less able to compete with their siblings and, consequently, the mortality of later hatched nestlings is generally high. While numerous hypotheses have been proposed to explain the possible adaptive significance of hatching asynchrony, there is a growing body of literature that suggest environmental conditions may affect the viability of eggs and promote incubation prior to clutch completion. Exposure to high ambient temperatures, in particular, has been shown to reduce hatching success for several species. Accordingly, the egg viability hypothesis posits that when ambient temperatures are high (i.e. above physiological zero, but below optimal developmental temperatures), incubation prior to clutch completion may preserve hatching success. Here, we examined support for the egg viability hypothesis in European starlings (*Sturnus vulgaris*) by investigating the effect of temperature on the incubation behavior of parents and the hatching success of eggs. By manipulating the exposure of eggs to ambient conditions in the presence and absence of parental incubation, we determined if hatching success of

eggs declines with exposure to high ambient temperatures ($>24^{\circ}\text{C}$). Additionally, we examined whether parents increase incubation effort when ambient temperatures were above physiological zero as predicted by the egg viability hypothesis. While we failed to find support for the egg viability hypothesis in starlings, our results are nonetheless informative. By using two separate strategies for analyzing temperature data from nests, we demonstrate that common data reduction techniques may be misleading. As such, we suggest future studies exercise caution when employing strategies intended to simplify the analysis of large data sets. Additionally, we report an interesting relationship between laying order and hatching order within experimental nests. When the onset of incubation was synchronized within a subset of nests, eggs tended to hatch in reverse order of laying. These results are consistent with results of other studies that have found that later laid eggs within clutches develop at a faster rate and hatch sooner than earlier laid eggs – reducing the degree of hatching asynchrony with the nest.

Introduction

Embryonic development in virtually all species of birds is dependent on parental incubation (Drent 1975) and, as a result, parents have the ability to behaviorally influence the development of young by varying their incubation effort over the course of the laying and incubation periods (Wiebe et al. 1998; Wang and Beissinger 2009). In many species of birds, the onset of incubation occurs prior to clutch completion, such that early-laid eggs within a clutch are sometimes incubated for several hours by the time the last egg is laid (Mead and Morton 1985; Lord et al. 2011; Podlas and Richner 2013) – providing the earlier laid eggs with a developmental head start. Consequently, eggs within clutches

frequently hatch asynchronously (Johnston et al. 2013), the degree of which varies among and within species (Zach 1982; Wang and Bessinger 2009), and in the order in which they were laid (Clotfelter et al. 2000). Since earlier-hatched nestlings are better able to monopolize food resources provided by parents (Cotton et al. 1999), nestlings of later laid eggs are placed at a competitive disadvantage and generally suffer reduced growth rates and increased mortality (Zach 1982; Stouffer and Power 1990; Forbes et al. 2001). Because of its pervasiveness throughout a wide range of avian taxa and fitness consequences for both parents and nestlings, hatching asynchrony has received considerable attention in the scientific literature. Several hypotheses – many of which are based on the assumption that selection has favored asynchronous hatching directly – have been proposed to explain the possible evolutionary significance of hatching asynchrony (reviewed in Stenning 1996). However, a separate and smaller set of hypotheses suggest that hatching asynchrony may be incidental and a consequence of selection for incubation prior to clutch completion. This latter group of hypotheses includes the ‘egg viability hypothesis’ that posits incubation during the laying stage may preserve hatching success within clutches when conditions (i.e. ambient temperatures) are not ideal (Arnold et al. 1987; Stolenson and Beissinger 1999).

While many factors – including proper humidity, light exposure, and egg turning – are important for the normal development of avian embryos (Walsberg and Schmidt 1992; Schalkwyk et al. 2000, Clark and Reed 2012), temperature is the most pivotal (Drent 1975). Within most species of birds, the optimal temperature for embryonic development occurs between 36-38° C, though in many species some degree of development in many species can occur at egg temperatures as low as 24° C – a threshold

known as a physiological zero (reviewed in Webb 1987). At temperatures below physiological zero, no development occurs and the viability of eggs declines slowly (Decuypere and Michels 1992). However, prolonged exposure to temperatures above physiological zero yet below optimal temperatures can result in abnormal development and reduced hatching success (Webb 1987; Deeming and Ferguson 1992; Stolenson and Beissinger 1999). According to the egg viability hypothesis, if ambient temperatures are above 24° C during the laying stage, incubation prior to clutch completion may limit exposure of eggs to suboptimal developmental temperatures and minimize reductions in the hatching success of those eggs. However, when ambient temperatures are below 24° C, birds can delay the onset of incubation until clutch completion without sacrificing hatching success of early laid eggs within a clutch.

While first put forth to explain hatching asynchrony in waterfowl (Arnold et al. 1987) and later applied to species breeding in the tropics and sub-tropics (Stolenson and Beissinger 1999; Beissinger et al. 2005) – where ambient temperatures generally exceed physiological zero – there is growing support for the egg viability hypothesis within temperate songbirds. The degree of hatching asynchrony within clutches has been shown to increase with ambient temperatures in Pied flycatchers (*Ficedula hypoleuca*; Slagsvold and Lifjeld 1989) and, more specifically, the onset of incubation occurs earlier at lower latitudes in tree swallows (*Tachycineta bicolor*), where ambient temperatures are warmer (Ardia et al. 2006). Additionally, female tree swallows increase the amount of time spent incubating eggs during the laying stage in response to the experimental heating of nest boxes (Ardia et al. 2009). Finally, by experimentally preventing parental incubation, ambient temperatures have been shown to reduce hatching success of eggs in black kites

(*Milvus migrans*, Viñuela 2000) and house sparrows (*Passer domesticus*, Veiga and Viñuela 1993). However, despite the mounting evidence that ambient temperatures can alter hatching success and incubation behavior, the egg viability hypothesis has received less attention than other hypotheses for hatching asynchrony, such as the brood reduction hypothesis (Lack 1954).

