**Red-winged blackbirds (*Agelaius phoeniceus*) with higher baseline glucocorticoids
also invest less in incubation and clutch mass**

Laura A. Schoenle^{*a}, Alana M. Dudek^a, Ignacio T. Moore^a, and Frances Bonier^{ab}

Abstract

Glucocorticoid hormones facilitate responses to environmental challenges by mediating diverse physiological and behavioral changes, including resource mobilization and altered reproductive effort. Elevated glucocorticoids might indicate that an individual is facing high levels of environmental challenges and thus, elevated concentrations might be associated with reduced fitness (CORT-fitness hypothesis). Alternatively, the energetic demands of reproduction might be a challenge that requires elevated glucocorticoids to mobilize resources to support reproductive effort, ultimately increasing reproductive investment and fitness (CORT-adaptation hypothesis). Investigations of glucocorticoid-fitness relationships have yielded mixed results. Variation in the direction of this relationship could be caused in part by differences in the contexts in which the relationship was assessed. Incorporating context, such as life history stage, could be key to understanding the role of glucocorticoids in influencing fitness outcomes. We investigated the relationship between corticosterone and reproductive effort and success within a single life history stage: incubation of eggs. In an observational study, we measured baseline corticosterone in incubating female red-winged blackbirds (*Agelaius phoeniceus*), monitored incubation behavior, and determined hatching success for each nest. Incubating birds with higher baseline corticosterone concentrations had more frequent, shorter incubation bouts and spent less time overall incubating their clutches of eggs than birds with lower corticosterone concentrations. Elevated corticosterone was also associated with lower clutch mass, but neither corticosterone nor incubation effort

were correlated with hatching success. Although experimental tests are needed to establish causation, these results suggest that during the incubation period, corticosterone might shift resource investment towards self-maintenance, and away from current reproductive effort.

Keywords: corticosterone; incubation; clutch mass; reproduction; fitness

Introduction

When an individual is faced with immediate or chronic environmental challenges, glucocorticoid hormones can mediate a suite of physiological and behavioral responses, including shifts in resource acquisition and mobilization, and reproductive behaviors (Romero, 2004; Sapolsky et al., 2000). The role of glucocorticoids in facilitating the response to environmental and social challenges has lead to interest in the relationship between these hormones and fitness (e.g., Breuner et al. 2008, John-Alder et al. 2009, Ebensperger et al. 2011, Vitousek et al. 2014, Madliger and Love 2016). Initially, elevated baseline glucocorticoid concentrations (i.e., levels not influenced by an immediate threat/acute stressor) were thought to signal that an individual was in poor condition or facing challenges (Arlettaz et al., 2007; Bonier et al., 2009a; Wikelski and Cooke, 2006). The CORT-fitness hypothesis is built on this assumption and asserts that glucocorticoids will negatively correlate with fitness and reproductive effort because individuals facing greater environmental challenges will have higher concentrations of circulating glucocorticoids (Bonier et al., 2009b). However, the relationship between

baseline glucocorticoids and survival and/or reproduction appears to be highly variable across contexts (Bonier et al., 2009a; Crespi et al., 2013). Even within a single species, the direction of the relationship between glucocorticoids and fitness can vary across life history stages (Bonier et al., 2009b; Ouyang et al., 2013, 2011), environmental conditions (Riechert et al., 2014; Schultner et al., 2013), or among individuals with different reproductive strategies (Lancaster et al., 2008). For example, in house sparrows (*Passer domesticus*), both males and females with low glucocorticoid concentrations prior to breeding and high glucocorticoid concentrations during breeding produced the most offspring (Ouyang et al., 2011).

To account for the variation in the direction of glucocorticoid-fitness relationships, Bonier et al. (2009a) proposed an extension of the CORT-fitness hypothesis: the CORT-adaptation hypothesis. The CORT-adaptation hypothesis suggests that if reproduction is energetically challenging, then glucocorticoids could mediate physiological and behavioral changes in support of meeting that challenge, resulting in increased reproductive effort and success (Bonier et al. 2009a). In support of this hypothesis, there is a growing body of evidence that glucocorticoids can be associated with increased reproductive success and thereby, fitness (e.g., Bonier et al. 2009a, Escribano-Avila et al. 2013, Riechert et al. 2014). We might expect to find support for the CORT-adaptation hypothesis when reproductive effort is the primary factor influencing individual variation in glucocorticoid concentrations, which is most likely during the most energetically-demanding stages of reproduction and/or in the individuals that invest most in reproduction. To determine whether glucocorticoid concentrations are tightly

linked to challenges associated with reproduction, studies testing the CORT-adaptation hypothesis should assess reproductive effort in addition to metrics of reproductive success. Life history stage must also be taken into account because the relationship between glucocorticoids and both reproductive effort and success can vary across life history stages (Bonier et al., 2009b; Ouyang et al., 2013, 2011).

Avian species are frequently used to address questions about reproductive effort and success because it is relatively easy to identify specific life history stages and monitor both parental effort and the reproductive outcome. In particular, the avian life history stage of incubation offers a unique opportunity to test the relationship between glucocorticoids and reproductive effort. In most avian species, an incubating bird spends its time either on the nest, regulating temperature and otherwise caring for its clutch of eggs, or away from the nest, fulfilling its own needs, often by foraging. This dichotomy in behavior means that reproductive effort can be easily quantified by measuring the time spent on or away from the nest.

To date, few studies have examined the relationship between corticosterone (the primary avian glucocorticoid) and incubation behavior, and most focus on seabird species that experience prolonged fasts during incubation. In seabirds, high corticosterone is associated with reduced effort during incubation and reproductive success. For example, both experimental and observational studies of the Adélie penguin (*Pygoscelis adeliae*) have found higher corticosterone to be associated with reduced nest attentiveness and increased likelihood of abandonment (Spée et al., 2011, 2010; Thierry et al., 2013).

