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DO PARENTS ADJUST INCUBATION BEHAVIOR AS A FUNCTION OF NEST ECTOPARASITES? AN EXPERIMENTAL ANALYSIS OF THE EFFECTS OF NEST MITES ON INCUBATION RHYTHMS IN BARN SWALLOWS

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GLOSSARY

Abiotic – Physical (non-biological) features of the environment (e.g., temperature, humidity, precipitation).

Altricial – Upon hatching, young rely heavily on parental care during early development.

Attentiveness – Refers to parental care at the nest during the incubation period (e.g., monitoring egg temperatures via brood patch contact, egg turning, balancing time on and off the nest).

Biotic – Biological features of the environment (e.g., parasites, bacteria, plants, decomposers).

Brood – The number of nestlings in a single nest.

Brood patch – Ventral area of hormone-induced feather loss to allow an increase in heat transfer to eggs during incubation.

Clutch – The number of eggs laid by a female within a single nest.

Cross-foster – An experimental procedure where half the young from two broods are switched between nests, sometimes in association with a particular treatment. This design is effective to analyze the relative influence of genetics and the environment on particular traits during early development.

Egg-turning – A parental behavior during incubation to help distribute temperature to all surfaces of the eggs.

Incubation period – The total number of days from the start of incubation (one day prior to clutch completion) to hatch date.

Off-bout – A period of time an incubating parent is off the nest.

On-bout – A period of time an incubating parent is on the nest transferring heat to their eggs.

Passerine – Refers to all songbirds (of the order Passeriformes) and includes more than half of all bird species.

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ABSTRACT

Avian incubation behavior involves a complex set of decisions to balance off-bouts of self-maintenance and on-bouts of egg temperature regulation. Nest ectoparasites may indirectly affect offspring fitness by directly altering the lengths and frequencies of parental on and off-bouts during incubation. Although parasites are known to impact parental care when nestlings are present, whether parental incubation behavior changes in response to nest ectoparasites is not well understood. To assess whether nest ectoparasites influence incubation behavior, I manipulated northern fowl mites (*Ornithonyssus sylviarum*) in barn swallow (*Hirundo rustica erythrogaster)* nests at the start of incubation. I compared disinfected and parasitized nests using model eggs with temperature data loggers to measure incubation rhythms and temperatures. The four main questions were 1) do parasites influence the duration of the incubation period 2) do parasites influence incubation rhythms 3) do parasites influence egg temperature and 4) does incubation behavior change across the incubation period. All variables of interest exhibited high variation across all three phases of incubation regardless of clutch size or treatment, indicating incubation is a highly flexible parental behavior. These results suggest parasites do not directly affect incubation behavior, however clutch size increases time on nest during the early stages of incubation. This suggests incubation is a dynamic behavior possibly constrained by parental fitness and other environmental factors, rather than nest ectoparasite intensity. Although ectoparasites are known to influence barn swallow territory choice and nestling provisioning, my results demonstrate that the presence of this ectoparasite does not influence incubation behavior.

INTRODUCTION

Avian incubation is a complex and dynamic behavior that requires continuous regulation by the incubating parent (Cooper and Voss 2013). During the incubation period, parents adjust their behavior to limit temperature variation of the developing embryos (Álvarez and Barba 2014; Ardia *et al.* 2006; Ardia *et al.* 2009; Cooper and Voss 2013). In order to develop successfully, avian embryos require near constancy of optimal temperatures which range from about 36 – 39 ° C (Reid *et al.* 2002). Parents must frequently attend to their clutches during the incubation period in order to sustain this temperature constancy (Ardia *et al.* 2009). Egg temperatures that drop below the minimum threshold of 26° C encounter suboptimal developmental conditions that can cause detrimental effects to the embryo (Webb 1987; Ardia *et al.* 2009). However, parental effort during incubation involves a series of important trade-offs that introduce many energetic constraints (Bryan and Bryant 1999; Reid *et al.* 2000; Pérez *et al.* 2008; Ardia *et al.* 2009; Cooper and Voss 2013). These constraints and tradeoffs can be seen in the frequent on and off-bouts, known as incubation rhythms**,** that are characteristic of incubation behavior across many avian taxa (Martin 2002; Martin and Schwabl 2008; Ardia *et al.* 2009; Cooper and Voss 2013; Álvarez and Barba 2014). During on-bouts, parents are investing in temperature regulation and optimal embryonic development, while off-bouts are dedicated to self-maintenance behavior including foraging and preening (Álvarez and Barba 2014; Ardia *et al.* 2009; Cooper and Voss 2013; MacDonald *et al.* 2012).

Parental care trade-offs are dynamic across the incubation period as adults constantly adjust their behavior to complement developmental changes of growing embryos. Parental behavior is predicted to change as the incubation period progresses and blood vessels begin developing. An increase in blood circulation properties as embryos age can affect how heat is

distributed from brood patch contact and then lost to the environment (Prinzinger and Dietz 1995; Turner 2002; Tzschentke and Rumpf 2011; Boulton and Cassey 2012; Cooper and Voss 2013; Tong *et al.* 2013). Previous studies have shown that females will modify their incubation rhythms in response to an increase in the rate of heat loss of aging embryos, in order to increase mean egg temperatures and minimize temperature variations. This results in a higher frequency of shorter on and off-bouts (Cooper and Voss 2013), which increases the time parents spend directing energy towards embryo development (Álvarez and Barba 2014; Cooper and Voss 2013). Thus, measuring incubation behavior at several different time points across development is important, and can account for deviations in temperature demands.

Female condition, experience, age and other fitness-related factors have all been shown to directly impact incubation behavior (Engstrand and Bryant 2002; Ardia *et al.* 2009; Jones 1987). However, it is not well understood how other aspects of the nest environment may influence incubation behavior, particularly those that can have important impacts on nestling survival and fitness. One such aspect is the effect of nest ectoparasites on incubation behavior.

Ectoparasites live and feed externally on their hosts (e.g., ticks, fleas, mites) and have been shown to cause many detrimental effects, including reduced survival, lower reproductive success, and decreased body condition (Clayton and Tompkins 1995; Møller 1990; Proctor and Owens 2000). Effects from parasites in wild bird-ectoparasite interactions can be age-specific and costly to young offspring that are confined to the nest (Martin *et al.* 2001). While such infections have been shown to influence parental investment in provisioning and sanitation (nest cleaning) behavior (Christe *et al.* 1996a; Hund *et al.* 2015a; Tripet and Richner 1997), it is relatively unexplored how the presence of nest ectoparasites may influence the fine scale regulation of incubation rhythms.

During incubation, parents can respond to the presence of ectoparasites through an increase in resource allocation to either 1) current reproduction or 2) parasite defense and future reproduction (Gallizzi *et al.* 2008; Linden and Møller 1989; Saino 2002; Richner and Triplet 1999). Thus an infected nest may indicate to incubating parents that their chicks are going to be of lower quality with reduced chances of survival and that they should save resources in order to invest more in the future reproduction of higher quality clutches (Hund *et al*. 2015b; Gallizzi *et al.* 2008; Møller 1990; Møller 1993), and allocate more time to self-maintenance behaviors (Møller 1990). Alternatively, parents in infected nests may invest more time to their clutch during incubation in order to hatch higher quality nestlings that can better deal with the impending costs that parasites impose. This allocation of resources to current reproduction has been seen in some studies measuring provisioning rates and parasites; adults increased parental care in nests with heavy parasite-loads (Hund *et al.* 2015a; Tripet and Richner 1997; Richner and Tripet 1999). Thus, incubation represents an interesting comparison in the allocation of parental care as ectoparasites will impose costs on nestlings that have yet to hatch, so tradeoffs between current and future reproduction are more temporally isolated than during the nestling period.

