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Prolactin is related to incubation constancy and egg temperature following a disturbance in a precocial bird

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ABSTRACT

To maximize fitness, parents may trade-off time and energy between parental care and self-maintenance. In vertebrates, prolactin and corticosterone are two important hormones that regulate parental investment because they stimulate parental care and mobilize energy, respectively. Further, concentrations of both hormones change in response to disturbances. One of the most important parental care behaviors in birds is incubation, since small changes in egg temperature have large effects on offspring. We investigated how prolactin and corticosterone may mediate parental incubation constancy (i.e., the daily amount of time spent incubating eggs) and regulation of egg temperature. We collected blood samples from female wood ducks (*Aix sponsa*) near the start and end of the incubation period to measure baseline and stress-induced (30 min after capture and restraint) hormone concentrations. We also quantified incubation constancy and egg temperature using artificial egg temperature loggers. As expected, prolactin decreased and corticosterone increased after 30 min of capture and restraint. Corticosterone concentrations (baseline and stress-induced) were negatively related to body mass, but were not related to incubation constancy. In contrast, prolactin concentrations (baseline and stress-induced) were higher at the end than the start of the incubation period, and stress-induced prolactin concentrations were positively related to incubation constancy following a nest disturbance (i.e., capture). Further, prolactin (baseline and stress-induced) concentrations were positively related to egg temperatures, but only after the disturbance. These results suggest that prolactin may be associated with the regulation of parental incubation constancy and resulting heat-transfer after a disturbance, which may ultimately affect offspring development.

1. Introduction

Parental care is necessary for successful offspring development in most bird species, and one of the most important aspects of avian parental care is egg incubation. Adequate regulation of egg temperature is crucial because small changes in average incubation temperatures not only affect hatch success, but also influence a range of fitness-related offspring traits (DuRant et al., 2013c) including thermoregulation (DuRant et al., 2013a), immune function (DuRant et al., 2012), growth rate (DuRant et al., 2010; Nord and Nilsson, 2011; Ospina et al., 2018; Wada et al., 2015), metabolic rate (Nord and Nilsson, 2011; Wada et al., 2015), performance (Hope et al., 2019; Hopkins et al., 2011), and behavior (Hope et al., 2018b, 2019). Further, there is evidence that incubation temperature affects long-term survival of offspring (Berntsen and Bech, 2016; Hepp and Kennamer, 2012; Nord and Nilsson, 2016). Thus, there should be selection pressure on parents to optimize incubation temperature. However, incubation is an active, energetically costly behavior compared to resting, and is time-consuming for parents (Nord and Williams, 2015; Thomson et al., 1998; Tinbergen and Williams, 2002), especially for species in which only one sex incubates. This might create a trade-off for parents in how they invest time and energy towards incubation behaviors and important self-maintenance behaviors, such as foraging. Further, this trade-off can be affected by various disturbances (e.g., predators, human disturbance; Fontaine and Martin, 2006; Ghalambor and Martin, 2002; Martin et al., 2015), which are particularly significant in relation to incubation

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because incubating birds spend a large portion of their time on the nest, which could make it difficult to avoid disturbances. Thus, a complete understanding of the trade-off between parental incubation behavior and self-maintenance is crucial to understand how different parental care strategies evolve and how species respond to disturbances during parental care. The latter is especially important as sources of anthropogenic disturbances (e.g., urban development, introduced predators) increase.

The energetic demands and behavioral decisions necessary to manage the trade-off between parental care and self-maintenance are mediated by various physiological signals, including hormones (Ricklefs and Wilkelski, 2002; Wingfield and Sapolsky, 2003). Prolactin and corticosterone are two hormones of particular importance because they stimulate parental care and mobilize energy reserves, respectively (Buntin, 1996; McEwen and Wingfield, 2003). During periods of avian parental care, baseline prolactin concentrations increase as a result of longer photoperiods (Dawson et al., 2001; Dawson and Sharp, 1998; Hall, 1986), and in response to nest, egg, and offspring stimuli (Hall, 1987, 1986; Leboucher et al., 1993; Sharp et al., 1998; Silver, 1984). Baseline corticosterone concentrations either increase or decrease during parental care, depending on the species and the type of care (Romero, 2002). However, when birds are faced with an acute disturbance, typically corticosterone concentrations increase and prolactin concentrations decrease, and the resulting hormone concentrations after the disturbance are commonly referred to as 'stress-induced corticosterone' and 'stress-induced prolactin', respectively (Angelier and Chastel, 2009; Sapolsky et al., 2000). These post-disturbance high concentrations of corticosterone and low concentrations of prolactin may be related to a shift in investment from parental care to immediate survival, which can manifest as reduced parental care behaviors or, in extreme cases, offspring abandonment (Groscolas et al., 2008; Ouyang et al., 2012; Spée et al., 2010). Thus, maintaining high prolactin concentrations and/or low corticosterone concentrations in the face of stressors could be a mechanism by which animals maintain parental care behaviors despite disturbances (prolactin: Angelier et al., 2016; Angelier and Chastel, 2009; corticosterone: Bókony et al., 2009; Holberton and Wingfield, 2003; O'Reilly and Wingfield, 2001; Wingfield et al., 1995).

