

Maternal glucocorticoids do not predict reproductive investment nor breeding success in a free-ranging bird.

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RUNNING TITLE: Glucocorticoids and fitness

KEYWORDS: stressor, corticosterone, fitness, avian, tree swallow

SUMMARY STATEMENT: Maternal stress hormone levels do not predict fitness of female tree swallows.

ABSTRACT

The hormone corticosterone (CORT)) has been hypothesized to be linked with fitness, but the directionality of the relationship is unclear. The “CORT-fitness hypothesis” proposes that high levels of CORT arise from challenging environmental conditions, resulting in lower reproductive success (a negative relationship). In contrast, the “CORT-adaptation hypothesis” suggests that, during energetically demanding periods, CORT will mediate physiological or behavioural changes that result in increased reproductive investment and success (a positive relationship). During two breeding seasons, we experimentally manipulated circulating CORT levels in female tree swallows (*Tachycineta bicolor*) prior to egg laying, and measured subsequent reproductive effort, breeding success, and maternal survival. When females were recaptured during egg incubation and again during the nestling stage, the CORT levels were similar among individuals in each treatment group, and maternal treatment had no effect on indices of fitness. By considering variation among females, we found weak support for the “CORT-adaptation hypothesis”; there was a non-significant positive relationship between CORT levels during incubation and hatching success. During the nestling stage CORT levels were unrelated to any measure of investment or success. Within the environmental context of our study, we found little support for any relationship between maternal glucocorticoid levels and indices fitness.

INTRODUCTION

Within and among species individuals vary in the strategies used to maximise fitness, by adjusting the relative effort put into current versus future reproductive events (Williams, 2005; Hansen et al., 2016). There is ample evidence that increased energy expenditure and effort during one breeding bout results in decreased reproductive success, probability of re-nesting, or survival in subsequent bouts (Nager, 2006; Crossin et al., 2013, 2016; Harms et al., 2014; Bleu, Gamelon & Sæther, 2016; Henderson et al., 2017).

Glucocorticoids (GCs) have been hypothesized to be a mediator of the trade-off between current and future reproduction (Wingfield et al., 1998; Bleu, Gamelon & Sæther, 2016; Hansen et al., 2016). GCs are metabolic hormones that fluctuate daily with feeding and other activities, and under resting conditions regulate energy balance (Landys, Ramenofsky & Wingfield, 2006; Wilcoxon et al., 2011; Hau & Goymann, 2015). In response to an environmental stressor, GC levels increase rapidly, resulting in increased availability of metabolic substrates, and adjustment of behaviors toward immediate survival (Wingfield & Sapolsky, 2003; Romero, 2004) while inhibiting reproductive behaviour and physiology (Sapolsky, Romero & Munck, 2000; Dantzer et al., 2014), i.e. the CORT-trade-off hypothesis (Patterson et al., 2014).

GCs are thought to play a role in translating environmental cues into adaptive physiological responses. In birds, the dominant GC is corticosterone (hereafter, CORT), and an elevation of baseline CORT levels may signal a poor quality environment or an individual in poor condition (Bonier et al., 2009b). Following this reasoning, Bonier et al (2009a) formulated “the CORT-fitness hypothesis,” which predicts that individuals with higher circulating CORT levels would have lower fitness. In support of this, higher baseline CORT levels have been

negatively associated with habitat quality, body condition, hatching success, and fledging success across various species, e.g. blue tits *Cyanistes caeruleus* (Henderson et al., 2017); tree swallows *Tachycineta bicolor* (Patterson, Winkler & Breuner, 2011); barn swallows *Hirundo rustica* (Saino et al., 2005); Adelie penguins *Pygoscelis adeliae* (Thierry, Ropert-coudert & Raclot, 2013).