Studies have shown that blood-feeding ectoparasites, such as haematophagus mites and hen fleas, (Møller 1990; Clayton and Tompkins 1995; Fitze *et al.* 2004) can influence nest attendance behavior during incubation, as adults may spend more time off the nest preening in order to remove parasites, thus investing more in self-maintenance due to physical agitation (Clayton and Tompkins 1995). By reducing the time spent at the nest, parents also reduce the risk of parasite transmission (Christe *et al.* 1996a; Møller 1990; Richner and Heeb 1995). Previous studies have also demonstrated that high ectoparasite loads can influence the duration of the incubation period. Møller (1990) found that nests with high tropical fowl mite

(*Ornithonyssus bursa*) densities lengthened the incubation period of European barn swallows (*Hirundo rustica rustica*) yet shortened the nestling period. Fitze (2004) also found that the presence of hen fleas (*Ceratophyllus gallinae*) in great tit nests (*Parus major*) prolonged both the incubation and nestling period. While studies have explored patterns with parasite infection through both nest attendance behaviors and the length of incubation, it remains unknown how parasites influence incubation behavior on a fine scale and if these changes are dynamic across embryo development.

To test how ectoparasites influence fine scale incubation behavior across the embryonic developmental period, I experimentally manipulated densities of the northern fowl mite (*O. sylviarum*) in the nests of barn swallows (*H. r. erythrogaster)* and used thermocouple eggs to measure incubation rhythm temperatures. Northern fowl mites (*O. sylviarum*) are haematophagus ectoparasites that live in the nest material of barn swallows and feed on nestlings. However, during incubation, mites lack access to nestlings and parents represent the only source of blood for mites.

How incubating adults adjust their behavior and regulate nest temperature in response to nest ectoparasite infections represents an important gap in knowledge in the field of incubation research. I looked at four main questions in order to test this (Table 1): 1) how do parasites influence the duration of the incubation period 2) how do parasites influence the frequency and length of incubation rhythms 3) how do parasites influence egg temperatures and 4) does incubation behavior change across all stages of incubation (early, middle, and late). Since previous research has shown that clutch size can impact incubation behavior, I included this variable in all empirical analyses.

Table 1 Hypotheses and predictions

Question	Hypothesis/prediction	Test	Evidence
How do parasites influence the duration of the incubation period?	H1. Parasites decrease the duration of incubation (parent's invest more: faster embryonic development) H2. Parasites increase the duration of incubation (parent's invest less: slower embryonic development) H3. Parasite manipulation does not affect the length of incubation and rate of embryonic development	Analyzed the length of incubation periods (number of days to hatch) based on treatment.	H1: NA H2: (Fitze *et al.* 2004; Martin and Schwabl 2008; Møller 1990; Møller 1993) H3: NA
How do parasites influence the duration and frequency of adult on-bouts (warming and equilibrium periods) and off-bouts?	H1. Parasites decrease the duration and increase the frequency of on and off-bouts (parents invest more in incubation) H2. Parasites increase the duration and decrease the frequency of on and off-bouts (parents invest more in self-maintenance) H3. Parasite manipulation does not affect the duration or frequency of on and off-bouts (parasites do not influence trade-off between self-maintenance and incubation)	Analyzed the length of off-bouts, the length of on-bouts (total time on nest; combined warming and equilibrium period lengths) and number of on-bouts, based on treatment.	No current studies examining the relationship between bout length and frequency to ectoparasite intensity
How do parasites influence nest temperatures?	H1. Parasites increase the mean egg temperature and lower temperature standard deviation (parent's invest more in incubation) H2. Parasites decrease the mean egg temperature and increase temperature standard deviation (parent's invest more in self-maintenance) H3. Parasite manipulation does not affect the mean temperature or standard deviation (parasites do not influence tradeoff between self-maintenance and incubation)	Analyzed the mean nest temperature and standard deviation in nest temperature based on treatment.	No current studies examining the relationship between nest temperature and temperature variation to ectoparasite intensity
How does incubation behavior due to parasites change across the incubation period based on different stages of embryonic development?	1. Parental behavioral response due to parasites changes over the course of the incubation period; predicts a change in nest attentiveness due to parasites across developmental stages (early, middle, and late) 2. Parental behavioral response due to parasites shows no difference over the course of the incubation period; predicts no change in nest attentiveness due to parasites across developmental stages (early, middle, and late)	Analyzed questions two and three across all three stages of embryonic development and compared (early, middle and late).	H1: Turner1997; Boulton and Cassey 2012; Cooper and Voss 2013; Martin and Schwabl 2008; Álvarez and Barba 2014) H2: NA

MATERIALS AND METHODS

Study System: North American barn swallow *(Hirundo rustica erythrogaster)*

      The North American barn swallow (*H. r. erythrogaster)* is a widespread migratory passerine. Barn swallows are relatively small (17 - 20 g) and breed in small to large colonies in the Northern hemisphere where they complete one to three breeding attempts per year (Scordato and Safran 2014; Hund *et al.* 2015a). Barn swallows exhibit biparental care of their nestlings (both males and females participate). Nestlings are altricial, which means they depend on parental care after hatching and can remain in the nest for 15 – 27 days, but typically fledge after an average of 17 days (Brown and Brown 1999). Barn swallows lay three to seven eggs per clutch and perform biparental intermittent incubation (early onset incubation, which begins prior to clutch completion upon laying the penultimate egg), although only females form brood patches and perform the majority of incubation. The incubation period can range from 12 – 17 days (Brown and Brown 1999).

Northern fowl mite (*Ornithonyssus sylviarum)*

      The northern fowl mite *(O. sylviarum)* is a blood-feeding (haematophagus) external parasite of the family *Macronyssidae* (parasitic mites) that lives in the nest material of North American barn swallows *(H. r. erythrogaster)* (Hund *et al.* 2015a; Proctor and Owens 2000). Fowl mites are the primary nest ectoparasite of *H. r. erythrogaster* and feed on altricial nestlings confined to the nest during development. These mites overwinter in nests and begin emerging upon the start of the breeding season (Hund *et al.* 2015b). Female mites on average lay two to five eggs and reproduction cycles are short; the entire life cycle from egg to reproducing adult is completed within a week (five to seven days) in the presence of available blood meals. Thus, the

mite population can change dramatically across the developmental period of nestlings with two to three generations of mites per nestling period (Richner and Heeb 1995; Proctor and Owens 2000; Szabo *et al.* 2002).

Field Methods

    I conducted a large field experiment across 24 barn swallow breeding colonies in Boulder County during the summer of 2016 (May – July). Adult birds were temporarily captured using mist nests and targeted night captures. Each adult was given a numbered aluminum leg band (United States Geological Survey) and a unique color combination consisting of colored leg bands and colored tape that allowed for individual identification at nest sites. Field observations were conducted to identify adults associated with a particular nest in order to define 1) the social mating pair, and 2) the pair's corresponding nest. All nests at study colonies were checked every three days to determine when pairs began lining their nests with feathers and active nests were then checked daily in order to obtain accurate dates for clutch initiations and clutch completions. Nests where eggs were being incubated were checked every three to four days to minimize disturbance and to monitor for predation events.