Although parental behavior has often been studied in relation to stress-induced corticosterone concentrations, relatively little is known about how it may be related to stress-induced prolactin concentrations (Angelier et al., 2016; Angelier and Chastel, 2009). The hypothesis that the suppression of the corticosterone stress-response serves to maintain parental behaviors is supported in some species, where lower stress-induced corticosterone concentrations are related to a greater investment in parental care behaviors (Ouyang et al., 2012; Silverin, 1986; Spée et al., 2011). However, some species exhibit greater stress-induced corticosterone concentrations as parental investment increases, suggesting that corticosterone mobilizes energy without causing abandonment (DuRant et al., 2013b; Perfito et al., 2002; Romero, 2002). Prolactin is classically known as the 'parental care hormone' (Buntin, 1996; Hall, 1986; Sockman et al., 2006; Vleck, 1998). Many studies show that baseline prolactin concentrations are positively related to parental effort (reviewed in Angelier et al., 2016), and that experimental reductions in circulating baseline prolactin concentrations lead to a reduced frequency of parental care behaviors, including incubation behavior (e.g., Angelier et al., 2009; Smiley and Adkins-Regan, 2018; Thierry et al., 2013). To date, however, there has only been one study investigating whether prolactin concentrations after a disturbance are related to parental behavior, which found that snow petrels (*Pagodroma nivea*) with low stress-induced prolactin concentrations were more likely to abandon their nest than those with higher concentrations (Angelier et al., 2015). Nothing is known about how stress-induced changes in prolactin may be related to subtle changes in the frequency of day-to-day parental care behaviors, or how these changes may affect developing offspring. Understanding the role of both corticosterone and prolactin in mediating changes in incubation will shed light on the mechanism by which animals modulate their behavior in reaction to disturbances during parental care.

In this study, we investigated whether prolactin and corticosterone are associated with parental incubation constancy (i.e., daily amount of time spent incubating eggs) and resultant changes to egg temperatures after birds are confronted with a disturbance during incubation. We investigated this question by studying a population of wood ducks (*Aix sponsa*) nesting in nest boxes. Because the current study was a part of a larger study investigating the effects of clutch size on the costs of incubation, we first manipulated clutch size. This manipulation also strengthened the current study because it resulted in a range of clutch sizes and decoupled investment in egg-laying from investment in incubation. We installed artificial egg temperature loggers in the nest boxes to quantify parental incubation constancy and egg temperatures throughout incubation. We captured hens at two timepoints during the ~ 30-day incubation period (early and late) and collected blood samples to determine baseline and stress-induced (after 30 min of a standardized capture/hold stressor) prolactin and corticosterone concentrations. We investigated the relationships among hormones, incubation constancy, and egg temperature both during the 24 h before and the 24 h after this disturbance because we expected that hormones and incubation constancy would change over a short time period in response to the disturbance. We hypothesized that the secretion of high prolactin concentrations during incubation, especially when faced with a disturbance, is an indicator of high parental investment (Angelier and Chastel, 2009). Thus, we predicted that both baseline and stress-induced prolactin concentrations would be positively correlated with incubation constancy and egg temperatures. Specifically, a positive relationship between stress-induced prolactin and incubation constancy would support the hypothesis that maintaining high prolactin concentrations despite a disturbance is an indicator of increased parental investment (Angelier and Chastel, 2009). We had two alternative hypotheses for corticosterone. First, elevated concentrations of corticosterone may help mediate incubation constancy through increasing energy availability (DuRant et al., 2013b), in which case we would predict that baseline and stress-induced corticosterone concentrations would be positively related to incubation constancy and egg temperatures. Alternatively, elevated concentrations of corticosterone may inhibit parental care behavior through shifting investment toward self-maintenance (e.g., O'Reilly and Wingfield, 2001; Ouyang et al., 2012; Silverin, 1986; Spée et al., 2011), in which case we would predict that there would be a negative relationship between corticosterone concentrations and incubation constancy and egg temperatures.

2. Methods

2.1. Study species and site

The wood duck is a dabbling duck that is widely-distributed throughout the United States and nests in tree cavities and nest boxes (Hepp and Bellrose, 2013). Females lay one egg per day and produce an average clutch size of 12 (Bellrose and Holm, 1994). Conspecific brood parasitism is common in both natural and artificial nest cavities (Roy Nielsen et al., 2006a; Semel et al., 1988; Semel and Sherman, 1986), affecting up to 85% of nests and resulting in parasitized clutch sizes that reach over 40 eggs in some populations (Hepp and Bellrose, 2013; Morse and Wight, 1969). Hatch success is high in parasitized clutches, and is normally only slightly lower, if at all, than in un-parasitized clutches (Roy Nielsen et al., 2006b; Semel et al., 1988).