Similar patterns were found in the common eider (*Somateria mollissima*), where

incubating females implanted with corticosterone were more likely to lose an egg to predation (typically due to low nest attentiveness) than control females (Criscuolo et al., 2005) and in an observational study, higher corticosterone was linked to reduced reproductive success in females that selected exposed nesting sites (D'Alba et al., 2011). However, the physiological mechanisms associated with incubation effort could be very different in species that rely on energy stores to support incubation bouts that last for hours or days (e.g. common eider [Bolduc and Guillemette, 2003], Adélie penguin [Derksen, 1977]) versus species, like most songbirds, that often incubate for less than thirty minutes at a time (Conway and Martin, 2000). Birds that frequently switch between incubating eggs and tending to their own needs are continuously trading off the time invested in reproduction (time on the nest) with the time invested in self-maintenance (time away from the nest). To the best of our knowledge, there have been no studies that successfully investigate the relationship between individual variation in corticosterone and incubation effort in a bird with relatively short incubation bouts (but see Lothery et al. 2014).

Here, we test the CORT-fitness hypothesis and its extension, the CORT-adaptation hypothesis in incubating female red-winged blackbirds (*Agelaius phoeniceus*), which regularly leave the nest to feed during incubation. Based on the CORT-fitness hypothesis, we predict that females with elevated circulating glucocorticoids should reduce investment in incubation and ultimately have lower reproductive success than individuals with lower glucocorticoid concentrations. Based on the CORT-adaptation hypothesis, we predict that if the energetic demands of reproduction drive variation in glucocorticoids

among incubating females, females with higher corticosterone will increase their incubation effort, resulting in higher reproductive success.

Methods

Study species and population

Red-winged blackbirds are polygynous, and multiple females will nest on the territory of a single male (Searcy and Yasukawa, 1995). Females are the sole incubator of eggs and hatchlings (Holcomb, 1974) and are not fed by their mate (Beletsky, 1996). As a result, the relationships between a female's corticosterone, incubation behavior, and nest success might be more tightly linked than in species with bi-parental incubation or mate feeding. In red-winged blackbirds, incubation effort decreases across the incubation period (Holcomb, 1974), therefore we included day of incubation in all analyses of incubation effort. We studied red-winged blackbirds nesting across six marshes at the Queen's University Biological Station (44°34'02.3" N, 76°19'28.4" W) in southeastern Ontario during May - June 2014 and 2015. All marshes provided similar nesting habitat and we found no difference in the birds' corticosterone, scaled mass index, fat score, incubation effort, clutch size or mass, or hatching success across marshes (all $P > 0.25$), and thus, we do not distinguish among marshes for our analyses. All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee and the Queen's University Animal Care Committee and were in compliance with US and Canadian national standards for the use of animals in research.

Trapping and Blood Collection

In 2014 and 2015, we captured 59 incubating female red-winged blackbirds in 70 capture events. We captured females between 5:30 - 10:30 AM on days 3-11 of the 12-day incubation period by flushing them into mist nets placed 1-3 m from the nest or by placing a single-celled walk-in (Potter) trap directly on top of the nest. Both traps and nets were set-up when the female was away from the nest. We estimated the day of incubation by counting the number of days after clutch completion or before the hatching date. Four individuals were excluded from any analyses including the day of incubation because we were unable to determine either the clutch completion date or hatch date.

Rapidly after capture, we punctured the brachial vein with a 26 gauge ½ inch needle and collected 300 µL of blood into heparinized capillary tubes. We recorded the exact time of sample collection after the bird entered the net or trap and used only samples collected within three minutes for analyses including corticosterone (Romero and Reed, 2005). We found no relationship between the log of baseline corticosterone and time from capture among the samples collected within 3 minutes ($\beta = 0.0004$, 95% CI = -0.002 – 0.003, $P = 0.72$, $N = 40$) or between log corticosterone and the time from net or trap set-up and capture ($\beta = 0.004$, 95% CI = -0.01 – 0.003, $P = 0.28$, $N = 28$). Therefore, we concluded that these samples were reasonable representations of baseline concentrations of corticosterone.

We fitted each individual with a unique combination of color bands and a numbered aluminum band from the Canadian Wildlife Service. We measured wing cord with a wing rule (to the nearest 1 mm), tarsus with calipers (to the nearest 0.1 mm), mass using a Pesola spring scale (to the nearest 0.5 g), and scored fat on a 0-5 scale (Wingfield

and Farner, 1978). We assessed the body condition and age of females because incubation effort could be related to condition or previous breeding experience, and physiology and life history strategy could change with age. First year red-winged blackbirds have delayed plumage development such that 1 year old females have completely brown epaulet feathers (upper marginal-coverts) and females over 1 year in age have colored epaulets ranging from orange to red (Johnsen et al., 1996; Yasukawa and Searcy, 1995). Based on epaulet color, we identified females as either 1 year old (i.e., in their first breeding season) or older (i.e., in their second or later breeding season). While one person processed the female, another person used a Pesola spring scale to weigh each egg in the nest (to the nearest 0.1 g). We monitored nests every other day after capturing the female until hatching to determine hatching success and identify predation events.

Incubation Monitoring

We placed temperature loggers (Thermochron iButton DS1921G-F5) in the nest of each female the day before the incubation assessment to monitor incubation behavior without requiring an observer near the nest (Hartman and Oring, 2006). We glued temperature loggers to a nail and placed the logger in the nest by pushing the nail through the nesting material to prevent the females from removing the logger. We set the temperature loggers to record temperatures at 1-minute intervals for about 34 hours (resolution to 0.5° C).

We monitored incubation behavior one or two days after we caught the female starting with the first off-bout of the day and ending 8 hours later. Monitoring incubation shortly after capture allows us to pair hormone concentrations closely with behavior

while simultaneously minimizing the effects of capture on incubation behavior. We defined an off-bout as a period with a drop in temperature of at least 1.5° C that began with a minimum cooling rate of 0.25° C per minute and an on-bout as a period with an increase in temperature of at least 1.5° C and a minimum initial warming rate of 0.25° C per minute. We selected these conservative temperature parameters to minimize the likelihood of missing off-bouts at warm ambient temperatures. For each incubation observation, we plotted the nest temperatures by time to verify visually that our decision rules mapped to reasonable on-off bout patterns. We validated our incubation monitoring protocol using 1 hour of direct visual observations of 5 nests and found our iButton data to provide a reliable estimate of true on-off bouts (see supplementary material, Fig. S1). We successfully monitored incubation in 46 nests after capture (observations from an additional 9 nests were discarded because we were unable to clearly identify regular on-off bouts using the decision rules described above).