    Experimental nests were paired by clutch completion date: for these nests incubation began at the same time and eggs were at the same developmental time point. On the morning of the third day of incubation, the largest and smallest eggs (mass) were exchanged across paired nests. Because eggs were reciprocally exchanged between nests, clutch size remained the same in all nests. This cross-fostering design was used for a larger project looking at gene expression and melanin coloration in feathers. Even though these data were collected from cross-fostered broods of mixed parentage, all experimental nests were subjected to the same cross-foster

techniques. This process has been previously performed within this system and shown no effect on parental care with mixed paternity (Hund *et al.* 2015a), thus the cross-foster should not confound these results.

Ectoparasite treatment

Each nest in this study was subjected to an experimental manipulation of a removal or addition of mites. Eggs were temporarily removed and nests were disinfected by heating them to 125° C with a heat gun to kill any naturally occurring parasites (Hund *et al.* 2015b). Nests were then allowed to cool before eggs were returned, and in total this process took around five minutes. One nest in each experimental pair was then randomly assigned to a parasite treatment and 100 live, field-collected mites, were added into the nest.

Thermocouple eggs

On day three of incubation, I deployed model eggs with thermocouple temperature data loggers (OM-EL-USB-TC, Omega Engineering, Inc.), into each experimental nest (here after referred to as omegga loggers). The omegga loggers were painted to mimic the appearance of real barn swallow eggs, and are equivalent in both size and shape (Fig. 1). These model eggs are filled with wire pulling lubricant that closely matches the thermal properties of the albumin, which surrounds the yolk during development, of a real egg and the thermocouple probe was carefully placed in the center of each egg. These data loggers collect fine scale temperature readings of the nest at one minute intervals. In order to prevent females from attempting to turn or center these model eggs, the omeggas were tied in place by passing thread attached to the egg through the nest and securing it directly below. Thermocouple eggs were placed in the nest

during the parasite manipulation and remained in the nest until shortly after the eggs hatched.

The omegga loggers were removed on day three of the nestling period. The thermocouple eggs

did not interfere with heat transfer as the gel inside mimics the conductive properties of real

eggs. Similar thermocouple eggs have been used in tree swallow (*Tachycineta bicolor*) nests to

study incubation (Ardia *et al.* 2009), which shows that the addition of artificial eggs does not

impact female incubation behavior or nest attendance. As one omegga was added to each

experimental nest, clutch size was increased by one consistently across treatments. Considering

the ability of some passerines, like barn swallows, to tolerate nest disturbances (Cooper and Voss

2013), thermocouples did not influence behaviors of interest in this study (incubation).

Fig. 1. *A thermocouple omega egg (circled) in a clutch of barn swallow eggs*

Of the 95 experimental nests with thermocouple eggs, 65 ($n_{disinfected} = 33$, $n_{parasitized} = 32$) survived through to hatching due to a high amount of predation in the early summer of 2016 (30 failed; $n_{predated} = 16$ ($n_{parasitized} = 7$, $n_{disinfected} = 9$); $n_{abandoned} = 14$ ($n_{parasitized} = 6$, $n_{disinfected} = 8$)). I excluded data taken before 2 pm on the day the thermocouple eggs were deployed and any

readings after the confirmed hatch date, during the brooding period. These incubation data were

separated into day and night accounting for daily sunrise and sunset times. Here, I present day-

time incubation data collected across the entire incubation period of each experimental nest.

I used data collected from the omegga loggers to monitor incubation across the

incubation period (from day three to hatch). Specifically, I analyzed incubation rhythms during

early, middle, and late stages of embryonic development (days: early: three – five, middle: six –

nine, and late: ten – hatch) (Joseph and Daniel 1954; Yamasaki 1988; Prinzinger and Dietz 1995;

Nichelmann 2004; Murray *et al.* 2013; Tong *et al.* 2012; Turner 1994). Analyses focus on the

pair of incubating parents as the sex of individual parents cannot be determined based on

temperature data alone. The majority of these data are likely from the incubating female, since

females alone develop brood patches and do most of the incubation (Brown and Brown, 1999).

Analysis Software

Rhythm 1.0 is a software program that allows for the conversion of omegga logger data

text files to a recognizable format for future input (Cooper *et al.* 2005). Rhythm can process

temperature logs from data recorded at any time interval and create two output files, one sound
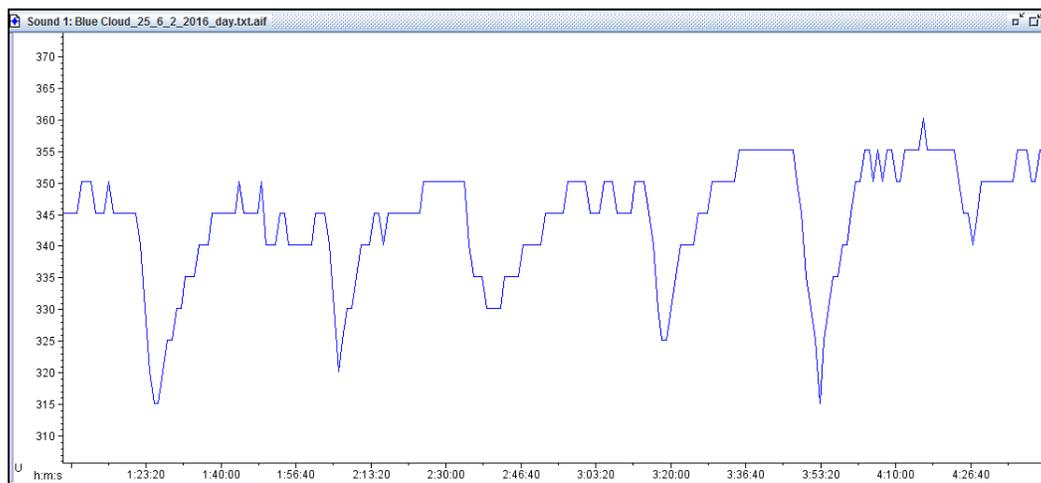
Fig. 2. *Temperature graph of nest 25 at Blue Cloud Ranch on day 5 of incubation: A decrease in slope indicates off-bout cooling period, an increase in slope indicates on-bout warming period and a plateau in slope indicates an on-bout equilibrium period.*

file (.aif) and one selection file (.sel). Rhythm is designed for incubation studies using Raven Pro

64 1.5 software (beta version) and automatically generates these files, which are necessary for

temperature log analyses of incubation rhythms, the visits to (on-bouts) and absences (off-bouts)

from the nest. By setting the detector settings in Rhythm, the two output files will automatically

detect egg cooling and re-warming periods (Cooper *et al.* 2005b). For fine scale analysis of the

entire incubation period, which ranged from 12 – 15 days, I set Rhythm at one minute intervals and precise detector settings. The detector settings were set at a minimum off-bout duration of two minutes, minimum depth of one degree, cooling and re-warming period slopes of one-quarter degree per minute, and an automatic timeout setting of ten minutes. These settings were informed by other studies using similar thermocouple eggs (Ardia *et al.* 2009; Cooper *et al.* 2005b; Cooper and Voss 2013).