Female wood ducks are solely responsible for incubation, which begins with partial night incubation about 4 days before the end of egg-laying. After clutch completion, females progress to full incubation, which lasts an average of 30 days (Hepp and Bellrose, 2013). Females have high incubation constancy and usually take one recess in the morning and one in the evening to forage (Hepp and Bellrose, 2013;

Manlove and Hepp, 2000), although behavior is variable among individuals (Bellrose and Holm, 1994; Manlove and Hepp, 2000). Ducklings are precocial and can feed themselves shortly after exiting the nest (Hepp and Bellrose, 2013), and thus egg incubation is one of the most important aspects of parental care in this species. Further, small changes in average incubation temperature have large effects on the morphology, physiology, behavior, and survival of wood duck offspring (DuRant et al., 2013c; Hepp and Kennamer, 2012), which suggests that females should be under selective pressure to optimize incubation temperature.

We studied a population of wood ducks breeding in nest boxes at the Department of Energy's Savannah River Site in South Carolina (33.1°N, 81.3°W) on a series of 12 ephemeral wetlands, where wood ducks have been monitored for over 35 years. We monitored nest boxes from 21 February – 20 June 2014. All methods were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC #11-056 and 14-083).

2.2. Clutch size manipulation

As part of a larger study, we manipulated clutch sizes. Once 3 – 5 eggs were laid, we began adding or removing one egg per day to achieve both reduced and augmented clutch sizes. Occasionally, to create very large clutch sizes (e.g., 33 eggs), we added more than one egg per day, which is similar to what happens in heavily parasitized nests in the field (Hepp and Bellrose, 2013; Morse and Wight, 1969). We ensured that the eggs that we added to a clutch were always of a similar lay-date to the eggs of that clutch. All eggs were moved before incubation had started. To measure incubation constancy and temperature, we replaced seven eggs in each nest with seven artificial (one stationary and six free-moving) eggs containing temperature loggers (see sections 2.4 and 2.5). Out of 40 total nests in this study, we installed free-moving loggers into 33 nests (this number was limited by the number of loggers). The other 7 nests contained only a stationary logger. Out of the 33 nests, we collected full temperature data from the six free-moving loggers in 29 nests, but due to technological difficulties, three nests contained five and one nest contained four functional loggers (i.e., the other 1–2 loggers did not successfully record temperature data). Final clutch sizes ranged from 8 to 33 eggs (mean ± SD = 17.6 ± 5.2 eggs; including artificial eggs) and clutch sizes were equally distributed among wetlands and across the breeding season.

2.3. Capture and blood sampling

We captured females at their nest boxes once during early (range = 3–8 days after the start of full incubation; mean ± SD = 5.3 ± 1.4 d) and once during late (range = 21–32 d; mean ± SD = 24.4 ± 1.9 d) incubation to take blood samples for baseline and stress-induced prolactin and corticosterone concentrations. To capture birds, we slowly and quietly approached the nest box while the hen was incubating, covered the opening of the nest box, and then removed the hen from the nest. All birds were captured after their morning recess between 9:09 and 15:57 h (mean ± SD = 12:07 ± 1:49 h). We collected blood from the femoral vein using a 25 G needle. We collected up to 300 μl of blood to measure baseline hormone concentrations and 200 μl for stress-induced hormone concentrations using 100 μl heparinized capillary tubes. For baseline samples, the blood that was collected the quickest was allocated to corticosterone analyses. Baseline samples were collected within ~ 4 min of covering the opening of the nest box (samples used for corticosterone were collected within: range = 2.7 – 4.0 min; mean ± SD = 3.5 ± 0.5 min; samples used for prolactin were collected within: range = 2.7 – 7.0 min; mean ± SD = 4.2 ± 1.2 min). There was no relationship between the amount of time it took to collect the blood sample and baseline corticosterone (r^2 = 0.01; p = 0.36) or baseline prolactin (r^2 = 0.04; p = 0.14). After the baseline sample, we placed a cloth hood over the hen's head and upper torso to cover its eyes and then placed the hen in a cloth bag for 30 min, as a standardized acute stressor (Wingfield et al., 1992). Then, blood samples for stress-induced hormone concentrations were taken, and we changed which leg the blood was collected from, if necessary. We then measured hen size (mass and tarsus length), and immediately placed the hen back on the nest. Blood samples were placed on ice in the field, and after transport to the laboratory they were centrifuged at 3.5*g* for 5 min and plasma was stored at −80 °C until it was analyzed.

2.4. Incubation constancy

To determine hen incubation constancy, we equipped each nest with a stationary temperature logger, which was contained within an artificial egg, as described in (Hope et al., 2018a). Briefly, we mounted one thermal probe from the logger in a stationary, but flexible, position in the middle of the clutch. This logger also had a second probe, which we attached to the inside wall of the nest box to record ambient temperature. We programmed the logger to simultaneously record ambient and egg temperatures every 2 min. This high temporal resolution enabled us to infer hen behaviors from the temperature data (e.g., beginning of full incubation, active incubation vs. recesses). We analyzed incubation behavior during the 24 h before capture and the 24 h after release for each time that a hen was captured. We used this short time window to investigate the short-term relationships among hormones and behavior because both hormone concentrations and behavior can rapidly respond to perturbations, and return to normal levels relatively quickly.