Weather data collection

We obtained ambient temperature data for the field site from weather stations at the Queen's University Biological Station. As we expected precipitation to influence incubation behavior, we noted whether or not it was raining on the day of the incubation observation. It rained during the incubation monitoring period for only 2 incubation observations, and thus, we placed a new logger in the nest and monitored the incubation temperatures 3 or 4 days post-capture to minimize the influence of variation in behavior due to inclement weather conditions. We performed our analyses both including and

excluding nests monitored more than 1 day after capture and including them did not influence the outcomes of the study.

Corticosterone Assay

We centrifuged the blood samples in capillary tubes at 6,000 RPM for 10 minutes within six hours of sample collection. We then separated the plasma from the red blood cells and froze the plasma at -20 °C until conducting the hormone assay. We quantified total corticosterone in plasma using a direct radioimmunoassay (Wingfield et al., 1992) after extracting the hormone with dichloromethane. In 2014, we performed a single extraction with 5 mL of dichloromethane and in 2015, to improve extraction efficiency, we performed a double extraction using 3 mL of dichloromethane per extraction. We used a corticosterone antibody from Esoterix Endocrinology (catalogue number B3-163) and labeled corticosterone from New England Nuclear Research Products (catalogue number NET-399). All samples from 2014 and 2015 were run in a single assay within each year. Samples were individually corrected for extraction efficiency, and average extraction efficiency was 75% and 78% in 2014 and 2015, respectively. The average detection limit was ~1.4 ng/mL in 2014 and ~1.0 ng/mL in 2015. In 2014, 3/22 samples fell beneath the detection limit and samples were assigned the concentration at that limit. In 2015, no samples fell below the detection limit. Within-assay variation of 4 replicates of known-concentration standards was 6.3% in 2014 and 9.7% in 2015. Inter-assay variation was 8.2%.

Statistical analyses

We performed all statistical analyses using R software, Version 3.2.4 (R Core Team 2016). We used only two-tailed tests and set the alpha level at 0.05. We used log (base 10)-transformed corticosterone for all analyses including the hormone to improve model fit. All means are reported as mean +/- standard error and unstandardized regression coefficients are reported as β . To avoid pseudo-replication, we used data from a single capture and its associated incubation observation from each female that was sampled during more than one breeding attempt. We selected the observation for which we had all physiological data or, if both observations were associated with a complete dataset, we used the first of the two observations.

Corticosterone, body condition, and age

To evaluate the relationship between corticosterone, body condition, and age we performed a linear mixed model and model selection including log corticosterone as the response variable, age, fat score, and scaled mass index as fixed effects, and year as a random effect. Scaled mass index is a condition index based on mass and a morphometric measure that accounts for the scaling relationship between different measures of body size (Peig and Green, 2009). To calculate the scaled mass index, we used wing cord as a measure of structural size because wing cord was the structural measure most strongly correlated with mass. We used second order Akaike's Information Criterion (AICc) to identify the best fit model from all possible candidate models, including a null model. For the full models and each of the top models obtained through model selection (within 2 AICc of the best-fit model), we tested the distribution of the residuals for normality with a Shapiro-Wilk normality test and visually inspected the relationship of the residuals and

predicted values to assess model fit. Model fit was good for all tested models. We used model averaging of the top models to estimate the effect sizes and p-values of the predictor variables.

To assess the relationship between female age and body condition, we used a Pearson's chi-square test for the analysis of the fat score-age relationship and a 2-sample t-test for the analysis of age and scaled mass index.

Principal components analysis (PCA) of incubation behavior

Using the data collected by the temperature loggers, we identified the following metrics to describe incubation behavior over the 8 hour incubation period: 1) the total time spent on the nest, 2) the number of incubation bouts, 3) the mean length of an on-bout, and 4) the mean length of an off-bout. The four metrics were correlated, and thus, we applied a principal components analysis (PCA) to a correlation matrix of the four incubation variables to reduce dimensionality. We performed a parallel analysis (Horn, 1965) with the `fa.parallel` function in the `{psych}` package and determined that two principal components should be retained from the PCA. We confirmed that two factors were appropriate by visually evaluating a scree plot (Everitt and Hothorn, 2011). The two principal components cumulatively explained 95.8% of the variance (PC1 = 59.3%, PC2 = 36.5%). A more positive PC1 value was associated with frequent, brief on-off bouts and less total time incubating (Table 1). To aid interpretation of the results, we multiplied the PC1 scores by -1, such that a more positive value of this transformed PC1 reflects greater investment in incubation. A more positive PC2 was associated with greater total time spent incubating, but briefer on-bouts (Table 1).

Corticosterone and incubation effort

To test the alternative predictions that corticosterone is either positively or negatively correlated with investment in incubation behavior, we used two linear mixed effects models (LMM) with -PC1 and PC2 as the response variables, trap type and year as random effects, and log corticosterone, day of incubation, minimum temperature, age, and the two-way interactions of log corticosterone with day of incubation and log corticosterone with minimum temperature as fixed effects. Corticosterone (but not scaled mass index or fat, all $P > 0.15$) differed between birds caught in nets and walk-in traps (see supplementary material, Fig. S2), so we evaluated additional models including trapping method and the interaction of trapping method and corticosterone as fixed effects. Neither of these additional fixed effects were significant predictors of behavior, and we continued analyses with trap type as a random effect. Including trap type and year as a random effect did not improve model fit, and thus we excluded them from the final models and instead used simple linear regression. We identified the top models using AICc and checked for model fit as described above. Model fit was good for models with -PC1 as a response variable and moderate when PC2 was the response variable (residuals were left skewed). We used model averaging of the top models to estimate the effect sizes and p-values of the predictor variables.