In contrast, a positive relationship is predicted by “the CORT-adaptation hypothesis.” This hypothesis suggests that because CORT can mediate the mobilization of fuels, causing changes in behaviour or physiology that can increase investment in reproduction, elevated CORT will lead to higher fitness during energetically demanding times (Wingfield & Sapolsky, 2003; Bonier et al., 2009a). Indeed, across a variety of species and life history strategies individuals with higher reproductive success have been reported to have higher CORT levels, e.g. eastern bluebirds *Sialia sialis* (Burtka, Lovern & Grindstaff, 2016); black-legged kittiwakes *Rissa tridactyla* (Chastel, Weimerskirch & Wing, 2005); petrels *Macronectes* spp (Crossin et al., 2013); western bluebirds *S. mexicana* (Kleist et al., 2018); mourning doves *Zenaida macroura* (Miller, Vleck & Otis, 2009).

In fact, there may exist no consistent relationship between CORT and fitness, due to a variety of factors masking directionality (Madliger & Love, 2016a). For example, a lack of relationship could be due to different functions of CORT; when resources are plentiful, elevated CORT could stimulate energy mobilization and parental provisioning; however, CORT could also be elevated in parents experiencing stressors (Vitousek, Jenkins & Safran, 2014). Even within a breeding season, different stages can have differing parental energetic requirements presumably requiring different levels of GC-mediated energy mobilization (Humphreys, Wanless & Bryant, 2006; Nager, 2006; Tulp et al., 2009; Sakaluk, Thompson & Bowers, 2018).

Most studies that have explored relations between CORT and fitness have been correlative. Although such studies are certainly informative, e.g. (Bonier et al., 2009b), studies that manipulate CORT levels directly, and explore the resultant parental response are also needed. Using tree swallows as a model organism, we attempted to elevate maternal CORT experimentally prior to egg-laying, and quantify subsequent variation in maternal reproductive investment and reproductive success over two breeding seasons. We asked two primary questions: (1) How does maternal CORT influence reproductive investment and success? (2) Does the directionality of the relationship between maternal CORT and reproductive investment and success change between the incubation and nestling provisioning stages, given the increased energy expenditure and brood value during chick provisioning? If the CORT-fitness hypothesis were supported, we expected to detect a negative relationship between maternal CORT and measures of reproductive investment and success. Conversely, if the CORT-adaptation hypothesis were supported, we expected to see a positive relationship between maternal CORT and reproductive investment and success.

MATERIALS AND METHODS

Study location and species

All protocols were approved by Trent University Animal Care Committee, with a handling, banding and collection permit provided by Canadian Wildlife Service, Environment Canada. Our study took place during spring and summer 2015 and 2016, using tree swallows, a small, migratory, aerial insectivore, that breeds across central and northern North America (Winkler et al., 2011). They are cavity nesters that readily occupy artificial nest boxes, and both sexes begin nest building in late April to early May, with laying occurring through May and June. Most

populations lay only one clutch of 5 or 6 eggs per season; the female then incubates the eggs for about 14 days. Chicks are fed by both parents and fledge at approximately 21 days post-hatch.

We had two field sites near Peterborough Ontario, Canada (University Nature Area: 44° 21' N, 78° 17' W; and Lakefield Township Sewage Lagoon: 44° 25' N, 78° 15' W). In 2015 and 2016, the Nature Area had 66 and 70 boxes, respectively; Sewage Lagoon had 50 and 52 nest boxes. The Nature Area consisted of open woodland with long grasses, shrubs, and scattered apple (*Malus pumila*), buckthorn (*Rhamnus cathartica*), red cedar (*Juniperus virginiana*), white cedar (*Thuja occidentalis*), and dogwood (*Cornus florida*). The immediate landscape around boxes at the Sewage Lagoon was exposed soil and grasses; the greater surrounding area was farmland consisting of both crop and pasture land. Nest boxes at the Sewage Lagoon were 5 to 10 metres from the water.