Here, we investigated the egg viability hypothesis in European starlings, a temperate, cavity-nesting songbird species that exhibits a moderate and variable degree of hatching asynchrony (Feare 1984; Cabe 1993). In conducting this research, we considered two key predictions of the egg viability hypothesis: (1) the hatching success of eggs declines with increased exposure to ambient temperatures above 24° C in the absence of parental incubation and (2) parents engage in incubation prior to clutch completion when ambient temperatures are high. To examine whether exposure to high ambient temperatures affected hatching success, we manipulated the exposure of eggs to ambient temperatures in the presence and absence of parental incubation. To determine whether parents increased incubation effort during the laying stage in response to high ambient temperatures, we used automated data loggers to monitor ambient and nest cup temperature over the course of the laying period. Additionally, because we effectively synchronized the onset of incubation for entire clutches within two of our treatment groups, we were able to examine the effect of asynchronous and synchronous incubation initiation on patterns of hatching, nestling growth and nestling survival – allowing us to consider support for the brood reeducation hypothesis (Lack 1954).

Methods

Study System and Experimental Design

This research was conducted in a long-term nest box colony of European starlings in Normal, IL (40.5221° N, 89.0127° W) between April and July of 2013. To investigate the effect of ambient temperatures on hatching success and incubation behavior, we manipulated the exposure of eggs to ambient temperatures in the presence or absence of parental incubation during the laying period.

During the breeding season, cedar nest boxes (for details see Pryor and Casto 2015) were visited daily and, upon discovery, nests were randomly assigned to one of three treatment groups: T_1 , T_2 , or T_3 . Eggs from T_1 nests were collected daily, shortly after laying, then stored in an empty nest box with a blocked entrance hole during daytime hours (0600 – 2000) for the duration of the laying period before being returned to their nest of origin. Because nighttime temperatures occasionally drop below 0° C towards the start of the breeding season, all T_1 eggs were relocated indoors at $\approx 21^\circ\text{C}$ each night (2000 – 0600) to prevent freezing. Thus, these eggs were exposed to daytime ambient conditions for the duration of the laying period and lacked any parental incubation. To control for the effect of storage time in the absence of high ambient temperatures, eggs within T_2 nests were collected daily, shortly after laying, but were stored indoors at $\approx 21^\circ\text{C}$ for the duration of the laying stage before being returned to their nest of origin once laying ceased. Finally, T_3 served as an unmanipulated control treatment. Eggs within T_3 nests remained in nests during the laying period. Because eggs within T_2 were held at a constant temperature, we anticipated less variation within the treatment and assigned nests to T_1 , T_2 , and T_3 at a 2:1:2 ratio.

General Field Methods

All eggs were weighed and marked with a non-toxic marker to indicate their nest of origin and position within the laying sequence. Eggs belonging to either experimental treatment (T_1 or T_2) were removed shortly after laying, replaced with painted wooden eggs, and stored accordingly. Clutches were considered complete when no new eggs were discovered during the daily nest check. Upon clutch completion, eggs belonging to experimental nests were returned to their original nest and wooden replacement eggs were removed.

Using clutch completion date to estimate the date of hatching for each nest, we monitored hatching in all nests. Starting on the projected hatch day, nests were checked every two hours from 0600 – 2000 until either all eggs hatched or no additional eggs hatched for 36 hours. During each nest check, newly hatched nestlings within a nest were uniquely marked by clipping a single toenail. Based on the contents of the nests, we were able to determine the hatching order of nestlings and infer from which egg each nestling had hatched in most cases. In situations in which multiple nestlings hatched between two successive nest checks, nestlings were assigned the same hatch order and randomly attributed to the eggs that had hatched (e.g. if the first and second laid egg had hatched between two consecutive nest checks, one nestling was randomly assigned to the first laid egg and the other nestling was assumed to have hatched from the second laid egg). To minimize disturbance at the nest during this period, nest checks were brief, usually lasting less than one minute. Consequently, rates of nest abandonment during the hatching period were low. We continued to monitor nests during the nestling stage to

check for evidence of brood reduction and fledging. All nestlings were weighed on brood day 5, 10, and 15 (the day on which the first nestling hatches = brood day 0).

Temperature Data Loggers

To examine incubation behavior, we monitored ambient and nest cup temperature using temperature data loggers (iButtons, Maxim Integrated Products; Sunnyvale, CA). Data loggers were installed in each nest and programmed to record temperature and time every 15 minutes. For each nest, data loggers were installed within the nest cup, on the underside of the nest box, and within the nest box approximately one inch from the top of the nest box. However, ambient temperatures within nest boxes were similar to ambient temperatures outside nest boxes, and thus we did not include ambient temperature data collected from inside the nest box in this study. To prevent females from ejecting or repositioning data loggers within the nest cup, data loggers were placed in cloth pouches and tied in place to fix their position within the nest cup. All other data loggers assigned to nests were held in place by hard plastic fobs (Embedded Data Systems; Lawrenceburg, KY) attached to the nest box. For nests assigned to the ambient treatment group, an additional data logger was kept with the eggs during the duration of the laying period.

By comparing ambient and nest temperatures (i.e. data loggers located on the underside boxes and within nest cups, respectively), we were able to determine the amount of heat capable of inducing embryonic development that was attributable to ambient conditions as well as that attributable to parental incubation. To quantify the amount of heat likely to result in embryonic development, we calculated degree hours above 24° C – based on prior estimates of physiological zero (reviewed in Webb 1987).