We used Rhythm/RAVEN software (Cooper and Mills, 2005) to analyze the temperature data to determine when the hen was on and off of the nest each day. We determined that the hen was off of the nest when there was a temperature drop of at least 2 °C that lasted for at least 13 min (Manlove and Hepp, 2000), or if there was a temperature drop of < 2 °C but the drop followed a clear daily pattern of off-bouts for that hen. From these data, we calculated the total number of hours that each hen was on the nest (i.e., incubation constancy) during the 24-hour periods surrounding capture.

2.5. Incubation temperature

To monitor incubation temperature, we used six free-moving artificial eggs containing data loggers as described in (Hope et al., 2018a). These artificial eggs mimic the cooling and warming properties of real eggs and were calibrated before using (methods fully described in Hope et al., 2018a). Temperature data from these six free-moving eggs, rather than those from the centrally located stationary logger, were used to calculate incubation temperature because they more accurately represented the temperature that real eggs experience as they are rotated by hens throughout the course of incubation (Hope et al., 2018a). We calculated the average incubation temperature for each egg, and then calculated the average of these values to obtain an overall average incubation temperature. We calculated this for each 24-hour period for which we had incubation constancy data.

2.6. Hormone assays

To determine the concentrations of prolactin and corticosterone in the blood samples, we used radioimmunoassays. The corticosterone assay was conducted at Virginia Tech, using the protocol of DuRant et al. (2010) and the B3-163 anti-corticosterone antibody (Esoterix Endocrinology). Mean plasma volumes (± SD) used to measure baseline and stress-induced hormone concentrations, respectively, were 48.2 ± 6.1 μl and 20.0 ± 0.4 μl. All samples were run in one assay and samples were run singly to increase the probability of detection. We corrected for the individual extraction efficiency of each sample in the final calculations. The mean extraction efficiency was 72.5%. Intra-

assay variation was 6.5% and the assay limit of detection was ~ 1 ng/ml (varied based on plasma volume and extraction efficiency of each sample). The prolactin assay was conducted at the Centre d'Etudes Biologiques de Chizé, using the protocol of (Cherel et al., 1994) and the antibody against chicken prolactin (supplied by Dr. A. F. Parlow, Harbor-UCLA Medical Center, Torrance, CA, USA). All samples were run in one assay and samples were run in duplicate, with 25 µl of plasma for each duplicate. The intra-assay variation was 9.1% and the limit of detection was 0.43 ng/ml.

2.7. Statistical analyses

All statistical analyses were conducted in R v. 3.3.1 (R Core Team, 2018) using the *lme4* package (Bates et al., 2015). We assured that all models met the assumptions of normal and homoscedastic residuals by examining fitted vs. residuals plots, normal quantile plots, and histograms of residuals. We reduced all models using stepwise backward elimination of non-significant terms. If not otherwise specified, we defined significance as $p < 0.05$. When two models contained the same response variable and thus were not independent, we used Bonferroni corrections to adjust p-values, which are noted below. We report p-values using type III Wald chi-square tests (*Anova*; *car* package; Fox and Weisberg, 2011) for each model. We only report terms that remained in the models, however results from all full and reduced models are reported in the Supplementary Material. For all analyses, bird ID was included as a random effect to account for multiple observations from each individual.

In our analyses, we used baseline and stress-induced hormone concentrations as variables, not the magnitude of change between these concentrations. In regard to corticosterone, the difference or percentage change in hormone concentrations is typically of little biological relevance because baseline and stress-induced concentrations of corticosterone bind to different receptors (type 1 and type 2), and thus are not completely comparable (Romero, 2004). In regard to prolactin, there is evidence that behavioral changes associated with prolactin may only be observed after crossing a certain threshold (Angelier et al., 2016; Boos et al., 2007; Spée et al., 2010). If this is the case, an individual that has crossed this threshold and one that has not could both have the same value for the magnitude of change in prolactin. Thus, for our study, examining baseline and stress-induced concentrations separately is most biologically relevant.

To determine whether prolactin or corticosterone varied throughout the incubation period or in response to a nest disturbance, we constructed two linear mixed-effects models. Prolactin and corticosterone were the dependent variables for the two models and both were log-transformed to meet model assumptions. For both models, stage of incubation (early or late), bleed (baseline or stress-induced), and their interaction were included as categorical predictors, bird ID was included as a random factor, and clutch size, day of year, time of day, and body condition (residuals of mass vs. tarsus linear regression) were included as continuous covariates in initial models. Stage of incubation, bleed, and time of day were retained in the final model structure for the model investigating prolactin. Bleed and hen body condition were retained in the final model structure for the model investigating corticosterone.