Corticosterone and metrics of reproduction

We assessed the relationships between corticosterone, -PC1, and PC2 with the reproductive metrics of clutch mass (cumulative mass of all of the eggs in one clutch), clutch size (number of eggs in one clutch), mean egg mass, and hatching success (the

proportion of eggs that hatched). Clutch mass reflects the overall resources and effort the female allocated to producing eggs, while clutch size and mean egg mass can provide information about whether females are investing in producing large eggs and/or many eggs. In addition, egg mass can predict nestling size, growth rate, and survival (e.g., Whittingham et al., 2007; Williams, 1994). Egg mass can change with time due to water loss, and thus, we included the day of incubation on which eggs were weighed in all models predicting clutch or egg mass. We used a t-test to determine how clutch mass related to clutch size. In our population, only one female laid 5 eggs (all others laid 3 or 4), and because it was not possible to estimate variance for the bird laying 5 eggs, we excluded it from analyses of clutch size, but included it in Fig. 2B and Fig. S4. When testing the relationships between incubation and reproduction, we analyzed the residuals of both -PC1 and PC2 controlling for day of incubation to control for variation in incubation behavior across the life history stage. We used a simple linear regression with clutch mass or mean egg mass as the response variable and either corticosterone, residual -PC1, or residual PC2 as the predictor variables. We used t-tests to assess whether or not there were differences in corticosterone, residual -PC1, or residual PC2 among birds laying 3 or 4 eggs. For hatching success, we used logistic regression with the same predictor variables. We visually inspected the relationship of the residuals and predicted values to assess model fit. We also compared each final model to the null model and unless mentioned in the results, $\Delta AICc > 2$.

Results

Corticosterone, body condition, and age

Baseline concentrations of corticosterone in female red-winged blackbirds ranged from 1.3 - 16.8 ng/mL (5.5 +/- 0.53 ng/mL, $N = 40$) and were higher in 2015 (7.05 +/- 0.94 ng/mL, $N = 18$) than in 2014 (4.32 +/- 0.46 ng/mL, $N = 22$). Neither age nor metrics of body condition were correlated with corticosterone. Although both fat and scaled mass index were included in two of the top four models (within 2 AICc of the best fit model) predicting corticosterone, they did not perform better than the null model (Table 2). Model averaging revealed no significant predictors for corticosterone (all $P > 0.12$).

There were no differences in fat score ($\chi^2 = 0.40$, $P = 0.53$, $N = 39$) or scaled mass index ($t = 0.17$, 95% CI = -1.67 – 1.97, $P = 0.87$, $N = 39$) between 1 year old females and females that were 2 or more years old.

Corticosterone and incubation effort

Females varied across all metrics of incubation (Table 3). The top model predicting incubation effort (-PC1) included both log corticosterone and the day of incubation (Table 4). Females with higher corticosterone (Fig. 1) and females sampled later during the incubation period (Fig. S3, supplementary material) had reduced incubation effort (Table 5). Minimum temperature, and the interactions between corticosterone and temperature and corticosterone and day of incubation did not predict incubation effort and were not retained in the top model (but see additional model rankings in Table 5). The two top models predicting PC2 included: 1) the null model and 2) a model containing only day of incubation, with PC2 decreasing across the incubation period. Model averaging revealed no significant predictors for PC2.

Corticosterone, incubation behavior, and reproductive output

Females with lower corticosterone laid heavier clutches than those with higher corticosterone ($\beta = -5.06$, 95% CI = -7.89 - -2.23, $P = 0.001$, $N = 30$) (Fig 2A). In addition, there was a trend for females that laid 4-egg clutches to have lower corticosterone than those laying 3-egg clutches ($t = 1.86$, 95% CI = -0.02 - 0.38, $P = 0.08$, $N = 39$) (Fig. 2B). Larger clutches were heavier than smaller clutches ($t = -3.25$, 95% CI = -3.93 - -0.88, $P = 0.003$, $N = 34$) (Fig. S4), suggesting that the number of eggs contributes differences in clutch mass. Corticosterone was not correlated with mean egg mass ($\beta = -0.38$, 95% CI = -1.23 - 0.48, $P = 0.37$, $N = 30$) (Fig. S5). Incubation effort (-PC1 and PC2) was unrelated to clutch size (-PC1: $t = -1.09$, 95% CI = -1.86 - 0.60, $P = 0.29$, $N = 36$; PC2: $t = -0.24$, 95% CI = -1.32 - 1.06, $P = 0.81$, $N = 36$), clutch mass (-PC1: $\beta = 0.52$, 95% CI = -0.22 - 1.26, $P = 0.16$, $N = 29$; PC2: $\beta = -0.42$, 95% CI = -1.59 - 0.74, $P = 0.46$, $N = 29$), or mean egg mass (-PC1: $\beta = -0.02$, 95% CI = -0.21 - 0.17, $P = 0.83$, $N = 29$; PC2: $\beta = 0.12$, 95% CI = -0.17 - 0.41, $P = 0.40$, $N = 29$). When testing the relationship between incubation effort and mean egg mass, the best fit models were the null models. We detected no relationship between incubation effort (-PC1 or PC2) or corticosterone and hatching success (-PC1: $\beta = -0.12$, 95% CI = 0.72 - 1.08, $P = 0.23$, $N = 33$; PC2: $\beta = 0.07$, 95% CI = 0.85 - 1.37, $P = 0.55$, $N = 33$; log corticosterone: $\beta = 0.16$, 95% CI = 0.45 - 3.08, $P = 0.75$, $N = 36$).

Discussion

As predicted by the CORT-fitness hypothesis, birds with higher corticosterone invested less in reproduction than birds with lower corticosterone, having both lower incubation effort and lighter clutches of eggs. Corticosterone could contribute to a

reduction in incubation effort by 1) increasing foraging behavior or 2) acting on the physiological mechanisms supporting incubation behavior. Corticosterone is associated with increased foraging behavior in several avian species (e.g. Astheimer et al. 1992, Lohmus et al. 2006, Angelier et al. 2007a, 2007b, 2008, Crossin et al. 2012, but see: Cottin et al. 2014). For example, red-eyed vireos (*Vireo olivaceus*) fed meal worms injected with corticosterone increased rates of feeding relative to control individuals (Lohmus et al., 2006). It follows that if a bird spends more time foraging, it must spend less time incubating. Thus, corticosterone could be mediating a trade-off favoring self-maintenance activities over reproduction by increasing foraging behavior (Breuner, 2011; Wingfield and Sapolsky, 2003). Corticosterone could also influence other hormones associated with reproduction. In birds, corticosterone can suppress circulating concentrations of prolactin (Angelier and Chastel, 2009; Angelier et al., 2009; Criscuolo et al., 2005, but see: Crossin et al., 2012), a hormone associated with increased parental investment in reproduction (Angelier and Chastel, 2009; Smiley and Adkins-Regan, 2016). If corticosterone suppresses prolactin in female red-winged blackbirds, we might expect to see a reduction in incubation effort associated with increased corticosterone.