We determined the number of degree hours above 24° C that were attributable to incubation behavior by comparing external ambient and nest cup temperatures and assuming that positive deviations of nest cup temperatures from ambient temperatures reflected parental incubation. To determine the number of degree hours above 24° C that were attributable to ambient temperature we determined the remaining degree hours above 24° C not due to parental incubation.

Statistical Analyses

To determine the effect of treatment on hatching, we compared the hatching success of eggs between treatments using logistic regression with nest included as a random effect. Additionally, we used logistic regression, with nest included as a random effect, to determine if the probability of hatching decreased as the number of degree hours above 24° C experienced by eggs within the T₁ treatment group increased.

To examine the effect of ambient temperature on incubation behavior during egg laying, we used temperature data collected from all nests and analyzed the relationship between ambient and nest cup temperatures using a two-pronged strategy. First, we used an analysis of covariance to examine the effect of treatment and the number of degree hours above 24° C attributable to ambient temperatures on the total number of degree hours above 24° C attributable to parental incubation. We did this by conducting two separate analyses, using temperature data collected over the course of the entire laying period as well as data that was collected on the penultimate day of laying only – since other analyses of our population have shown that most incubation prior to clutch completion occurs during the penultimate day of laying (JT Hanser; unpublished data).

Additionally, because eggs within both T_1 and T_2 nests were collected and replaced with wooden eggs, they were effectively the same treatment from the standpoint of the parents. Consequently, we collapsed T_1 and T_2 into one treatment and compared it to T_3 in order to examine the effect of real versus wooden eggs on incubation behavior.

For the second analysis, we used repeated-measures mixed-models to examine the effect of ambient temperature and time of day on nest cup temperature. Models were constructed using a multicoinor harmonic regression technique, in which the periodicity of time series data is represented by the summation of multiple pairs of sine and cosine functions (Mikulich et al. 2003; Fernández et al 2009). The inclusion of multiple pairs of sine and cosine functions – each pair having a different period – improves the fit of the model and allows for deviations from a perfect sinusoidal wave (Fernández et al. 2009). As a result, in addition to the fundamental 24-hour period, we constructed models that also included periods of shorter lengths (12, 8, 6, 4.8 hour cycles). Because temperature readings are serially correlated, we specified an autoregressive moving-average [ARMA(1,1)] covariance structure (Jennrich and Schluchter 1986, Ives et al. 2010).

Since most incubation prior to clutch completion occurs on the penultimate day of laying (JT Hanser; unpublished data), we expected ambient temperature would most strongly influence parental incubation on that day. Thus, for simplicity, we restricted this analysis to the penultimate day of laying. Similar to our first analysis, we included treatment in our model by pooling T_1 and T_2 nests into one treatment to consider the effect of wooden eggs versus real eggs on incubation behavior. To control for the possibility that patterns in nest cup temperature differed as a result of clutch size, we also included clutch size in the model. Models were later evaluated by comparing corrected

AIC (AICc) values, where a lower AICc value indicates greater support for a model (Burnham et al. 2011).

To determine the relationship between hatching order and laying order, we fit simple linear regressions to individual nests to quantify the relationship between laying order and hatching order. Because clutch and brood sizes are variable (Cable 1993), we converted raw laying and hatching order to relative measures ranging from 0 to 1. This meant that the relative laying order of first laid eggs was equal to 0 and the relative laying of laid eggs was equal to 1, while intermediately laid eggs were distributed evenly between 0 and 1. Relative hatching order was calculated similarly. Additionally, to examine differences in the relationship between laying order and hatching order between treatments, we compared the slope parameters of individual within-nest regressions line using a Kruskal–Wallis analysis of variance on ranks.

Finally, after establishing that the degree of hatching asynchrony differed between treatments, we examined the effect of asynchronous and synchronous hatching on nestling growth and survival across treatments. Nestling survival was analyzed using logistic regression with nest included as a random effect, while nestling growth data was analyzed using a repeated measures mixed model. All statistical analyses were performed in SAS (version 9.3).

Results

The Effect of Ambient Temperature on Hatching Success and Incubation Behavior

Treatment did not have a significant effect on the probability of eggs hatching ($F_{2,54.22}=2.90$, $p=0.0636$). Moreover, prior to clutch completion, the total number of degree hours

above 24° C did not affect the hatching success of eggs within T₁ nests ($F_{1,46.06}=0.94$, $p=0.3373$). Because the actual physiological zero temperature may be expected to differ between species, we considered additional thresholds. However, neither the total number of degree hours above 26° C ($F_{1,43.63}=1.03$, $p=0.3125$) nor the total number of degree hours above 28° C ($F_{1,39.33}$, $p=0.3855$) had a significant effect on the hatching success of eggs within T₁ nests.

Treatment and the number of degree hours above 24° C attributable to ambient temperature had a significant effect on incubation behavior for the entire laying period as well as for the penultimate day of laying (Table 4). The total number of degree hours above 24° C attributable to parental incubation increased with the number of degree hours above 24° C attributable to ambient conditions over entire laying period ($R^2=0.318$) as well as on the penultimate day of laying only ($R^2=0.419$). Unexpectedly, treatment also had a significant effect in both analyses (Table 4). Over the entire laying period, parents that incubated real eggs (i.e. those from T₃ nests) applied more heat (i.e. degree hours above 24° C) than parents that incubated wooden eggs during the laying stage (i.e. those from T₁ and T₂ nests) (Real: Mean=9.25 deg. hrs above 24° C, SD=0.90; Wooden: Mean=6.31 deg. hrs above 24° C, SD=0.74 deg. hrs above 24° C). Similarly, during the penultimate day of laying, parents that incubated real eggs (i.e. T₃ nests) applied more heat than parents that incubated wooden eggs during the laying stage (Real: Mean=6.50 deg. hrs above 24° C, SD=0.59; Wooden: Mean=4.06 deg. hrs above 24° C, SD=0.47 deg. hrs above 24° C). For both analyses, the number of degree hours above 24° C that

could be attributed to parental incubation was square root transformed to meet the assumptions of normality.