To determine if incubation constancy (hours spent on the nest during a 24 h period) varied throughout the incubation period or as a result of a nest disturbance, we constructed one linear mixed-effects model. Incubation constancy was the dependent variable and was cube-transformed to meet model assumptions. The stage of incubation (early or late), the time period in respect to the day of capture (either 24 h before capture or 24 h after release), and their interaction were included as categorical predictors. Bird ID was included as a random factor. Clutch size, day of year, time of day, and body condition were included as continuous covariates in the initial model. Stage of incubation, the time period in respect to the day of capture, and time of day were retained in the final model structure.

To investigate whether there were individual relationships among baseline/stress-induced prolactin or corticosterone and incubation constancy (time spent on the nest) during either the 24 h before capture or the 24 h after release, we constructed four linear mixed-effects models with bird ID as the random factor. For the first two models, incubation constancy during the 24 h before capture was the dependent variable. Since these two models used the same response variable, significance was defined as $p < 0.025$. For the second two models, incubation constancy during the 24 h after release was the dependent variable and was cube-transformed to meet the model assumptions. Similarly, because these two models used the same response variable, significance was defined as $p < 0.025$. For one model of each set, baseline prolactin and baseline corticosterone were included as independent variables and in the other, stress-induced prolactin and stress-induced corticosterone were included as independent variables. To avoid issues of autocorrelation, baseline and stress-induced concentrations of each hormone were not included in the same models because they were correlated with each other (prolactin: r(spearman) = 0.75, $p < 0.001$; corticosterone: r(spearman): 0.34, $p = 0.005$). For the same reason, body condition and stage of incubation (early or late) were not included in these models because they were significantly related to corticosterone and prolactin, respectively (see Results). For all models, clutch size, day of year, and time of capture were included as initial covariates. The terms that were retained in each of these four models are reported in Tables A.2 and A.3.

Lastly, we investigated whether hormone concentrations were related to incubation temperature by constructing four linear mixed-effects models with bird ID as the random factor. Two models included incubation temperature during the 24 h before capture as the dependent variable and the other two models included incubation temperature during the 24 h after release as the dependent variable. Incubation temperature during the 24 h after release was cube-transformed to meet model assumptions. Because the same response variable was used for two models, significance was defined as $p < 0.025$ for these models. One model in each set contained baseline prolactin and corticosterone concentrations as predictors, and the other model contained stress-induced prolactin and corticosterone as predictors. All models contained average ambient temperature during the 24 h period and clutch size as initial covariates. To avoid issues of autocorrelation, body condition and stage of incubation (early or late) were not included in these models. All incubation temperatures were calculated from the free-moving temperature loggers, and thus those nests without free-moving temperature loggers were excluded from these analyses. The terms that were retained in each of these four models are reported in Tables A.2 and A.3.

3. Results

3.1. Factors related to hormone concentrations

Plasma prolactin concentrations were affected by capture/handling and differed with the stage of incubation (early or late). Specifically, prolactin concentrations were lower after 30 min of capture and handling stress than they were at baseline ($X^2 = 41.2$, $p < 0.001$; $N_{individuals} = 35$, $N_{observations} = 124$; Fig. 1A; Table A.1). In addition, prolactin concentrations were greater later during the incubation period compared to earlier ($X^2 = 36.0$, $p < 0.001$; Fig. 1A; Table A.1). The time of day of capture also explained some of the variation in prolactin, where prolactin concentrations were slightly higher later in the day ($X^2 = 3.86$, $p = 0.0496$; Table A.1).

In contrast to prolactin concentrations, corticosterone concentrations remained similar throughout early and late incubation (Fig. 1C; Table A.1), but were related to hen body condition. Body condition was negatively related to both baseline and stress-induced corticosterone concentrations ($X^2 = 10.9$, $p < 0.001$; Table A.1). Further, as