Raven Pro 64 1.5 is a sound analysis software used to study animal communication. Raven analyses can also be used to display a time series of temperature data measured over regular time intervals (Cooper *et al.* 2005b). Raven can analyze daily temperature graphs (Fig. 2) from the entire incubation period by sequentially opening up the Rhythm 1.0 output files and automatically selecting egg cooling periods and re-warming periods performed by the incubating adult. Once selections are completed, temperature graphs require manual edits to ensure selections are representative of parental behavior. Low temperature levels reflect the energy cost of re-warming eggs, which is why correct detection of incubation rhythms are essential for proper temperature analysis (Ardia *et al.* 2009).

Data Validation

In order to validate the incubation behaviors characterized by the temperature loggers and Raven Pro 64 1.5 analyses, I collected observational data on a subset of nests during the incubation period. Incubation observations consisted of hour-long visual observations with binoculars in a camouflaged area in order to record the movement of parents to and from the nest. Field observations were then compared to the corresponding temperature graphs to test for concordance between personal observations and temperature logger data of on and off-bouts.

Separation of models

Mixed-effects linear models were run in accordance with potential changes during altricial embryonic developmental processes over the course of the incubation period. Different thermal properties of eggs effect the rate of heat loss, which changes throughout embryonic development as eggs develop blood vessels that aid in heat distributions (Turner 2002). I divided models into early, middle and late stages of incubation to account for potential changes in incubation behavior due to embryo development. I also looked at totals across the incubation period in order to compare the total length (duration) of the incubation period.

I separated models into incubation stages based on the following information. Early stage embryonic development (day one through five) is characterized by completion of the majority of morphogenesis (differentiation of tissues and organs). Thus, early stage incubation in my models is day three through five, since we added thermocouple eggs on the third day of incubation. Middle stage incubation (day six through nine) is grouped by the growth of existing tissues and limb development with few new structural formations. Late stage incubation (day ten to hatch) is the last changes the embryo completes in preparation for hatch, such as growth of feather germs, completion of eyelids, and cornification (hardening) of skin and nails (Daniel 1954; Yamasaki 1988; Nichelmann 2004; Murray *et al.* 2013). I divided each stage of incubation based on studies that previously examined the embryological staging of altricial species similar to *H. r. erythrogaster* (Daniel 1954; Murray *et al.* 2013; Yamasaki and Tonosaki 1988).

Statistical analysis

All statistical analyses were performed with the statistical package R, version 3.3.2 (R core Team 2016), and the 'nlme' package (Pinheiro *et al.* 2017) to analyze data. Fine scale

temperature data was collected from experimental barn swallow nests (N = 65) at different sites (N = 14) every one minute (collection range: 12 – 15 days). I built mixed-effects linear models for the following response variables: mean temperature, minimum temperature, standard deviation in temperature, number of warming bouts, mean length of warming bouts, mean length of off-bouts, number of equilibrium bouts, mean length of equilibrium bouts, and mean total time on the nest (averaging the total time, warming bouts plus equilibrium bouts, for each day). I looked at these response variables for each period during incubation, early, middle, and late (early: three to five; middle: six to nine; late: ten to (12-15); total: three to hatch) (refer to *methods: separation of models*). For each model I report p-values, beta values with confidence intervals and F-values. I created models for total time on nest and mean length of time on nest for the combined warming and equilibrium bouts, as this was the total on-bout during incubation. When necessary, I log transformed data to fit assumptions of normality; this was done for the mean length of equilibrium bouts during early, middle, and late stage incubation, and the mean time on nest during mid-stage incubation; all other response variables were normally distributed. I also looked at the length of time each nest took to hatch (measured as the number of days between the start of incubation and hatching). Each model contained treatment and clutch size as fixed effects with date and site as nested random effects to control for potential non-independence between nests at the same breeding site and variation in ambient temperature, as well as variation in sample size across site and date. I included clutch size as a fixed effect because it can directly influence incubation efficiency during intermittent incubation of altricial young (Dobbs *et al.* 2006). Given the structure of my data, I kept the random effects in all models even if they were not significant. The parasite treatment included 32 nests and the

disinfected treatment included 33 nests. Clutch size ranged from three to six eggs and nests took between 12 and 17 days to hatch.

RESULTS

Observational comparisons and settings for logger data collection

Field observations of incubating adults compared to corresponding raw data temperature graphs in Raven Pro 64 1.5 revealed a temperature detection delay of only two to four minutes. This delay was equivalent across all incubation observations (N = 16) and allowed for implementation of a strict editing process in Raven Pro 64 1.5 to automatically selected temperature logs unrepresentative of parental behavior. These rules include 1) only select off-bouts of one degree or more when there is a preceding warming period and 2) only edit the beginning of off-bouts if there is a one-half degree decrease in the equilibrium period. A selected decrease or increase in temperature below one degree should not be selected since incubation behavior cannot be accounted for. These small temperature fluctuations could be parents adjusting their brood patch contact with the clutch, egg-turning processes or actual movement off the nest. Editing rules ensure that all data is uniform and different parental behaviors are accounted for. Temperature graphs demonstrated a large amount of variation over the course of the incubation period regardless of treatment.

Question 1: How do ectoparasites influence the duration of the incubation period?

I looked at the length of incubation (number of days to hatch) for the total incubation period of each nest in order to test question one (Table 1). My results support the third hypothesis of question one (Q1; H3); I found no effect of parasite manipulation or clutch size on

the length of incubation period. Specifically, the number of days to hatch exhibits no significant effect from treatment or clutch size (see statistical values, Table 2d).

Question 2 and 4: How do parasites influence the duration and frequency of on-bouts and off-bouts and how does this effect change across the different stages of incubation?

Mean length off-bout

I looked at the mean length of off-bouts for the total incubation period of each nest (average length of bouts per day averaged across total or stage of incubation) and across all three stages of incubation (early, middle, and late) in order to test questions two and four (Table 1). My results support the third hypothesis of question two (Q2; H3), and the second hypothesis of question four (Q4; H2); I found no effect from parasite manipulation or clutch size on the length of off-bouts in early, middle or late stages of incubation. Specifically, the length of off-bouts did not differ significantly between treatment group or clutch size in any stage of incubation or for the total incubation period (see statistical values in Table 2a, 2b, 2c, and 2d).

Mean length of warming periods

I looked at the mean length of warming periods for the total incubation period of each nest (average length of periods per day averaged across total or stage of incubation) and across all three stages of incubation (early, middle, and late) in order to test questions two and four (Table 1). My results support the third hypothesis of question two (Q2; H3), and the first hypothesis of question four (Q4; H1); I found no effect from parasite manipulation on the length of warming periods in early, middle or late stages of incubation, however I found an effect from clutch size in the early stage and the total incubation period. Specifically, the length of warming periods did not differ significantly between treatment groups in middle stage or late stage incubation (see

statistical values, Table 2b and 2c) and had a non-significant trend with clutch size (see statistical values, Table 2b and 2c) for middle and late stages. Results show that length of warming periods have a significant effect of a positive trend with clutch size in the early phase of incubation and across the total incubation period (see statistical values for mean length of warming periods for clutch size, Table 2a and 2d). This indicates nests with larger clutches have longer warming periods throughout both the total incubation period and early stages of incubation (see Fig. 4a and 6a).