Using a repeated-measures mixed-model approach, we constructed five competing models describing the effect of ambient temperature and time of day on nest cup temperature. AICc values of the various models are summarized in Table 4. Comparison of AICc values revealed that model 5, containing periods of 24, 12, 8, 6, and 4.8 hrs in length, provided the best fit. Within the final model (i.e. model 5), egg type (wooden eggs vs real eggs) did not have a significant effect on nest cup temperature ($F_{1,37}=1.87$, $p=0.1800$). Similarly, the effect of clutch size was not significant ($F_{1,37}=0.91$, $p=0.3452$). As such, both clutch size and egg type were removed from the final model. The final model depicting the effect of time of day and ambient temperature can be seen in Figure 4.

The Relationship Between Laying Order and Hatching Order

While eggs tended to hatch in the order in which they were laid in T_3 nests, hatching order was the reverse of laying order in T_1 and T_2 nests (Figure 5). Slope parameters of within-clutch regression lines were significantly different between treatments ($F_{2,39}=45.55$, $p<0.0001$), indicating that the relationship between laying order and hatching order in T_1 and T_2 nests was different from that in T_3 nests.

The Effect of Treatment on Hatching Asynchrony, Brood Reduction, and Nestling Growth

Treatment had a significant effect on the degree of hatching asynchrony within nests ($F_{2,50}=5.89$, $p=0.0051$), with the degree of hatching asynchrony within T_1 nests being less

than T_3 nests ($p=0.0016$; Tukey-Kramer adjustment for multiple comparisons). However, the degree of hatching asynchrony within T_2 nests was not significantly different from either T_1 ($p=0.5976$) or T_3 ($p=0.1692$). As such, we only considered T_1 and T_3 when examining the effect of asynchronous and synchronous hatching on nestling growth and survival.

In T_3 nests (i.e. more asynchronously hatching nests) hatching order had a significant effect on nestling survival, such that later hatched nestlings were less likely to survive ($F_{1,83}=6.71$, $p=0.0295$). Similarly, the probability of a nestling successfully fledgling declined as the hatching lag (i.e. the time elapsed between the first hatched egg within a nest and the hatching of the focal egg) increased ($F_{1,83}=6.38$, $p=0.0134$). However, overall, the probability of survival for nestlings within T_1 and T_3 nests did not differ ($F_{1,39.1}=1.27$, $p=0.2659$). Treatment also had a significant effect on mass of last hatched nestlings, such that the mass of last hatched nestlings within T_1 was less than the mass of last hatched nestlings within T_3 nests over the course of nestling development (Treatment: $F_{1,14}=6.27$, $p=0.0253$; Nestling Age: $F_{2,28}=184.17$; $p<0.0001$; Treatment*Nestling Age: $F_{2,28}=0.68$, $p=0.4929$).

Discussion

In this study we investigated the egg viability hypothesis in starlings by manipulating the conditions to which eggs were exposed during the laying period. In doing so, we examined two key predictions of the egg viability hypothesis by considering the effect of ambient temperatures on the hatching success of eggs and incubation behavior of parents. Our data demonstrate that the viability of eggs did not decline with exposure to high

ambient temperatures, even after we considered multiple potential thresholds for physiological zero. Additionally, while our initial analysis suggested incubation effort – the number of degree hours above 24° C that could be attributed to parental incubation – increased with the degree hours above 24° C attributable to ambient conditions, a subsequent analysis indicated otherwise (Figure 4). According to our second analysis, while ambient temperature has a positive effect on nest cup temperature during the morning and late evening of the penultimate day of laying, ambient temperatures are typically below physiological zero during these times. However, during the afternoon, when ambient temperatures are occasionally above 24° C, ambient temperature does not have a positive effect on nest cup temperature. Instead, nest cup temperature remains relatively constant regardless of ambient temperature during most of the penultimate day of laying, suggesting that parents incubate less as ambient temperatures increase during the afternoon. As a result, we failed to find support for either prediction of the egg viability hypothesis in this study.

Instead, our results may be consistent with another hypothesis related to hatching asynchrony, the brood reduction hypothesis (Lack 1954). While nestling survival within more synchronously hatching (T_1 nests) and more asynchronously hatching nests (T_3 nests) did not differ, later hatched nestlings were less likely to survive within the more asynchronously hatching nests. Moreover, last hatched nestlings within asynchronously hatching nests weighed less than last hatched nestlings within synchronously hatching nests. These data suggest that while levels of brood reduction were similar between treatments, brood reduction within asynchronously hatching nests was more likely to affect last hatched nestlings that were smaller than their counterparts within

synchronously hatched nests. Thus, brood reduction within asynchronous nests may have been less “costly” than brood reduction within synchronous nests by enabling parents to eliminate the nestling in which they had invested the least. However, limited food availability is a major component of the brood reduction hypothesis (Lack 1954; Viñuela 2000) and, having not directly manipulated or quantified food availability, it is difficult to draw strong conclusions about support for the brood reduction hypothesis from our current data.

Within studies of incubation behavior, the use of iButtons and other automated temperature data loggers is becoming increasingly popular (e.g. Cooper et al. 2005; Ardia et al. 2009; Johnson et al. 2013). However, while this technology offers researchers the ability to amass large amounts of data with relative ease, managing and analyzing large data sets can be difficult (Jennrich and Schluchter 1986, Lynch 2008, Hampton et al. 2013). As a result, studies have typically relied on data reduction techniques to simplify analysis and examine broad patterns in incubation behavior. For example, rather than examining temperature data as a time series data set, some studies have used automated data loggers to determine the total amount of effective heat transferred to nests by parents or the amount of time spent incubating eggs prior to clutch completion (e.g. Badyaev et al. 2003, Ardia et al. 2006, Johnson et al. 2013). While sufficient and appropriate in many situations, data reduction methods result in the loss of information and may limit the ability of researchers to detect more nuanced patterns of incubation in some circumstances.