Reducing current reproductive investment could increase the likelihood of successful reproductive bouts in the future (Love et al., 2014), and as a result, might increase lifetime fitness in organisms that can reproduce multiple times throughout their lives. Red-winged blackbird females have an average lifespan of 2.14 years (Yasukawa and Searcy, 1995), and after reaching adulthood (1 year), breed for an average of 2.4 +/- 1.9 years (Beletsky, 1996). Therefore, corticosterone could mediate an adaptive trade-off

response between current and future reproduction by stimulating foraging and/or regulating incubation behavior. As the probability of having future reproductive opportunities could change with age, we might expect reproductive investment to vary with age as well. However, there was no difference in incubation effort between females that were one year old as compared to females that were two or more years old. Females might make decisions about reproductive investment based on an assessment of their condition and the likelihood of successfully fledging young in the current bout, both of which are not necessarily age-independent.

Alternatively, corticosterone concentrations could increase in response to environmental challenges that also reduce reproductive investment, creating a correlation between corticosterone and reproduction that does not reflect causation. Given that corticosterone is expected to increase with energetic demand, it is unlikely that a reduction in incubation effort or investment in eggs would cause an increase in corticosterone. Individuals in poorer condition might be expected to both increase corticosterone and reduce investment in incubation and their clutch. Although we found no support for relationships between body condition and corticosterone, minute-to-minute energetic status might not be reflected in our condition metrics and could underlie the negative correlations between corticosterone and investment in reproduction. Frequent disturbances at the nest could also explain more frequent on-off bouts, a reduction in the time spent incubating, and higher corticosterone concentrations. We did not observe differences in these variables across field sites, but individual nests may vary in exposure to levels of disturbances, possibly from potential predators like snakes or raccoons.

Two studies have addressed the relationship between corticosterone and incubation effort in songbirds, but neither was able to assess the link between individual variation in these traits. Lothery et al. (2014) found no relationship between corticosterone and incubation effort in an observational study of the house wren (*Troglodytes aedon*). However, birds with higher corticosterone were more likely to abandon after capture (39/71 nests abandoned), resulting in a biased sample. Ouyang et al. (2013a) experimentally elevated corticosterone in great tits (*Parus major*) and found a positive relationship between corticosterone and incubation effort, which is the opposite of our observation in red-winged blackbirds. One explanation for this difference is that manipulations with exogenous hormone might induce different effects from endogenous increases in hormone concentrations (Dantzer et al., 2016; Sopinka et al., 2015). The extended duration and constant release of corticosterone from the silastic implants used by Ouyang et al. could alter how an individual produces or responds to the hormone or influence other physiological or behavioral controls of incubation (Dantzer et al., 2016), like androgen levels (de Jong et al., 2016; Oring et al., 1989). Alternatively, the difference in the direction of the relationship between corticosterone and incubation effort might be explained by differences in brood value, which is the value of a single reproductive bout relative to the lifetime fitness of an average individual. Female great tits have larger clutches [great tit, average ~8.53 eggs (Boyce and Perrins 1987); red-winged blackbird, average ~3.28 eggs (Yasukawa and Searcy 1995)] and fewer reproductive opportunities [average lifespan: great tit, 1.4 – 1.91 years (Perrins, 2004); red-winged blackbird, 2.14 years (Yasukawa and Searcy, 1995)] than red-winged blackbirds. As a result, female great

tits have a faster pace of life and their brood value is higher than the brood value of female red-winged blackbirds. Positive relationships between corticosterone and reproductive effort (CORT-adaptation hypothesis) might occur more often in species in which the value of a current reproductive bout is high (Bókonyi et al., 2009) if individuals are heavily investing in reproduction. The negative relationship between corticosterone and incubation behavior observed in female red-winged blackbirds mirrored that observed in various seabird species (Criscuolo et al., 2005; Spée et al., 2011, 2010; Thierry et al., 2013) that have a relatively slow pace-of-life and low brood value for each reproductive bout.

The cost of incubation relative to other challenges also might contribute to the direction of the relationship between glucocorticoids and both reproductive effort and success. Sub-optimal environmental conditions can enhance or reveal glucocorticoid-fitness relationships. For example, in Adélie penguins, the negative effect of corticosterone on incubation effort was enhanced during severe weather events (Thierry et al., 2013). In our study, we observed a negative relationship between corticosterone and incubation behavior, but no relationship between corticosterone and hatching success. Our moderate sample size and low variation in hatching success might have prevented us from identifying the relationships, if they exist, or a corticosterone-hatching success relationship might not exist under relatively benign conditions. In both 2014 and 2015, the weather was mild throughout the breeding season, and good conditions may have improved the chances of a successful reproductive bout regardless of incubation effort.

In conclusion, our study suggests that in incubating female red-winged blackbirds, corticosterone might mediate a reduction in investment in the current reproductive bout. An experimental manipulation of corticosterone is required to determine if corticosterone causes changes in reproductive investment, both in terms of clutch mass and incubation effort. Although our findings generally align with the predictions of the CORT-fitness hypothesis, we saw no evidence that birds with higher corticosterone were facing greater challenges in terms of their body condition. In fact, we must take care not to assume corticosterone indicates poor condition. Further investigation of the CORT-fitness and CORT-adaptation hypotheses should assess individual variation in glucocorticoids over multiple life history stages. To understand the extent to which glucocorticoid-fitness relationships are context-dependent (i.e. linked to other factors like brood value and the environment), future work should sample across multiple years, sites of varying quality, and latitudes. Ultimately, explaining the link between glucocorticoids and fitness will require a combined analysis of observational and experimental studies collecting hormone and fitness data from a diversity of life history stages and contexts.