Experimental manipulation of maternal corticosterone levels

Nest boxes were monitored daily beginning 6 May in both years. When nests were about 75% formed (when cup-shaped or when feathers were present), we captured females using cardboard trapdoors over the nest box opening, or by surprising birds sitting in nest boxes. In 2015, nine females were caught at night (between 2200 and 2400 hours) by surprising birds sitting in nest boxes (no females were found in nest boxes at night in 2016). Upon capture, females were randomly assigned to CORT or sham treatment groups (Table 1). We sterilized the skin of the right flank with 70% ethanol, made a 4mm subcutaneous incision, and inserted a sterilized 10mm silastic tube (ID 1.47mm and OD 1.96mm, Dow Corning 508-006) filled with crystalline CORT (Sigma Aldrich C2505) that was sealed with silicone sealant (732 Dow Corning) at both ends (CORT treatment). To each implant we added a single hole using a 30 G needle (Ouyang et al.,

2013). Sham treatment birds received sterilized empty implants. Empty implants weighed approximately 0.02g and held an average of $0.007\text{g} \pm 0.0007\text{g}$ of CORT. Once the implant was inserted, the incision in the skin was sealed with a drop of 3M Vetbond (no. 1469SB). Each female was then aged as second year, SY, or after-second year, ASY (Pyle et al., 1987). Flattened wing length was measured with a standard ruler with a wing stop ($\pm 1\text{mm}$), mass was measured with a Pesola spring scale ($\pm 0.25\text{g}$). All birds (including any males caught inadvertently) were banded with a federal aluminum numbered leg band (Canadian Wildlife Service) and released. Birds were held for no more than 10 minutes before release. In 2016 and 2017, any previously banded female from 2015 or 2016 was counted as a returned bird in the return rate analysis regardless of whether they hatched a clutch that year.

We allocated females to the Control group if they were not caught prior to laying, either because they did not enter the nest box while it had a trap, or because they began laying earlier than we expected. Although these females did not receive an implant before egg laying they were handled and measured beginning during incubation (sample sizes in Table 1).

Nest and egg monitoring

Nest boxes were monitored daily throughout the nest-building and laying period, and when eggs were discovered, eggs were numbered with a black marker and weighed ($\pm 0.01\text{ g}$) using a digital balance. One female had a lay date of 4 June, which was greater than 3 standard deviations from the population mean (21 May). We did not consider this female further because we suspected it was re-nesting after a failed first attempt. All other nests were included in statistical analyses.

Nestling measurements

Beginning on day 12 of incubation (incubation day 0 = first day no new eggs were laid, and eggs were warm to the touch), nest boxes were checked twice daily. The hatch day of the first nestling was defined as day 0 for that nest. It was not possible to match nestlings to egg identity. We marked the talons of nestlings with coloured nail polish to distinguish individuals, until we banded them on day 10 post-hatch with aluminum numbered leg bands (Canadian Wildlife Service). Nestlings were weighed at hatch with an egg scale ($\pm 0.01\text{g}$), and on days 3, 7, 10, 13, and 14 post-hatch with a Pesola spring scale ($\pm 0.25\text{g}$). Beginning on day 18 post-hatch, we checked nest boxes daily by partially opening the door to determine fledging success. To guard against pre-mature fledging, the nest box opening was blocked for 1 minute after checking, and when the blocking was removed the box was observed for 5 min from a distance of a few metres; no instances of premature fledging were observed.

Blood sampling procedure

We recaptured adult females in nest boxes between day 2 and 5 of incubation (both years) and between day 3 and 6 post-hatch during chick rearing (in 2016 only) between 0600 and 1200 hours. Upon capture, we collected a 100 μl blood sample from the brachial vein using a micro-capillary tube within three minutes of the female entering the nest box. The mean time taken to draw blood ($\pm\text{SE}$) was $118 \pm 5\text{s}$ ($N=57$) during incubation, and $115 \pm 8\text{s}$ ($N=33$) during the nestling stage. Blood samples were kept on ice for up to eight hours. Samples were then centrifuged for four minutes at 19,200 $\times g$ (Thermo IEC Micro-MB) before plasma and red blood cells were frozen separately at -80°C . Prior to release, we recorded female body mass and marked the tail feathers and right primaries with a spot of white acrylic paint to distinguish

females from males during subsequent behavioural observations (Whittingham, Dunn & Clotfelter, 2003; Bonier, Moore & Robertson, 2011). If the female had not been captured previously (i.e., she was to become a Control female), upon first capture during incubation we recorded her head-bill length and wing length, and banded her.