Within this study, we employed a data reduction method alongside a more comprehensive analysis. While the data reduction method suggested that parents

increased incubation effort in response to ambient temperatures above 24° C, a more detailed analysis demonstrated otherwise. More specifically, our more detailed analysis revealed that nest cup temperature does not increase with ambient temperature on the afternoon of the penultimate day of laying – when ambient temperatures are highest (Figure 4). Rather, despite increases in ambient temperature – which should increase the temperature within the nest box – temperature within the nest cup remains relatively constant during the day, suggesting parents reduce incubation effort as ambient temperatures increase. The positive association between incubation effort and ambient temperatures above 24° C may reflect limitations within our simplified analysis. First, for days when ambient temperatures frequently exceed 24° C, ambient temperatures during the morning and evening hours may also be greater – though not necessarily above 24° C. However, because nest cup temperature increases with ambient temperatures during these hours, an increase in ambient temperatures during the morning and evening on warmer day may cause nest cup temperatures to rise above 24° C – despite ambient temperatures during these hours not exceeding 24° C. Additionally, the positive association between incubation effort and ambient temperatures above 24° C is also likely due to the confounding effect of ambient temperature and the metric of parental incubation used (i.e. degree hours above 24° C attributed to parental incubation). For example, if ambient temperature is greater than 24° C, any additional heat applied by parents would inherently increase the degree hours above 24° C that were attributed to parental incubation. However, when ambient temperatures are below 24° C, not all additional heat applied to eggs would be attributed to parental incubation – since parents may maintain eggs at

temperatures above ambient conditions yet below 24° C. As a result, we suggest future studies that employ the use of automated temperature data loggers or that deal with similarly large data sets exercise caution when designing incubation metrics and reducing data as a means of simplifying analysis.

Unexpectedly, while egg type was not a significant factor in our more detailed analysis of incubation behavior, it had a significant effect on the total number of degree hours above 24° C that could be attributed to parental incubation (Table 4). On average, the number of the degree hours above 24° C that could be attributed to parental incubation was greater in nests that contained real eggs. This effect was statistically significant when the data from the entire laying period was analyzed as well as when the analysis was restricted to the penultimate day of laying. This result may simply be due to differences in the thermal properties of real and wooden eggs. For example, the specific heat of dried wood ranges from 1.3-1.5 kJ·kg⁻¹·K⁻¹ (reviewed in Simpson and TenWolde 1999), while the specific heat of the yolk (approx. 3.0 kJ·kg⁻¹·K⁻¹) and albumen (approx. 3.9 kJ·kg⁻¹·K⁻¹) are considerably greater (Romanoff & Romanoff 1949). These values suggest that it takes more heat to raise the temperature of real eggs than wooden replicas. Thus, in the absence of any changes in incubation behavior of parents prior to clutch completion, we should expect wooden eggs to be warmer on average than real eggs and data loggers positioned at the base of the nest cup – just beneath the eggs – to record higher nest cup temperatures. Alternatively, the effect may be more complex and involve changes in the incubation behavior of parents as a result of differences in the thermal properties of wooden and real eggs. In Belding's Savannah sparrows, the experimental heating of eggs after completion of the clutch caused parents to decrease their incubation

attentiveness (Davis et al. 1984). If starlings similarly modulate incubation behavior during the laying stage as a result of egg temperature, then parents of nests containing wooden eggs – which heat faster – may increase the frequency of off-bouts or decrease the duration of on-bouts. However, it is not entirely clear how this interaction would produce the observed effect of warmer temperatures in nest containing wooden eggs.

Interestingly, while eggs generally hatched in the order in which they were laid in T_3 nests much like other studies (e.g. Clotfelter et al. 2000), hatching order was reversed in nests in which the onset of incubation was synchronized (Figure 5; T_1 and T_2). Several recent studies have reported that eggs across the laying sequence differ in the rate at which they develop, such that later laid eggs within a clutch develop more rapidly and hatch sooner than earlier laid eggs (Nicolai et al. 2004; Muck and Nager 2006; Boonstra et al. 2010; Hadfield et al. 2013). While we were unable to control for the effect of storage time on the duration of incubation required for eggs to hatch, our results are consistent with other studies that suggest intrinsic differences in eggs across the laying period may reduce the duration of incubation required for hatching in eggs laid latter in the laying sequence. If true, these results contradict early models of hatching asynchrony that assume embryonic developmental rates are identical across all eggs in a clutch and that hatching asynchrony is due solely to differences in the onset of incubation-induced development (e.g. Clark and Wilson 1981). Instead, differences in the rate of embryonic development across the laying sequence may also affect hatching patterns within nests – potentially minimizing the degree of hatching asynchrony within clutches, and calling into question whether it is truly adaptive. Future research should investigate possible mechanisms that may mediate such hatching patterns within birds. For example, several

maternally derived steroids hormones are found in the egg yolk (e.g. Schwabl 1993) and multiple studies have demonstrated their potential to influence offspring phenotype (reviewed in Gil 2003). Moreover, some reports suggest that yolk testosterone may influence the rate of embryonic development (Eising et al. 2001; Eising and Groothuis 2003; but see Sockman and Schwabl 2000) and one study has experimentally demonstrated that yolk testosterone increases the size of the hatching muscle within red-winged blackbird nestlings (*Agelaius phoeniceus*; Lipar and Ketterson 2000), which may facilitate earlier hatching.