Mean length of equilibrium periods

I looked at the mean length of equilibrium periods (log transformed for each incubation stage only) for the total incubation period of each nest (average length of periods per day averaged across total or stage of incubation) and across all three stages of incubation (early, middle, and late) in order to test questions two and four (Table 1). My results support the third hypothesis of question two (Q2; H3), and the first hypothesis of question four (Q4; H1); I found no effect from parasite manipulation on the length of warming periods in early, middle or late stages of incubation, however I found an effect from clutch size in the early stage only. Specifically, the length of equilibrium periods did not differ significantly between treatment groups or clutch size in middle stages, late stages or total incubation period (see statistical values, Table 2b, 2c, and 2d). Results show that length of equilibrium periods have a significant effect of a positive trend with clutch size in the early phase of incubation (see statistical values for mean length of equilibrium for clutch size, Table 2a). This indicates parents with larger clutches increase incubation behavioral effort through higher nest attendance early-on when compared to middle and late phases of incubation (see Fig. 4a, 5a and 6a).

Number of warming periods

I looked at the number of warming periods for the total incubation period of each nest (average number per day averaged across total or stage of incubation) and across all three stages of incubation (early, middle, and late) in order to test questions two and four (Table 1). My results support the third hypothesis of question two (Q2; H3) and the second hypothesis of question four (Q4; H2); I found no effect from parasite manipulation or clutch size on the number of warming periods in early, middle or late stages of incubation. Specifically, the number of warming periods did not differ significantly between treatment group or clutch size in any stage of incubation or for the total incubation period (see statistical values, Table 2a, 2b, 2c, and 2d).

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Mean total time on nest

I looked at the mean total time on nest for the total incubation period (log transformed for middle stage incubation only) of each nest (average length of time on nest per day averaged

across total or stage of incubation) and across all three stages of incubation (early, middle, and late) in order to test questions two and four (Table 1). My results support the third hypothesis of question two (Q2; H3), and the first hypothesis of question four (Q4; H1); I found no effect from parasite manipulation on the mean total time on nest in early, middle or late stages of incubation, however I found an effect from clutch size in the early stages of incubation. Specifically, the mean total time on nest did not differ significantly between treatment groups or clutch size in middle stage, late stage or total incubation (see statistical values, Fig. 2b, 2c, and 2d). Results show that mean total time on nest during the early stages of incubation was predicted by clutch size (see statistical values for mean total time on nest for clutch size, Fig. 2a).This indicates parents with larger clutches increase incubation effort through higher nest attendance early-on when compared to middle and late phases of incubation (see Fig. 4a, 5a, and 6a).

Question 3 and 4: How do parasites influence mean nest temperatures during the incubation period and how does this effect change across the different stages of incubation?

Mean nest temperature

I looked at the mean nest temperature for the total incubation period of each nest (average temperature per day averaged across total or stage of incubation) and across all three stages of incubation (early, middle, and late) in order to test questions three and four (Table 1). My results support the third hypothesis of question three (Q3; H3) and the second hypothesis of question four (Q4; H2); I found no effect from parasite manipulation or clutch size on the mean temperature in early, middle or late stages of incubation. Specifically, mean nest temperature did not differ significantly between treatment group or clutch size in any stage of incubation or for the total incubation period (see statistical values, Table 2a, 2b, 2c and 2d).

Minimum nest temperature

I looked at the minimum nest temperature for the total incubation period of each nest (average minimum temperature per day averaged across total or stage of incubation) and across all three stages of incubation (early, middle, and late) in order to test questions three and four (Table 1). My results support the third hypothesis of question three (Q3; H3) and the second hypothesis of question four (Q4; H2); I found no effect from parasite manipulation or clutch size on the minimum nest temperature in early, middle or late stages of incubation. Specifically, minimum nest temperature did not differ significantly between treatment group or clutch size in any stage of incubation or for the total incubation period (see statistical values, Table 2a, 2b, 2c, and 2d).

Standard deviation in of nest temperature

I looked at the standard deviation of nest temperature for the total incubation period of each nest (average deviation in temperature per day averaged across total or stage of incubation) and across all three stages of incubation (early, middle, and late) in order to test questions three and four (Table 1). My results support the third hypothesis of question three (Q3; H3) and the second hypothesis of question four (Q4; H2); I found no effect from parasite manipulation or clutch size on the variation in temperature of early, middle or late stages of incubation. Specifically, the standard deviation of nest temperature did not differ significantly between treatment group or clutch size in any stage of incubation or for the total incubation period (see statistical values in Table 2a, 2b, 2c, and 2d).