We collected nestling blood samples (50µl) from the brachial vein on days 7 or 8, and 13 post-hatch. Samples taken on day 7 or 8 post-hatch were for molecular sexing and were added to 1ml of lysis buffer in the field and subsequently stored at -20°C. Samples collected on day 13 were centrifuged and plasma was stored at -80°C (as part of a separate study).

Adult behavioural observation

On day 7 or 8 post-hatch between 0830 and 1400 hours, nest boxes were observed from a distance of 10 m for 1 hr (Lendvai et al., 2015), during which we counted the number of visits made by males and females to the nest box. This was the maximum distance at which it was still possible to distinguish the sex of the adult entering the box through binoculars. Observations made mid-day have been shown to provide the best estimates of feeding rate, although 1-hour observations periods done at any time of day predict total daily feeding rates (Lendvai et al., 2015)

Lab procedures

Corticosterone radioimmunoassay

Plasma samples were analyzed for total corticosterone in duplicate using a ¹²⁵I radioimmunoassay (MP Biomedicals #07120103) following the manufacturer's instructions (Washburn et al., 2002). Plasma was diluted 1:25 (10µl of plasma plus 240µl of assay buffer).

Samples that were not detectable were set to the lowest point on the standard curve (3.125 ng/ml), following Hogle and Burness (2014). We did not extract plasma because a serial dilution of non-extracted plasma pooled from five individuals was parallel to the standard curve. A total of 23 individual assays was performed. The average inter-assay variation was 7.99%; the intra-assay variation was 10.7%.

Molecular sexing protocol

Nestling blood samples taken on day 7 or 8 post-hatch were used for genetic sexing using the CHD1W and CHD1Z genes (Fridolfsson & Ellegren, 1999; Hogle & Burness, 2014). DNA extraction was done using DNEasy blood and tissue kits (Qiagen 69506). A touchdown PCR procedure was used with 10ul volumes consisting of 1.2ul 10X buffer, 0.4ul MgCl₂, 1.0ul dNTP, 0.25ul BSA, 0.2ul each of primers 2550 and 2718 (Fridolfsson and Ellegren, 1999), 1.0ul Taq polymerase, 3.75ul H₂O, and 2ul DNA in an Eppendorf thermocycler. Initial denaturing began at 94°C for 5 min followed by a touchdown sequence where the annealing temperature was lowered 1°C per cycle from 94° to 50°C. A further 24 cycles were run with a denaturing temperature of 94°C for 30 s, annealing temperature of 40°C for 30 s and extension of 72°C for 30 s, followed by a final extension at 72°C for 2 min after the last cycle. PCR products were separated in a 3% agarose gel stained with ethidium bromide and run in 1X TBE buffer. Each gel was run with known male and female adult samples for comparison (N=273 chicks).

Statistical analyses

All data will be deposited in the DataDryad data repository. We used R version 3.4.3 (2017) to run all analyses, and statistical significance was claimed at $P < 0.05$. During field work we were

generally blind to the experimental treatment, but not during statistical analysis. Sample sizes were determined by the number of breeding individuals in our study population that could be captured. To improve normality, all CORT values were \log_e transformed; all other metrics were untransformed. Raw means are reported \pm SE. Sample sizes varied among analyses because we were not always able to collect all measurements from all individuals. We included ‘year’ as a factor only in analyses of maternal CORT during incubation (CORTinc), because during the nesting phase (CORTnest) we measured CORT in one year only (2016).

We constructed our statistical models including only main effects that were of likely biological importance; as such, not all two-way interactions were included. We report outputs from global statistical models. Because we had explicit hypotheses, and because none of our response variables was correlated, we did not to use a post-hoc correction for the number of tests performed (Perneger, 1998; Streiner, 2015).

Morphological and hormonal measures of adult females

We ran preliminary tests to determine whether females that had been assigned to CORT or Sham treatment groups differed in pre-implant body mass (measured at time of implant; females in the Control group were not captured prior to incubation and thus there was no pre-implant mass measurement). To test for possible differences in body size among treatments, we compared a female’s wing length (measured pre-laying in the CORT and Sham treatments, and during early incubation in the Control females). Finally, we tested for differences in clutch initiation date in Julian days among treatments. Separate linear models (LM) were run with female pre-laying mass, wing length, and clutch initiation date as the response variable, and treatment (CORT, Sham, Control), site (Nature areas, Sewage Lagoon), year (2015, 2016), and age (Second year,

SY; After second year, ASY) as the predictors. We did not include any interactions terms as they were not of *a priori* interest.