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Table 4. Analysis of covariance for the effect of treatment (real eggs vs wooden eggs) and number of degree hours above 24° C due to ambient conditions on incubation effort of parents. Type three sums of squares solutions for treatment and number of degree hours above 24° C that could be attributed to ambient condition are also shown.

	Source	df	Sum of Squares	Mean Square	F Value	p
<i>Entire Laying Period</i>	Model	2	227.03	113.52	8.63	0.0008
	Error	37	486.42	13.15		
	Total	39	713.45			
	Treatment	1	82.80	82.81	82.81	0.0166
	Degree Hours Above 24° C	1	160.11	160.11	12.18	0.0013
<i>Penultimate Day of Laying</i>	Model	2	145.58	72.79	13.34	<0.0001
	Error	37	201.86	5.46		
	Total	39	347.44			
	Treatment	1	56.21	56.21	10.30	0.0027
	Degree Hours Above 24° C	1	107.24	107.24	19.66	<0.0001

Table 5. Summary of fit statistics¹ for the competing models describing the interaction between ambient temperature and time of day on nest cup temperature, including comparison of AICc values.

Model	Length of Periods Included within Model	-2 LL ²	AIC	AICc	Δ AICc
1	24	14937.7	149743.7	14943.8	129.9
2	24, 12	14865.7	14871.7	14871.7	57.8
3	24, 12, 8	14843.0	14849.0	14849.0	35.1
4	24, 12, 8, 6	14816.2	14822.2	14822.2	8.3
5	24, 18, 8, 6, 4.8	14807.9	14813.9	14813.9	0.0

¹Lower AICc value indicates a better fit.

²-2 LL = -2 Log Likelihood

Figure 4. *The effect of time of day and ambient temperature on nest cup temperature on the penultimate day of egg laying.*

Figure 5. *The relationship between relative laying order and relative hatching order for: (a) T_1 , (b) T_2 , and (c) T_3 nests. Light grey lines represent individual nests, while the dark lines represent the average relationship between laying order and hatching order for each treatment – determined by averaging the regression parameters (i.e. slope & intercept) for nests within each treatment.*

CHAPTER III

THE EFFECTS OF STORAGE TIME AND YOLK TESTOSTERONE

ON HATCHING ASYNCHRONY IN EUROPEAN STARLINGS

(*STURNUS VULGARIS*)

Abstract

Within most species of birds, eggs tend to hatch asynchronously and in the order in which they are laid. However, several studies have reported differences in the developmental rates of eggs within clutches, such that later laid eggs within a clutch develop more quickly than earlier laid eggs. Recently, we manipulated the onset of incubation in a subset of European starling (*Sturnus vulgaris*) nests by removing each egg shortly after laying and returning eggs to their original nest after clutch completion. While laying order predicted hatching order within unmanipulated nests, hatching order was reversed in nests in which the initiation of incubation was synchronized. These results are, consistent with the idea that later laid eggs within a clutch require a shorter period of incubation before hatching – minimizing the degree of hatching asynchrony within a nest. While their effects are not fully understood, yolk steroids, testosterone in particular, represent a potential mechanism through which females may be able to adjust the developmental rates of embryos. Here, we examine whether the differential transfer of testosterone (T) to egg yolks across the laying sequence influences the development of

embryos and limits the degree of hatching asynchrony. Alternatively, because of a limitation within our prior experiment, we also considered the effect of storage time on the duration of incubation prior to hatching. While several hypotheses have been proposed to explain the adaptive significance of hatching asynchrony, empirical support for these hypotheses is often mixed. Additionally, while many hypotheses suggest that parents benefit directly from asynchronous hatching, others have suggested that hatching asynchrony represents an incidental consequence of either a physiological constraint controlling incubation behavior or selective pressures not directly related to asynchronous hatching and the formation of nestling size hierarchies. In such cases, parents and offspring may benefit from minimizing the degree of hatching asynchrony within clutches.

Introduction

In contrast to the assumption of an early mathematical model of hatching asynchrony – that the rate of embryonic development and hence the duration of incubation are equal for all eggs within a clutch (i.e., variable t_2 in Clark and Wilson 1981) – several recent studies across avian taxa have found that later laid eggs develop faster and hatch sooner than earlier laid eggs (Nicolai et al. 2004; Muck and Nager 2006; Boonstra et al. 2010; Hadfield et al. 2013). Similarly, when the onset of incubation was experimentally manipulated in European starlings by removing eggs from a subset of nests until clutch completion, hatching patterns were also affected. While eggs hatched in the order in which they were laid within control nests, eggs hatched in reverse order when the onset of incubation was synchronized (JT Hanser; see Chapter 2). Taken together, these reports

suggest that in a diverse array of avian species eggs across the laying order differ intrinsically, such that those laid later within a clutch require shorter periods of incubation before hatching. By altering hatching patterns, these intrinsic differences may mitigate costs associated with asynchronous hatching, including reduced rates of growth, increased rates of mortality, and reduced body condition at the time of fledging amongst later-hatched nestlings (Zach 1982; Stouffer and Power 1990; Forbes et al. 2001).

While the mechanism underlying these results is not known, several maternally derived steroid hormones are found within the yolks of avian eggs (Schwabl 1993) and may enable females to influence within-clutch patterns of development and hatching (Gorman and Williams 2005). Evidence suggests that these hormones are metabolized by developing embryos during incubation (von Engelhardt et al. 2009; Paitz et al. 2011; Paitz and Casto 2012) and are capable of influencing development and offspring phenotype (reviewed in Gil 2003). Yolk testosterone, in particular, has received considerable attention and, in some species, has been shown to increase the rate embryonic development and reduce the length of incubation (Eising et al. 2001; Eising and Groothuis 2003; but see Sockman and Schwabl 2000; von Engelhardt et al. 2005). Thus, the differential allocation of testosterone (T) across the laying sequence may cause later laid eggs within clutches to hatch earlier - limiting hatching asynchrony within species in which the concentration of yolk T increases across the laying order, such as starlings (Lipar 2001; Pilz et al. 2003).