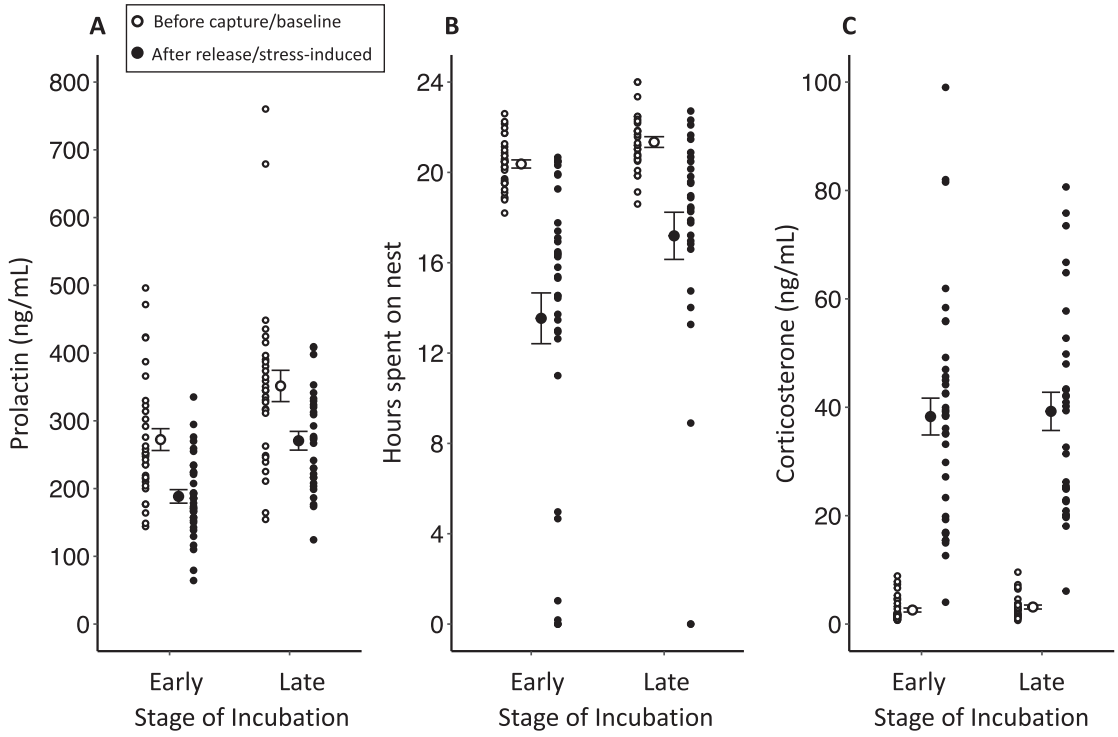

Fig. 1. Prolactin concentrations (A) of incubating wood duck hens decrease after 30 min of capture stress (open = baseline; closed = stress-induced) and are higher during late rather than early incubation. The amount of time that a hen spends incubating (B) is higher during the 24 h before capture than during the 24 h after release (open = before; closed = after), and hens spend more time on the nest during late compared to early incubation. Corticosterone concentrations (C) increase after 30 min of capture stress (open = baseline; closed = stress-induced), but are similar during early and late incubation. Large points are mean ± 1 SE. Prolactin and corticosterone were log-transformed and incubation constancy was cube-transformed for statistical tests, but raw values are plotted for clarity.

expected, corticosterone concentrations were higher after 30 min of capture/handling than they were at baseline (X^2 = 940, p < 0.001; $N_{individuals}$ = 37, $N_{observations}$ = 132; Fig. 1C; Table A.1).

3.2. Factors related to incubation constancy

Incubation constancy was affected by capture/handling and differed with the stage of incubation. On average, hens spent 27% less time on the nest during the 24 h after release than the 24 h before capture (X^2 = 103, p < 0.001; $N_{individuals}$ = 35, $N_{observations}$ = 128; Fig. 1B; Table A.1) and spent more time on the nest later during the incubation period compared to earlier (X^2 = 20.9, p < 0.001; Fig. 1B; Table A.1). Further, the time of day of capture also explained some of the variation in incubation constancy. The total time that birds spent on the nest during the 48 h of our study was slightly greater for birds that were captured later in the day compared to those captured earlier in the day (X^2 = 5.40, p = 0.020; Table A.1).

3.3. Relationships among hormones and incubation constancy

Stress-induced prolactin was related to incubation constancy after the disturbance, but there were no other relationships among hormones and incubation constancy. Stress-induced prolactin was positively related to the amount of time that hens spent incubating during the 24 h after release (X^2 = 6.18, p = 0.013; $N_{individuals}$ = 35, $N_{observations}$ = 63; Fig. 2; Table A.3). However, baseline prolactin was not related to incubation constancy during the 24 h after release (Table A.2), and neither stress-induced nor baseline prolactin concentrations were related to incubation constancy before capture (Tables A.2 and A.3). Corticosterone concentrations were not related to incubation constancy (Tables A.2 and A.3). Further, prolactin and corticosterone were not correlated either at baseline (r(spearman) = 0.05, p = 0.71) or stress-induced concentrations (r(spearman) = -0.06, p = 0.61).

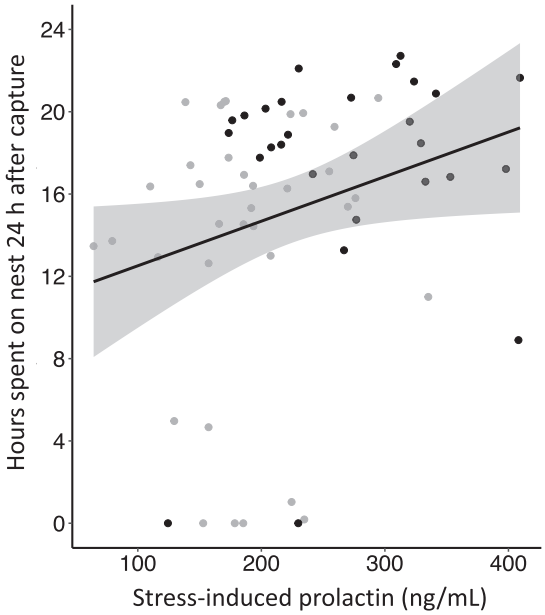

Fig. 2. Stress-induced prolactin concentrations are positively related to the amount of time that wood duck hens spend incubating during the 24 h following capture. Hens were captured both during early (gray points) and late (black points) incubation. The shaded areas show ± 1 SE. Time spent incubating was cube-transformed for statistical tests, but raw values are plotted for clarity.