Effect of Treatment on Three Response Variables
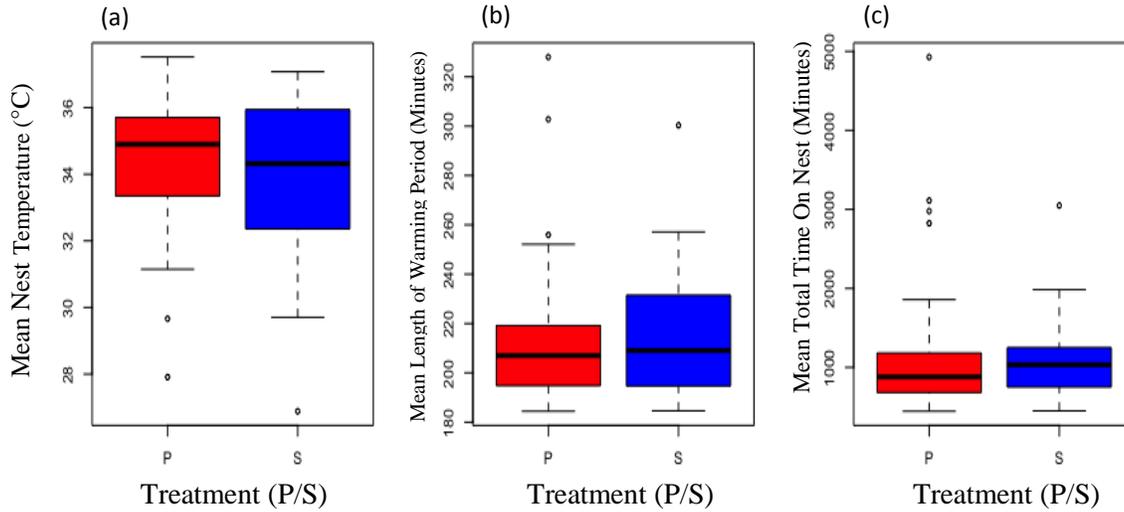
Fig. 3. *Box plots show no effect of treatment (parasitized (P) or disinfected(S)) on incubation behaviors (values averaged across the entire incubation period). Values for (a) mean temperatures, (b) mean length of warming periods, and (c) mean total time on nest. Plots made with raw data, statistics reported in test with mixed-effects linear models with treatment and clutch size as fixed effects and date and site as random effects.*
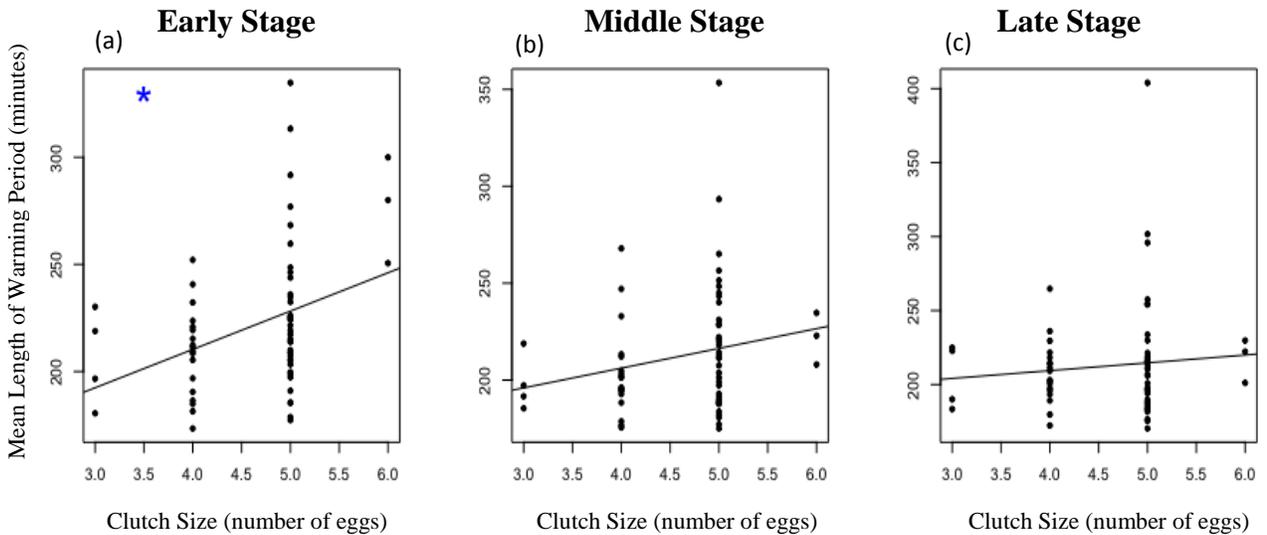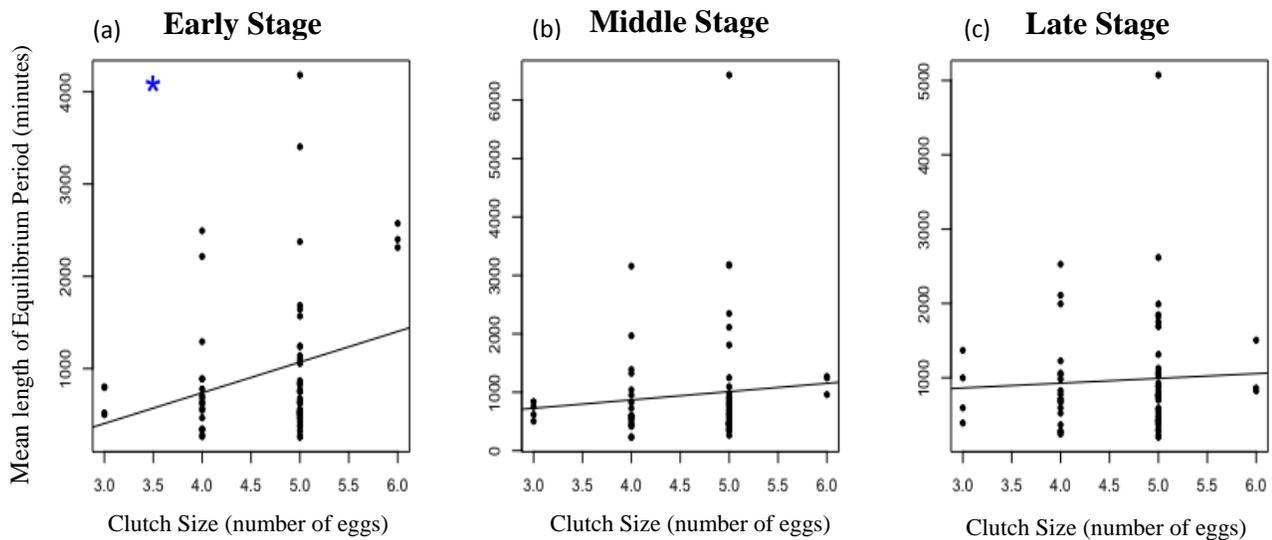

Fig. 4. *Scatter plots showing mean warming bout length and clutch size for the three stages of incubation: (a) early, (b) middle, and (c) late. Significant effect of longer mean warming period lengths during early stage incubation. Plots created with raw data, statistics reported in text with mixed-effects linear models with treatment and clutch size as fixed effects and date and site as random effects. *P<0.05*

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Period	Model	Treatment			Clutch Size		
		F	*β*	*p*	*F*	*β*	*p*
Early (a)	Mean temperature	1.35	-0.65 (-1.83 – 0.53)	0.26	0.03	0.09 (-0.90 – 1.07)	0.86
	Minimum temperature	0.35	-0.43 (-1.95 – 1.10)	0.56	1.51	0.72 (-0.51 – 1.94)	0.24
	Temperature Standard Deviation	0.03	-0.03 (-0.34 – 0.29)	0.87	1.00	-0.13 (-0.41 – 0.14)	0.33
	Number warming periods	0.32	-1.42 (-6.70 – 3.85)	0.58	2.20	-2.81 (-6.81 – 1.19)	0.16
	Mean length warming	0.06	-1.94 (-18.29 – 14.41)	0.81	9.38	17.81 (5.54 – 30.07)	**0.007**[*]
	Mean length off-bout	0.01	-5.66 (-134.96 – 123.63)	0.93	0.28	26.62 (-79.38 – 132.63)	0.60
	Number equilibrium periods	0.32	-1.42 (-6.70 – 3.85)	0.58	2.20	-2.81 (-6.81 – 1.19)	0.16
	Mean length equilibrium	0.83	0.14 (-0.19 – 0.47)	0.38	5.56	0.28 (0.03 – 0.53)	**0.03**[*]
	Mean total time on nest	0.62	146.75 (-246.10 – 539.59)	0.44	8.47	421.85 (116.05 – 727.65)	**0.01**[*]
Middle (b)	Mean temperature	3.10	-0.90 (-1.97 – 0.18)	0.10	0.07	0.11 (-0.79 – 1.01)	0.80
	Minimum temperature	0.45	-0.52 (-2.17 – 1.12)	0.51	1.12	0.65 (-0.65 – 1.95)	0.30
	Temperature Standard Deviation		0.06 (-0.33 – 0.46)		1.61	-0.12 (-0.45 – 0.21)	
	Number warming periods	0.68	-2.52 (-8.94 – 3.91)	0.42	1.61	-3.12 (-8.30 – 2.07)	0.22
	Mean length warming	0.04	-1.27 (-15.48 – 12.94)	0.85	3.01	9.74 (-2.10 – 21.59)	0.10
	Mean length off-bout	0.01	-4.72 (-135.33 – 125.90)	0.94	0.25	25.84 (-84.18 – 135.86)	0.63
	Number equilibrium periods	0.68	-2.52 (-8.94 – 3.91)	0.42	1.61	-3.12 (-8.30 – 2.07)	0.22
	Mean length equilibrium	0.0005	-0.003 (-0.32 – 0.31)	0.98	0.89	0.12 (-0.14 – 0.37)	0.36
	Mean total time on nest	0.62	-154.32 (-569.42 – 260.78)	0.44	0.65	140.05 (-225.24 – 505.35)	0.43
Late (c)	Mean temperature	2.04	-0.65 (-1.60 – 0.31)	0.17	0.12	0.13 (-0.66 – 0.92)	0.73
	Minimum temperature	0.27	-0.41 (-2.10 – 1.28)	0.61	1.05	0.62 (-0.65 – 1.88)	0.32
	Temperature Standard Deviation	0.18	0.10 (-0.39 – 0.58)	0.68	0.43	-0.11 (-0.48 – 0.25)	0.52