Acknowledgments

This work was supported by National Science Foundation grant IOS-1145625 to FB and ITM. LS was supported by the Virginia Tech Institute for Critical Technology and Applied Science and an EPA STAR fellowship (FP-917686). AD was supported by a Summer Undergraduate Research Fellowship from the Virginia Tech Fralin Life Science Institute and an Atlantic Coast Conference Creativity and Innovation grant. We would

like to thank Sophie Gong, Meredith Kernbach, Pria St. John, and Allyshia Van Tol for invaluable assistance in the field and the Queen's University Biological Station for logistical support. Two anonymous reviewers provided comments that improved the manuscript.

References

- Angelier, F., Bost, C.A., Giraudeau, M., Bouteloup, G., Dano, S., Chastel, O., 2008. Corticosterone and foraging behavior in a diving seabird: The Adélie penguin, *Pygoscelis adeliae*. Gen. Comp. Endocrinol. 156, 134–144.
- Angelier, F., Chastel, O., 2009. Stress, prolactin and parental investment in birds: A review. Gen. Comp. Endocrinol. 163, 142–148.
- Angelier, F., Clement-Chastel, C., Gabrielsen, G.W., Chastel, O., 2009. How does corticosterone affect parental behaviour and reproductive success? A study of prolactin in black-legged kittiwakes. Funct. Ecol. 23, 784–793.
- Angelier, F., Clement-Chastel, C., Gabrielson, G., Chastel, O., 2007a. Corticosterone and time–activity budget: An experiment with Black-legged kittiwakes. Horm. Behav. 52, 482–491.
- Angelier, F., Shaffer, S.A., Weimerskirch, H., Trouvé, C., Chastel, O., 2007b. Corticosterone and foraging behavior in a pelagic seabird. Physiol. Biochem. Zool. 80, 283–292.
- Arlettaz, R., Patthey, P., Baltic, M., Leu, T., Schaub, M., Palme, R., Jenni-Eiermann, S., 2007. Spreading free-riding snow sports represent a novel serious threat for wildlife.

Proc. R. Soc. B 274, 1219–1224.

Astheimer, L.B., Buttemer, W.A., Wingfield, J.C., 1992. Interactions of Corticosterone with Feeding, Activity and Metabolism in Passerine Birds. *Ornis Scand.* 23, 355–365.

Beletsky, L.D., 1996. The red-winged blackbird: the biology of a strongly polygynous songbird. Academic Press, San Diego, CA.

Bókony, V., Lendvai, A.Z., Liker, A., Angelier, F., Wingfield, J.C., Chastel, O., 2009. Stress response and the value of reproduction: are birds prudent parents? *Am. Nat.* 173, 589–98.

Bolduc, F., Guillemette, M., 2003. Incubation constancy and mass loss in the common eider *Somateria mollissima*. *Ibis*. 145, 329–332.

Bonier, F., Martin, P.R., Moore, I.T., Wingfield, J.C., 2009a. Do baseline glucocorticoids predict fitness? *Trends Ecol. Evol.* 24, 634–642.

Bonier, F., Moore, I.T., Martin, P.R., Robertson, R.J., 2009b. The relationship between fitness and baseline glucocorticoids in a passerine bird. *Gen. Comp. Endocrinol.* 163, 208–213.

Boyce, M.S., Perrins, C.M., 1987. Optimizing great tit clutch size in a fluctuating environment. *Ecology* 68, 142–153.

Breuner, C.W., 2011. Stress and Reproduction in Birds. *Horm. Reprod. Vertebr.* 4, 129–151.

Breuner, C.W., Patterson, S.H., Hahn, T.P., 2008. In search of relationships between the acute adrenocortical response and fitness. *Gen. Comp. Endocrinol.* 157, 288–95.

- Conway, C.J., Martin, T.E., 2000. Evolution of passerine incubation behavior: influence of food, temperature, and nest predation. *Evolution*. 54, 670–685.
- Cottin, M., Macintosh, A.J.J., Kato, A., Takahashi, A., Debin, M., Raclot, T., Ropert-coudert, Y., 2014. Corticosterone administration leads to a transient alteration of foraging behaviour and complexity in a diving seabird 496, 249–262.
- Crespi, E.J., Williams, T.D., Jessop, T.S., Delehanty, B., 2013. Life history and the ecology of stress: how do glucocorticoid hormones influence life-history variation in animals? *Funct. Ecol.* 27, 93–106.
- Criscuolo, F., Chastel, O., Bertile, F., Gabrielsen, G.W., Le Maho, Y., Raclot, T., 2005. Corticosterone alone does not trigger a short term behavioural shift in incubating female common eiders *Somateria mollissima*, but does modify long term reproductive success. *J. Avian Biol.* 36, 306–312.
- Crossin, G.T., Trathan, P.N., Phillips, R.A., Gorman, K.B., Dawson, A., Sakamoto, K.Q., Williams, T.D., 2012. Corticosterone predicts foraging behavior and parental care in macaroni penguins. *Am. Nat.* 180, E31–E41.
- D’Alba, L., Spencer, K.A., Nager, R.G., Monaghan, P., 2011. State dependent effects of elevated hormone: Nest site quality, corticosterone levels and reproductive performance in the common eider. *Gen. Comp. Endocrinol.* 172, 218–224.
- Dantzer, B., Westrick, S.E., van Kesteren, F., 2016. Relationships between endocrine traits and life histories in wild animals: insights, problems, and potential pitfalls. *Integr. Comp. Biol.* 56, 185–197.
- de Jong, B., Lens, L., Amininasab, S.M., van Oers, K., Darras, V.M., Eens, M., Pinxten,