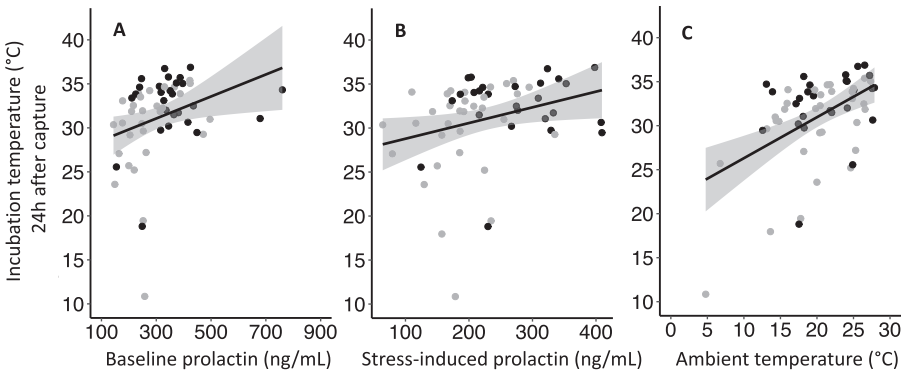

Fig. 3. Average incubation temperature during the 24 h following capture is positively related to (A) baseline prolactin concentrations, (B) stress-induced prolactin concentrations, and (C) average ambient temperature. Hens were captured both during early (gray points) and late (black points) incubation. The shaded areas show ± 1 SE. Incubation temperature was cube-transformed for statistical tests, but raw values are plotted for clarity. Statistical models were constructed using multiple predictors (Tables A.2 and A.3), but 2-way relationships are plotted for ease of interpretation.

3.4. Relationships among hormones and incubation temperature

Prolactin concentrations were related to incubation temperature during the 24 h after release, but not before capture. During the 24 h after release, average incubation temperature was positively related to both baseline ($X^2 = 7.94$, $p = 0.005$; Fig. 3A; $N_{observations} = 56$; $N_{individuals} = 31$; Table A.2) and stress-induced ($X^2 = 5.49$, $p = 0.019$; Fig. 3B; $N_{observations} = 58$; $N_{individuals} = 32$; Table A.3) prolactin concentrations. However, during the 24 h before capture, there were no relationships between incubation temperature and prolactin concentrations (Tables A.2 and A.3). Further, corticosterone was not related to incubation temperature (Tables A.2 and A.3). Ambient temperature was positively related to incubation temperature both before capture and after release (all $p < 0.01$; Tables A.2 and A.3; Fig. 3C). Clutch size was negatively related to incubation temperature before capture ($p < 0.001$; Tables A.2 and A.3), but not after release (Tables A.2 and A.3).

4. Discussion

In this study, we found evidence that inter-individual variation in prolactin concentrations could be important for the regulation of incubation constancy and egg temperature after a disturbance. Although prolactin is classically known as the parental care hormone (Buntin, 1996; Hall, 1986; Sockman et al., 2006; Vleck, 1998), surprisingly little is known about how it is related to parental decisions in a life-history context (Angelier et al., 2016). We found that both prolactin concentrations and incubation constancy increased as the incubation period progressed, but decreased after a nest disturbance. Further, incubating wood ducks with higher concentrations of stress-induced prolactin spent more time on the nest incubating during the 24 h after a nest disturbance (capture) than those with lower concentrations. Additionally, both baseline and stress-induced prolactin concentrations were positively related to egg incubation temperature during the 24 h that followed the disturbance, but not during the 24 h before. In contrast, corticosterone concentrations were not related to incubation constancy or temperature in any case. By revealing a possible role of prolactin in governing commitment to incubation after a stressor, this study improves our understanding of the proximate mechanisms that underlie how animals make decisions when faced with disturbances during parental care.

The individual relationships that we observed between prolactin, incubation constancy, and incubation temperature provide insight into how this hormone may be related to the regulation of incubation. First, we only found relationships among prolactin and incubation constancy and egg temperature during the 24 h after a disturbance, not before. Before the disturbance, there was little variation in incubation constancy among individuals (Fig. 1B). Thus it may be expected, statistically, that there would not be a significant relationship between these small behavioral differences and hormone concentrations. However, we found that incubation constancy was much more variable among individuals following a disturbance. Moreover, the concentration of prolactin that a parent maintained in the face of a disturbance was positively associated with both the time spent incubating and egg temperatures after that disturbance. This suggests that stress-induced prolactin concentrations may mediate parental behavior decisions when faced with environmental disturbances, which then may influence egg temperatures. In contrast, individual correlations revealed that baseline prolactin concentrations were not related to incubation constancy, but they were positively related to egg temperatures after the disturbance. It is possible that baseline prolactin could indicate the parent's ability to transfer heat (e.g., regulation of vascularization of the brood patch; Clapp et al., 2012; Jones, 1971) once they resume incubation, rather than the time spent incubating, after the disturbance. Together, these results provide evidence that small differences in prolactin concentrations among individuals could be an indicator of parental investment after being faced with a disturbance.