	Number warming periods	0.26	-1.62 (-8.39 – 5.15)	0.62	0.70	-2.17 (-7.62 – 3.28)	0.41
	Mean length warming	1.05	9.27 (-9.78 – 28.32)	0.32	0.71	5.69 (-8.60 – 19.99)	0.41
	Mean length off-bout	0.08	-18.77 (-160.35 – 122.81)	0.78	0.16	-20.89 (-132.17 – 90.38)	0.70
	Number equilibrium periods	0.26	-1.62 (-8.39 – 5.15)	0.62	0.70	-2.17 (-7.62 – 3.28)	0.41
	Mean length equilibrium	0.0001	-1.67 (-359.11 – 355.77)	0.99	0.15	55.36 (-245.55 – 356.26)	0.70
	Mean total time on nest	0.0007	4.55 (-371.32 – 380.43)	0.98	0.20	65.38 (-247.34 – 378.09)	0.67
Total (d)	Mean temperature	2.22	-0.74 (-1.78 – 0.31)	0.15	0.07	0.11 (-0.76 – 0.98)	0.80
	Minimum temperature	0.33	-0.42 (-1.98 – 1.14)	0.57	1.40	0.68 (-0.53 – 1.89)	0.25
	Temperature Standard Deviation	0.07	0.05 (-0.33 – 0.43)	0.79	0.84	-0.13 (-0.44 – 0.17)	0.37
	Number warming periods	0.39	-1.76 (-7.71 – 4.19)	0.54	1.40	-2.58 (-7.20 – 2.03)	0.25
	Mean length warming	0.12	2.41 (-12.31 – 17.14)	0.73	4.48	11.38 (0.03 – 22.72)	**0.05***
	Mean length off-bout	0.01	-5.44 (-127.52 – 116.65)	0.93	0.04	9.22 (-91.54 – 109.97)	0.85
	Number equilibrium periods	0.39	-1.76 (-7.71 – 4.19)	0.54	1.40	-2.58 (-7.20 – 2.03)	0.25
	Mean length equilibrium	0.03	-30.20 (-379.54 – 319.14)	0.86	1.15	148.91 (-144.41 – 442.23)	0.30
	Mean total time on nest	0.03	-28.68 -388.22 – 330.85	0.87	1.22	157.85 (-143.79 – 459.50)	0.29
	Number of days to hatch	0.07	0.07 (-0.47 – 0.60)	0.80	0.00002	-0.001 (-0.42 – 0.42)	0.99

Table 2. *Results of mixed-effects linear models for incubation collected during the (**a**) early period (three to five days), (**b**) middle period (six to nine days), (**c**) late period (ten to hatch) of incubation and (**d**) total incubation periods (mean value per day averaged across the entire period). All models contain treatment and clutch size as fixed effects and date and site as random effects. *P < 0.05*

Fig. 5. *Scatter plots showing mean equilibrium period length and clutch size for three stages of incubation: (a) early, (b) middle, and (c) late. Significant effect of longer mean equilibrium period lengths during early stage incubation. Plots created with raw data, statistics reported in text with mixed-effects linear models with treatment and clutch size as fixed effects and date and site as random effects.*P<0.05*
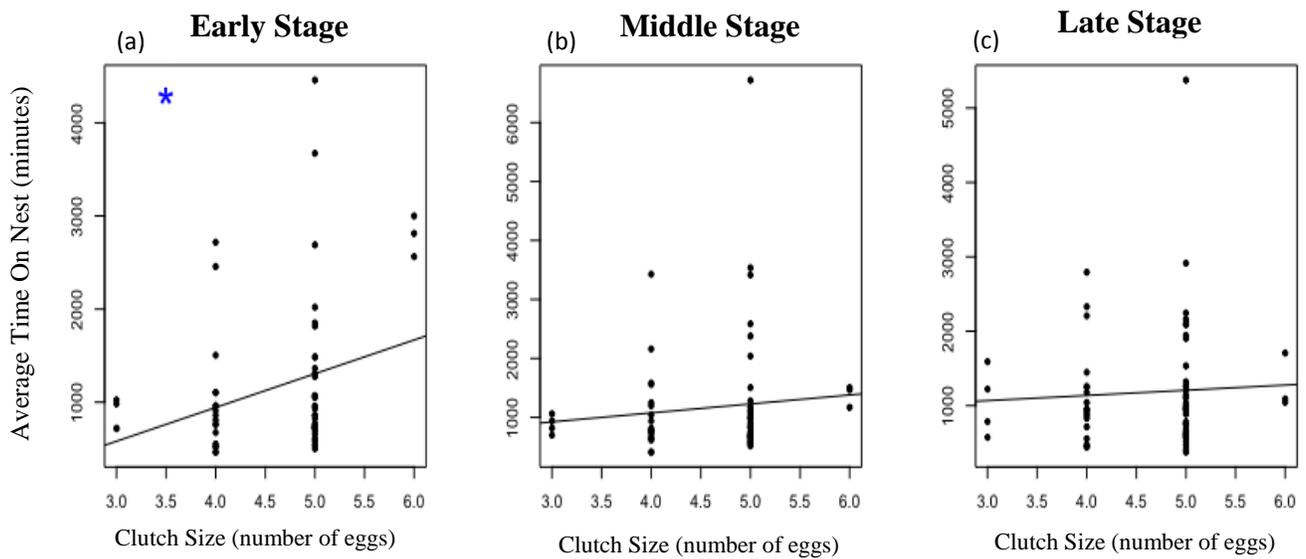
Fig. 6. *Scatter plots showing mean time on nest (values averaged across the entire incubation period) and clutch size for three stages of incubation: (a) early, (b) middle, and (c) late. Significant effect of longer mean time on nest during early stage incubation. Plots created with raw data, statistics reported in text with mixed-effects linear models with treatment and clutch size as fixed effects and date and site as random effects. *P<0.05*

Frequency Distributions of Three Response Variables
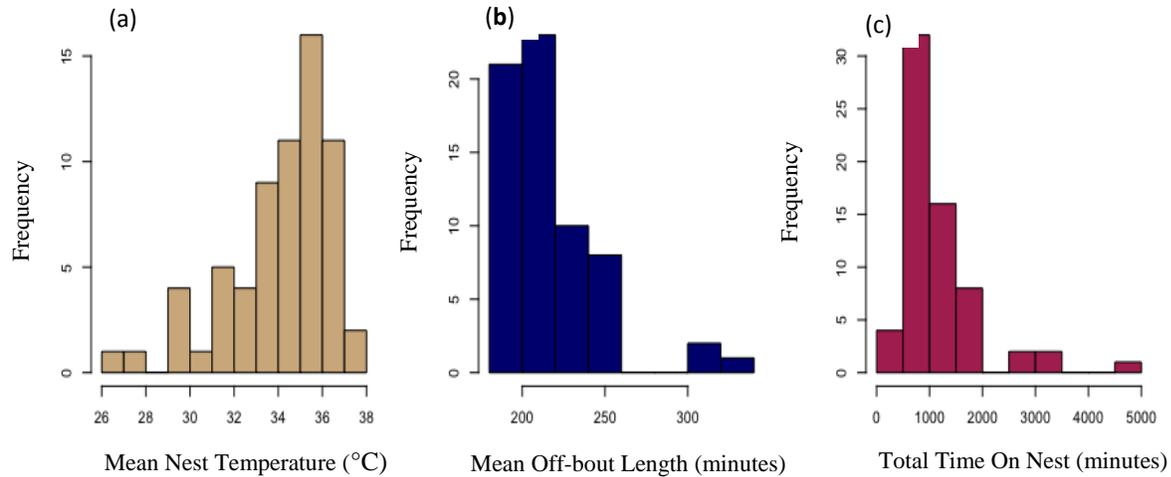
Fig. 7. *Three response variables (mean temperature, mean warming period length, mean total time on nest) show a large range in values across entire incubation periods. Frequency distributions for (**a**) mean nest temperature, (**b**) mean length of warming period, and (**c**) total mean time on nest (values averaged across the entire incubation period).*