If yolk T influences the length of incubation duration required for hatching, T may achieve this effect by altering the development of the muscular system underlying hatching behavior in embryos. In birds, hatching is a prolonged process involving the

coordination of several muscle groups wherein embryos systematically weaken the eggshell by rotating within the egg and repeatedly exerting pressure against the shell (Oppenheim 1972; Bond et al. 1988). Known as the hatching muscle, the musculus complexus is a large dorsally located muscle of the neck responsible for providing the force necessary to break through the shell during hatching. During development, an influx of lymph from a pair of adjacent lymph nodes causes the muscle to swell and exert pressure against the eggshell wall resulting in the formation of cracks around the circumference of the broad end of the egg (reviewed in Smail 1964). In red-winged blackbirds, yolk T has been experimentally shown to increase the mass of the musculus complexus in nestlings when measured two days after hatching (Lipar and Ketterson 2000). Thus, by increasing the mass of the hatching muscle, yolk T may alter hatching patterns by advancing the start or reducing the duration of hatching.

Alternatively, yolk T may influence embryonic development and hatching patterns in starlings by facilitating gas exchange. In avian embryos, gas exchange is limited by diffusion across the eggshell and chorioallantoic membrane. During the course of development, the metabolic demands of avian embryos increase several fold (Vleck et al. 1979) and the increased demand for oxygen is met by changes in the chorioallantoic membrane that allow for greater diffusion of oxygen (reviewed in Mortola 2009), including increases in hematocrit and hemoglobin concentrations (Tazawa 1971; Tazawa 1980). Prior work by our lab has demonstrated that most yolk T in starling eggs is converted to another hormone, etiocholanolone, during the first half of embryonic development (Paitz et al. 2011). While poorly understood, etiocholanolone has been demonstrated to stimulate erythropoiesis during early development in chicken embryos

(Levere et al. 1967). Thus, by increasing red blood cell production, yolk T may influence the rate of embryonic development and, subsequently, hatching patterns in starlings.

Here, we investigate the potential of yolk T to influence hatching patterns in starlings. By experimentally manipulating the concentration of T available to embryos, we aim to determine whether yolk T alters the period of incubation required before hatching and, if so, whether this change can be explained by an increase in the relative mass of the hatching muscle and/or an increase in the production of red blood cells. Moreover, by pharmacologically blocking androgen receptors, we examine potential androgenic and non-androgenic effects of prenatal T. If T acts via androgen receptors to decrease the incubation period, hatching should be delayed in eggs treated with an androgen receptor antagonist compared to control eggs. However, if T acts via a non-androgenic metabolite, eggs treated with an androgen receptor antagonist should not differ from control eggs.

Additionally, because within Chapter 2 we were unable to control for the effect of storage time within our experiment in which we synchronized the onset of incubation in European starlings, we consider the effect of storage time on the duration of incubation required before hatching. Storage time has been shown to lengthen the period of incubation required before hatching in chickens (Tona et al 2003a; Tona et al. 2003b). However, eggs used in those studies were stored substantially longer than the eggs used in our study and, even within chickens, shorter periods of storage may not lengthen incubation duration (Reis et al. 1997). Nonetheless, by storing eggs for varying lengths of time prior to artificial incubation, we examine whether short periods of storage have the

potential to influence hatching patterns in starlings and explain the reversed hatching order observed when onset of incubation is synchronized.

Methods

Study System and General Methods

All research was conducted in a long-term nest box colony of European starlings in Normal, IL (40.5221° N, 89.0127° W) between April and July of 2014. During the breeding season, nest boxes were checked shortly after laying each day and newly discovered nests were assigned to an experiment.

For both experiments, nests were visited daily and all newly laid eggs were collected, replaced with wooden decoys, and transported to our laboratory at Illinois State University where they were incubated using artificial incubators. All eggs were incubated at approximately 37.5° C at 60% humidity until they either hatched or until it was determined that the embryo had died. Eggs were rotated periodically during incubation and, starting on the projected hatch day, hatching was monitored every hour between 0700 – 2000. Upon hatching, nestlings were weighed, measured, and killed by decapitation. Afterwards, the musculus complexus was dissected, weighed, dried, and reweighed. Additionally, blood was collected to determine hematocrit, and assess hemoglobin concentration via the cyanomethemoglobin method (Drabkin and Austin 1932).

Females were allowed to incubate decoy eggs for 7 to 10 days, before being captured, banded, weighed, and measured. After capturing females, decoy eggs were

removed and birds were allowed to re-nest. Subsequent, re-nesting attempts by females were also included in the study, though not necessarily in the same experiment.

Experiment 1: Effect of Testosterone

To examine the effect of testosterone on the period of incubation required for hatching, we injected eggs with vehicle, testosterone, or flutamide (F) – a non-steroidal androgen receptor antagonist (Neri et al. 1972). Within this experiment, nests were assigned in alternating fashion to one of two treatments: (1) T treatment or (2) F treatment. Within T-treated clutches, first laid eggs were injected with 0.234ng of T in vehicle (5µL USP-grade sesame oil), while last laid eggs were injected with 5µl of vehicle. Within F-treated clutches, last laid eggs were injected with 0.045mg of F in vehicle (5µL USP-grade sesame oil), a non-steroidal androgen receptor antagonist (Neri et al. 1972) in vehicle, while first laid eggs will be injected with vehicle.