- R., Komdeur, J., Groothuis, T.G.G., 2016. Effects of experimentally sustained elevated testosterone on incubation behaviour and reproductive success in female great tits (*Parus major*). *Gen. Comp. Endocrinol.* 230–231, 38–47.
- Derksen, D. V., 1977. A quantitative analysis of the incubation behavior of the Adélie penguin. *Auk* 94, 552–566.
- Ebensperger, L.A., Ramírez-Estrada, J., León, C., Castro, R.A., Tolhuysen, L.O., Sobrero, R., Quirici, V., Burger, J.R., Soto-Gamboa, M., Hayes, L.D., 2011. Sociality, glucocorticoids and direct fitness in the communally rearing rodent, *Octodon degus*. *Horm. Behav.* 60, 346–352.
- Escribano-Avila, G., Pettorelli, N., Virgós, E., Lara-romero, C., Lozano, J., Barja, I., Cuadra, F.S., Puerta, M., 2013. Testing Cort-Fitness and Cort-Adaptation hypotheses in a habitat suitability gradient for roe deer. *Acta Oecologica* 53, 38–48.
- Everitt, B., Hothorn, T., 2011. *An Introduction to Applied Multivariate Statistics*. Springer, New York, NY.
- Hartman, C.A., Oring, L.W., 2006. An inexpensive method for remotely monitoring nest activity. *J. Field Ornithol.* 77, 418–424.
- Holcomb, L.C., 1974. Incubation constancy in the red-winged blackbird. *Wilson Bull.* 86, 450–460.
- Horn, J.L., 1965. A rationale and test for the number of factors in factor analysis. *Psychometrika* 30, 179–185.
- John-Alder, H.B., Cox, R.M., Haenel, G.J., Smith, L.C., 2009. Hormones, performance and fitness: Natural history and endocrine experiments on a lizard (*Sceloporus*

undulatus). Integr. Comp. Biol. 49, 393–407.

Johnsen, T.S., Hengeveld, J.D., Blank, J.L., Yasukawa, K., Nolan Jr., V., 1996. Epaulet

Brightness and Condition in Female Red-Winged Blackbirds. Auk 113, 356–362.

Lancaster, L.T., Hazard, L.C., Clobert, J., Sinervo, B., 2008. Corticosterone manipulation

reveals differences in hierarchical organization of multidimensional reproductive

trade-offs in r-strategist and K-strategist females. J. Evol. Biol. 21, 556–565.

Lohmus, M., Sundstrom, L.F., Moore, F.R., 2006. Non-invasive corticosterone treatment

changes foraging intensity in red-eyed vireos *Vireo olivaceus*. J. Avian Biol. 37,

523–526.

Lothery, C.J., Thompson, C.F., Lawler, M.L., Sakaluk, S.K., 2014. Food supplementation

fails to reveal a trade-off between incubation and self-maintenance in female house

wrens. PLoS One 9, e106260.

Love, O.P., Madliger, C.L., Bourgeon, S., Semeniuk, C.A.D., Williams, T.D., 2014.

Evidence for baseline glucocorticoids as mediators of reproductive investment in a

wild bird. Gen. Comp. Endocrinol. 199, 65–69.

Madliger, C.L., Love, O.P., 2016. Do baseline glucocorticoids simultaneously represent

fitness and environmental quality in an aerial insectivore? Oikos 1–14.

Oring, L.W., Fivizzani, A.J., El Halawani, M.E., 1989. Testosterone-induced inhibition of

incubation in the spotted sandpiper (*Actitis macularia*). Horm. Behav. 23, 412–423.

Ouyang, J.Q., Sharp, P.J., Dawson, A., Quetting, M., Hau, M., 2011. Hormone levels

predict individual differences in reproductive success in a passerine bird. Proc. R.

Soc. B Biol. Sci. 278, 2537–2545.

- Ouyang, J.Q., Sharp, P.J., Quetting, M., Hau, M., 2013. Endocrine phenotype, reproductive success and survival in the great tit, *Parus major*. *J. Evol. Biol.* 26, 1988–1998.
- Peig, J., Green, A.J., 2009. New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. *Oikos* 118, 1883–1891.
- Perrins, C.M., 2004. Great Tit (*Parus major*), in: Cramps, S., Simmons, K.E.L. (Eds.), BWPi: Birds of the Western Palearctic Interactive (DVD-ROM). Oxford University Press.
- Riechert, J., Becker, P.H., Chastel, O., 2014. Predicting reproductive success from hormone concentrations in the common tern (*Sterna hirundo*) while considering food abundance. *Oecologia* 176, 715–27.
- Romero, L.M., 2004. Physiological stress in ecology: lessons from biomedical research. *Trends Ecol. Evol.* 19, 249–255.
- Romero, L.M., Reed, J.M., 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* 140, 73–79.
- Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr. Rev.* 21, 55–89.
- Schultner, J., Kitaysky, A.S., Gabrielsen, G.W., Hatch, S.A., Bech, C., 2013. Differential reproductive responses to stress reveal the role of life-history strategies within a species. *Proc. R. Soc. B* 280.

- Searcy, W.A., Yasukawa, K., 1995. Polygyny and sexual selection in red-winged blackbirds. Princeton University Press, Princeton, NJ.
- Smiley, K.O., Adkins-Regan, E., 2016. Prolactin is related to individual differences in parental behavior and reproductive success in a biparental passerine, the zebra finch (*Taeniopygia guttata*). Gen. Comp. Endocrinol. 234, 88–94.
- Sopinka, N.M., Patterson, L.D., Redfern, J.C., Pleizier, N.K., Belanger, C.B., Midwood, J.D., Crossin, G.T., Cooke, S.J., 2015. Manipulating glucocorticoids in wild animals: basic and applied perspectives. Conserv. Physiol. 3, 1–16.
- Spée, M., Beaulieu, M.M., Dervaux, A., Chastel, O., Le Maho, Y., Raclot, T., Spée, M., Beaulieu, M.M., Dervaux, A., Chastel, O., Le Maho, Y., Raclot, T., 2010. Should I stay or should I go? Hormonal control of nest abandonment in a long-lived bird, the Adélie penguin. Horm. Behav. 58, 762–768.
- Spée, M., Marchal, L., Lazin, D., Le Maho, Y., Chastel, O., Beaulieu, M., Raclot, T., 2011. Exogenous corticosterone and nest abandonment: a study in a long-lived bird, the Adélie penguin. Horm. Behav. 60, 362–370.
- Thierry, A.M., Massemin, S., Handrich, Y., Raclot, T., 2013. Elevated corticosterone levels and severe weather conditions decrease parental investment of incubating Adélie penguins. Horm. Behav. 63, 475–483.
- Vitousek, M.N., Jenkins, B.R., Safran, R.J., 2014. Stress and success: individual differences in the glucocorticoid stress response predict behavior and fitness under high predation risk. Horm. Behav. 66, 812–819.
- Whittingham, L.A., Dunn, P.O., Lifjeld, J.T., 2007. Egg mass influences nestling quality

in tree swallows, but there is no differential allocation in relation to laying order or sex. *Condor* 109, 585–594.