DISCUSSION

In this study I experimentally manipulated ectoparasite levels within barn swallow nests to test hypotheses about how mite infections influence incubation behavior. I was able to collect data on fine scale incubation behavior by placing thermocouple model eggs into experimental nests. Overall, the results of this study demonstrate that incubation behavior is highly variable. I did not find a significant influence of mites on various aspects of incubation behavior, however I did detect an influence of clutch size on parental behavior during the early stage of the incubation period. Previous studies in this system have shown that mites influence settlement behavior, where males avoid nests with mites, and impact nestling survival (Hund et al, *in prep.*). Mites also affect parental care behavior during the nestling phase (Hund *et al.* 2015a). However,

before this study there was no evidence for how mites affect barn swallows fine scale incubation behavior.

There are several possible explanations for why I did not see a change in parental behavior in response to ectoparasites. One could be that females may lack the ability to respond to less favorable environmental conditions once settled at a nest, due to a heightened maternal drive to incubate. During incubation, females allocate a large amount of resources to maximize fitness through the number of eggs produced (Engstrand and Bryant 2002). Ideally females lay a clutch size she can support based on her ability to predict what the level of parasite load will be after hatch (Richner and Heeb 1995). However, I manipulated parasites after females had settled in a nest and had completed their clutch, which essentially traps them at their nest choice and clutch size. With only one to three breeding attempts per year in a short-lived species with age-independent mortality, this represents a short time frame of intensified maternal instincts that is crucial for females to successfully maximize reproductive output and fitness. This suggests that females may be energetically constrained by the need to reproduce during the breeding season and by the resources already invested into eggs, so a behavioral response to ectoparasites that reduced incubation would only be harmful to their own fitness. Thus, the ability to tolerate parasites instead of stimulating a behavioral response during incubation, may be more advantageous to an individual's current and future reproductive fitness.

Another possible explanation to why I did not see an effect from ectoparasites on incubation behavior is the lack of parasite activity during this period, as access to blood-meals is very limited during incubation. Northern fowl mites feed off of the blood of nestlings, however in the presence of eggs, mites face a period of malnourishment of length determined by parental incubation efficiency. The parasites used during experimental manipulations of nests were also

collected from mite populations post-overwintering, indicating that these mites were probably in poor condition after an extended fast. However, this also indicates that mites are extremely resilient to their environment and can survive weeks without a solid blood-meal (Proctor and Owens 2000). Most importantly, mites cannot feed on eggs and there are few studies to determine if fowl mites can actually feed on adults (northern fowl mites are rarely found on adults in this system and there is no evidence of sores or swellings from mite feeding, as are obvious on infected nestlings). There have been studies that found an effect of ectoparasites on incubation behavior (Fitze *et al.* 2004; Møller 1990; Møller 1993) so it remains unclear if or how parents might change behaviors through egg temperature analysis in response to blood mites.

It is also possible that there is an infection threshold that is required in order to see a behavioral response, and that the experimental manipulation was below this threshold. Although previous studies demonstrate incubation behavioral responses from experimental manipulation below this intensity (Møller 1990 added 50 tropical fowl mites to barn swallow nests), natural mite intensities found in infected overwintering nests and infected nests during the breeding season are frequently much higher than this experimental manipulation. Thus, the experimental manipulation of parasite-load may have been too low to decouple other fitness-related factors that could otherwise impact parental behavior and it would be interesting for future research to manipulate mites at several different levels to see if there is a threshold effect.

While I found no effect of treatment, I did see an effect of clutch size. Early in the incubation period, nests with larger clutches had higher nest attentiveness then nests with smaller clutches: parents with more eggs spent more time on the nest (combined average warming and equilibrium bouts). Interestingly, I only found this pattern early in the incubation period, but not in the middle or late stages, though there was a trend in this direction. This suggests that larger

clutches may take more investment during the early stages of embryonic development compared to the later stages. Cooper and Voss (2013) found a similar effect from large clutch sizes; Black-capped chickadees (*Poecile atricapillus*) illustrated decreasing incubation on-bout lengths as embryos developed, which indicates on-bout duration may be limited by a decline in female energy reserves towards the end of incubation. These results also highlight the need to look at incubation as a dynamic behavior, because if I had only measured behavior as an average of the entire incubation period, or at a single time point, I would not have understood this pattern.

This result is actually the opposite of what some models predict; the demands for parental care through heat transfer may actually increase rather than decrease from early to late embryo development (Turner 2002; Turner 1997). During early-stage incubation eggs only lose heat from surfaces where the heat was applied, as conductive heat transfer from contact with the brood patch. In middle and late-stage incubation, eggs rate of heat loss increases as blood circulation helps distribute heat to all surfaces of eggs, which is then lost to all areas the egg is in contact with (e.g., surrounding air, brood patch, thermocouple egg) (Turner 2002; Boulton and Cassey 2012). This model predicts an increase in parental energy expenditure as incubation progresses due to an increase in the cost of maintaining optimal egg temperatures (Turner 1991). Although this is contrary to my results, this further indicates that there is variation in the rates of embryonic development and lengths of incubation across avian taxa, with additional variation within subspecies of differing maternal strategies of incubation (Martin 2002; Martin and Schwabl 2008).

Given the high variation in behavior I saw across incubation, I would encourage future studies to measure incubation behavior at different stages of the incubation period and to explore

other factors that may be driving this variation, such as female body condition, age, experience, or mate quality.

CONCLUSION

I examined the incubation behavior of breeding barn swallows as a function of an ectoparasite manipulation experiment. Although no direct effect of the ectoparasite treatment on behavior was observed, my experiment confirmed that incubation is a highly dynamic behavior and clutch size has an effect on the degree of parental investment. Many observations during the incubation period were performed in order to validate my predictions for behavioral responses to nest ectoparasites based off time-series data. Future research on variations in incubation behavior like nest attentiveness, egg positioning and degree of movement, will further illuminate the allocation of time and energetic trade-offs in parental behavior during incubation and how these may be subject to change over the course of embryonic development. From this study, there seems to be little evidence that parents respond to ectoparasite infections during incubation. This may be explained by other complex evolutionary constraints (e.g., trade-offs to reproduction, maternal instinct, threshold clutch size rule) or other environmental interactions (e.g., ambient temperature, predation rates) that may be more important in shaping incubation behavior.

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