Because the difference in testosterone levels is greatest between first and last laid eggs, we restricted our manipulations to the first and last laid egg within each clutch. By injecting first laid eggs with a dose of testosterone that was equivalent to the mean difference in testosterone between first and last laid eggs (JM Casto; unpublished data), we simulated testosterone levels that were similar to those typically found within last laid eggs. Likewise, by dosing last laid eggs – which exhibit the highest levels of testosterone – with flutamide, we attempted to approximate reduced androgen influence similar to that of first laid eggs.

To ensure an appropriate flutamide dose, we used an amount that had previously been shown to be effective in red-winged blackbirds (Lipar and Ketterson 2000) and

scaled the dose to reflect the difference in the average mass of starling eggs compared to red-winged blackbird eggs. Additionally, because clutch size varies within starlings (Cabe 1993), restricting our manipulations to the first and last laid egg within a clutch allowed us to control for the relative position within the laying sequence. To create a hole through which we could inject solutions into eggs, we used a 26G needle to bore a small hole in the eggshell towards the narrow end of the egg. In order to minimize disturbance to the embryo and any potential contamination, all solutions were injected into the albumen of the egg and the site of injection was sterilized with rubbing alcohol and sealed afterwards with superglue.

Initially, eggs in this experiment were incubated in a still-air incubator (Series II Model 3110, Forma Scientific Inc., Marietta, OH) and manually rotated at least once every two hours between 0700 – 2000. However, after it became clear that hatching success within this incubator was unacceptably low ($<<50\%$), eggs were subsequently incubated in a forced-air incubator (Model 1-36VL, Percival Scientific Inc., Boone, IA) and manually rotated at least once every two hours between 0700 – 2000. Finally, because hatching success was still unsatisfactorily low ($<<50\%$), we employed the use of small forced-air incubators (TX7 automatic egg turning incubator, Lyon Technologies Inc., Chula Vista, CA) that rotated eggs automatically approximately once per hour.

Experiment 2: Effect of Storage Time

To determine the effect of storage time on the period of incubation required before hatching, we manipulated the length of time that eggs were stored prior to incubation. Accordingly, upon discovery, nests were randomly assigned to one of two treatment

groups: (1) Stored and (2) Not Stored. Within nests that were assigned to the Not Stored treatment group, eggs were placed in an incubator on the same day they were collected. Conversely, within nests that were assigned to the Stored treatment group, eggs were stored at 21° C and 60% humidity until the completion of the clutch. After clutch completion, all eggs were moved to an incubator. All eggs within this experiment were incubated within small forced air, dome incubators (TX7 automatic egg turning incubator, Lyon Technologies Inc., Chula Vista, CA) that automatically rotated eggs every hour.

Experiment 3: Effect of Egg Injection Protocol (pilot study)

To examine the effect of our egg injection protocol on hatching success, we used a modified Latin square design and injected eggs with a solution containing testosterone, flutamide, or a vehicle. Afterwards, we returned eggs to their original nests so that they could be naturally incubated. Doses of testosterone and flutamide within this experiment were the same as those used in Experiment 1; however, we injected all eggs within each clutch – not just first and last laid eggs. Likewise, egg injections were performed similarly to those in experiment 1.

We monitored these nests periodically, approximately once every three days, throughout incubation to determine hatching success. Because nests were monitored infrequently, we were occasionally unable to determine the precise number of eggs that hatched within a clutch. This uncertainty is likely due to the behavior of parents who occasionally remove unhatched eggs and dead nesting's from their nests. Because of this

uncertainty regarding the exact number of eggs that hatched, we report a minimum and maximum estimate for hatching success for eggs within this treatment.

Results

Experiment 1: Effect of Testosterone

Out of the 160 eggs that were collected, injected, and incubated, only 1 egg hatched.

Experiment 2: Effect of Storage Time

Out of the 78 eggs that were collected and incubated, only 4 eggs hatched.

Experiment 3: Effect of Egg Injection

Out of the 26 eggs that were injected and incubated by their parents, between 15 and 20 eggs hatched.

Discussion

Poor hatching success within both of our experiments prevented any analysis of the effect of testosterone or storage time on incubation duration. As part of a separate study in which eggs were injected and returned to their original nest so that parents could incubate the eggs naturally, hatching success ranged from approximately 58-77% percent. While lower than the hatching success of eggs in nature (JT Hanser, see below), these results - combined with the low hatching success within our experiment that examined the effect of storage time - suggest that the limited hatching success within this study was not due to our egg injection protocol. Instead, these results suggest that the incubation regime

used within our experiments was inappropriate. Hatching success in studies that employ artificial incubators is frequently lower than hatching success in nature (Batt and Cornwell 1972; Arnold et al. 1987), where as many as 90% of eggs hatch in starlings (JT Hanser; unpublished data). Temperature, humidity, light exposure, and the frequency at which eggs are rotated can all affect embryonic development and hatching success (Walsberg and Schmidt 1992; Schalkwyk et al. 2000; Hepp et al. 2006; Clark and Reed 2012), though temperature is the most critical factor for hatching success (Drent 1975). While we were not aware of the study at the time of this research, at least one paper in European starlings suggests that the optimal temperature for incubation may be 35 °C, and that temperatures slightly below that can also be relatively effective (Reid et al. 2002). Thus, it is possible that the temperature used within the artificial incubator was too high. However, without more information, it is difficult to determine the exact source of the low hatching success in this study.

While the results of this study were, understandably, disappointing they do offer a silver lining for future research interested in the effects of maternally deposited yolk steroids. Hatching success of injected eggs that were incubated by parents, though lower than levels observed in nature, was sufficiently high enough to allow experimental manipulation of hormone levels in future research, assuming that the research design allows eggs to be incubated by parents.

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