Our results involving corticosterone also shed light on the relationships between corticosterone, prolactin, and parental care in this species. In contrast to prolactin, corticosterone concentrations were not related to incubation constancy. Thus, we did not find support for either of our alternative hypotheses concerning corticosterone, which suggests that corticosterone neither mediates upregulation nor downregulation of incubation constancy in this species. Further, we found no relationship between prolactin and corticosterone. This is surprising in light of studies that show that experimentally increased corticosterone concentrations within the physiological range result in decreased prolactin concentrations, which then lead to decreased parental care behavior (e.g., Angelier et al., 2009). However, a review of the literature shows that the relationship between prolactin and corticosterone is complex and context- and species-dependent (reviewed in Angelier et al., 2013), and it is possible that these two hormones mediate different aspects of the behavioral and physiological stress response in some cases (Angelier and Chastel, 2009). Indeed, we found a negative relationship between corticosterone concentrations and female body condition, suggesting that a primary role of corticosterone during wood duck incubation may be to physiologically regulate energy use. Altogether, our results suggest that, when wood ducks face disturbances during incubation, prolactin may mediate the parental care response (including behavior and heat-transfer) while corticosterone may mediate the allocation of energy reserves throughout the entire body (Angelier and Chastel, 2009).

It is possible that the relationship that we found between stress-induced prolactin and incubation constancy (Fig. 2) was influenced by either the stage of incubation or the presence of visual or tactile stimuli from the eggs. Both prolactin and the amount of time that the hen spent incubating increased as the incubation period progressed and cannot be disentangled in our field study. Thus, the correlation that we found between incubation constancy and stress-induced prolactin concentrations after the disturbance may have been driven by incubation stage. However, if this relationship was solely driven by the relationship with

incubation stage, we would have also expected to find relationships between stress-induced prolactin and incubation constancy before the disturbance, and between baseline prolactin and incubation constancy both before and after the disturbance, none of which were the case. Additionally, classic studies show that prolactin is positively related to stimuli provided by visual cues and tactile contact with nests and eggs (Book et al., 1991; Hall, 1987, 1986; Leboucher et al., 1993; Sharp et al., 1998; Silver, 1984). Thus, it is possible that a decrease in prolactin concentrations in response to our standard experimental capture and restraint protocol may be, in part, a result of a lack of nest-related stimuli. However, it is unlikely that this is the sole explanation for a capture-related decrease in prolactin because there is evidence that stressors themselves influence prolactin concentrations (Angelier et al., 2016, 2015; Angelier and Chastel, 2009; Delehanty et al., 1997; Riechert et al., 2014). Further, if visual cues and tactile contact with eggs were driving hormone concentrations, we might have expected to find a positive relationship between clutch size and prolactin concentrations. However, clutch size was not a significant covariate in any of our models investigating hormone concentrations or incubation constancy. Taken together, our results call for future studies on incubation constancy where prolactin is manipulated to decouple the potential influence of sensory and breeding stage factors from prolactin concentrations.

Our study provides evidence that the behaviors of incubating parents after being faced with a disturbance may be related to prolactin concentrations. From a proximate perspective, this suggests that the plasma concentration of prolactin that an individual maintains after a stressor may play a role in regulating incubation constancy after a disturbance, and may have consequences for developing offspring via altered egg temperatures. However, the ultimate reason for why prolactin concentrations, incubation constancy, and temperature vary among individuals remains unknown. Because decreased incubation constancy and incubation temperature can have negative fitness consequences for offspring, baseline and stress-induced prolactin concentrations of parents and their related behavioral and energetic responses should be under selective pressure. Thus, the finding that individuals have different physiological and behavioral responses to the same disturbance, some of which may be disadvantageous for offspring development, suggests that individuals face other constraints or trade-offs. Future studies could further investigate the role of prolactin concentrations in a life history context by manipulating prolactin and determining if plasma concentrations are related to incubation constancy following repeated disturbance, probability of nest abandonment, thermal conditions of eggs over the entire incubation period, and offspring quality. Because disturbances that affect animals are becoming more common in the current era of rapid global change (e.g., urbanization, extreme weather events), it is important to fully understand how parents respond physiologically and behaviorally to disturbances.

CRediT authorship contribution statement

Sydney F. Hope: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Funding acquisition. **Sarah E. DuRant:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Frédéric Angelier:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **John J. Hallagan:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Ignacio T. Moore:** Methodology, Resources, Writing - review & editing. **Charline Parenteau:** Methodology, Investigation. **Robert A. Kennamer:** Methodology, Resources, Writing - review & editing. **William A. Hopkins:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Competing interests

The authors have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2020.113488.

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