Wikelski, M., Cooke, S.J., 2006. Conservation physiology. *Trends Ecol. Evol.* 21, 38–46.

Williams, T.D., 1994. Intraspecific variation in egg size and egg composition in birds: effects on offspring fitness. *Biol. Rev.* 68, 35–59.

Wingfield, J.C., Farner, D.S., 1978. The endocrinology of a natural breeding population of the White-Crowned Sparrow (*Zonotrichia leucophrys pugetensis*). *Physiol. Zool.* 51, 188–205.

Wingfield, J.C., Sapolsky, R.M., 2003. Reproduction and resistance to stress: when and how. *J. Neuroendocrinol.* 15, 711–724.

Wingfield, J.C., Vleck, C.M., Moore, M.C., 1992. Seasonal changes of the adrenocortical response to stress in birds of the Sonoran Desert. *J. Exp. Zool.* 264, 419–428.

Yasukawa, K., Searcy, W.A., 1995. Red-winged blackbird (*Agelaius phoeniceus*), in: Poole, A. (Ed.), *The Birds of North America Online*. Cornell Lab of Ornithology, Ithaca.

Tables

Table 1: Factor loadings of incubation behavior variables from 41 females onto two principal components.

	PC1	PC2
Eigenvalue	2.37	1.46
Variance Explained	59.3%	36.5%

Loadings

Total Time Incubating (min)	-0.290	0.730
Mean on-bout length (min)	-0.385	-0.646
Mean off-bout length (min)	-0.621	0.184
Number of incubation bouts	0.618	0.126

Table 2: Top models (including the null model) testing for the relationship between body condition metrics, age, and corticosterone. Models include year as a random effect ($N = 38$).

Model Rank	K ¹	AICc ²	Effects ³	Δ AICc ⁴
1	2	10.8	SMI	0
2	2	10.94	Fat	0.14
3	1	11.23	Null (1)	0.43
4	3	11.47	SMI + Fat	0.67

¹K indicates the number of parameters in the model. ²AICc is the second order Akaike's Information Criterion. ³Variable abbreviations of Effects: *SMI* is scaled mass index and *Fat* is the score for furcular fat stores (scale 1-5). ⁴ Δ AICc is the difference in AICc from the best-fit candidate model.

Table 3: Summary statistics for female incubation behavior ($N = 41$). We monitored incubation behavior for 480 minutes beginning with the first off-bout of the day.

Incubation Metric	Mean +/- SE	Range (Min-Max)
Total time spent on the nest (minutes)	278.7 +/- 5.3	195 - 349
Number of incubation bouts	22.0 +/- 0.97	9 - 41
On-bout length (minutes)	13.8 +/- 0.7	6.1 – 25.1
Off-bout length (minutes)	9.7 +/- 0.6	5.4 – 31.7

Table 4: Top ten models and the null model testing for the relationship between corticosterone and incubation behavior, measured as principal component 1 ($N = 35$).

Model Rank	K ¹	AICc ²	Effects ³	Δ AICc ⁴
1	3	125.41	LogCORT + IncDay	0
2	4	127.68	LogCORT + IncDay + Age	2.27
3	4	127.90	LogCORT + IncDay + MinTemp	2.49
4	4	128.11	LogCORT + IncDay + LogCORT* IncDay	2.70
5	2	129.70	IncDay	4.29
6	5	130.29	LogCORT + IncDay + Age + MinTemp	4.88
7	5	130.61	LogCORT + IncDay + Age + LogCORT* IncDay	5.20
8	5	130.66	LogCORT + IncDay + MinTemp + LogCORT* IncDay	5.25
9	5	130.68	LogCORT + IncDay + MinTemp + LogCORT*MinTemp	5.27
10	3	132.10	IncDay + Age	6.69
17	1	134.85	Null (1)	9.44

¹K indicates the number of parameters in the model. ²AICc is the second order Akaike's Information Criterion. ³Variable abbreviations of Effects: *LogCORT* is log-transformed corticosterone, *IncDay* is the day of incubation on which we monitored incubation, *Year* is the year of data collection (2014 or 2015), *Age* is whether a female is one year of age or older, *MinTemp* is the minimum temperature during the incubation observation, * indicates an interaction between two variables. ⁴ Δ AICc is the difference in AICc from the best fit candidate model.

Table 5: Parameter estimates from the top simple linear regression model predicting incubation behavior. Bolded values highlight statistically significant parameters.

Parameter	β^1	β'^2	SE ³	p value
Log corticosterone	-2.31	-0.38	0.14	0.01
Day of incubation	-0.35	-0.49	0.15	0.002

¹ β is the regression coefficient. ² β' is the standardized regression coefficient. ³SE is the standard error for the standardized model.

Figures

Figure 1.

Figure 2.

Figure Captions

Figure 1 Females with higher corticosterone concentrations have increased incubation effort. Incubation effort is residual -PC1 controlling for the day of incubation on which the bird's behavior was monitored. Higher incubation effort indicates more total time spent on the nest, longer incubation bout lengths, and fewer bouts. Regression line is based on the top model predicting incubation effort ($\beta = -2.3$, 95% CI = -4.09 - -0.52, $R^2 = 0.34$, $P = 0.01$, $N = 35$).

Figure 2. Females with low corticosterone laid heavier clutches (A), and tended to lay more eggs (B), than females with higher corticosterone. (A) Residual clutch mass controlling for the number of days since clutch completion is plotted against the log of corticosterone. Regression line is based on a linear model including residual clutch mass as the response variable and corticosterone as the predictor ($\beta = -5.04$, 95% CI = -7.81 - -2.27, $R^2 = 0.33$, $P = 0.0009$, $N = 30$). (B) Birds that laid 4 eggs tended to have lower corticosterone than birds that laid 3 eggs, but the difference was not significant ($t = 1.86$, 95% CI = -0.02 - 0.38, $P = 0.08$, $N = 39$). The single female that laid 5 eggs followed a similar pattern, but because there was only one clutch of that size, it could not be included in the analysis.