Implanted females recaptured during incubation may have differed phenotypically from individuals that abandoned their nests (and were never recaptured). To test this, we ran a generalized linear model (GLM) with binomial errors, with recapture status (recaptured/non-recaptured) as the dependent variable, and treatment and year as the fixed effects. To test whether the total number of individuals that subsequently laid eggs differed between the CORT and Sham maternal treatment groups, we used a chi-square test (because Control females were only captured post-egg laying, they were not included in this analysis).

Maternal baseline corticosterone during incubation and nestling stages

To test whether treatment affected maternal CORT levels within each breeding stage (incubation and nestling), we used linear models (LM) with either CORT during incubation (from hereafter CORTinc) or CORT during the nestling stage (CORTnest) as the response variable and maternal treatment, age, site, sample time (time from initial contact with bird to end of blood sample), and clutch initiation date (in Julian days) as fixed effects. We had no *a priori* predictions regarding interactions, so none was included in the models.

We analyzed CORTinc and CORTnest separately because CORTnest was only measured in 2016. Baseline CORTinc measurements (N=56) had one suspected outlier (121.22 ng/ml) removed prior to analysis. This value was > 3 standard deviations from the mean; considerably higher than the 0.5 to 14 ng/ml range reported for previously (Franceschini et al., 2008; Ouyang et al., 2011; Patterson, Winkler & Breuner, 2011; Madliger et al., 2015). Preliminary analyses

were run with and without this outlier, and although no difference was found in the pattern of significance of parameters, we chose to exclude it.

Measures of female reproductive investment

As indices of maternal investment during incubation we used clutch mass (summed mass of individual eggs at laying), and during the nestling phase we used maternal nest box visitation rate and nestling growth rate. To test whether a female's clutch mass correlated with her CORT levels, we used a linear model (LM) with clutch mass as the response variable and CORTinc, maternal treatment, age, site, and year as main effects. To explore investment during the nestling stage, we used a LM with the number of nest box visits per chick per hour (by the female) as the response variable and CORTnest, treatment, maternal age, site, and male nest box visits per chick per hour as fixed effects. Finally, we calculated nestling growth rate per day during the linear growth phase (Burness, Ydenberg & Hochachka, 2001) as the difference in individual mass between days 3 and 7 post-hatch, divided by 4 days. We used a linear mixed model (LMM, lmer in R package lme4) with individual chick mass gain per day as the response variable, and nest ID. Fixed effects were CORTnest, maternal treatment, maternal age, site, nestling sex. Additionally, we included an interaction between nestling sex and CORTnest.

Measures of female reproductive success

To test for a relationship between CORTinc and indices of reproductive success, we used a generalized linear mixed model (GLMM; glmer in R package lme4) with binomial errors, with either hatching or fledging success as the response variable (0 or 1 for each chick) and CORTinc, maternal treatment, age, site, and year as fixed effects, and nest ID as a random effect. To

explore the relationship between CORTnest and post-hatching reproductive success, we examined individual nestling mass at day 14 post-hatch and fledging success as indices of reproductive success. To test whether nestling mass differed with maternal CORT or treatment, we used a LMM with nestling mass at day 14 as the response variable and CORTnest, maternal treatment, maternal age, and site as fixed effects (year was not included because CORTnest was measured in 2016 only), and Nest ID as a random effect. Finally, to test whether fledging success differed with maternal CORT or treatment, fledging success (0 or 1 for each chick) was used as the response variable in a GLMM with binomial errors with maternal treatment, maternal age, site, and CORTnest as fixed effects, and Nest ID as a random effect. No interaction terms were included in these analyses.

Measures of female survival

We estimated female survival by using the return rates of adult females to the study sites the following spring and comparing this with CORTinc or CORTnest during the previous year in separate models. Return rate (either 0 or 1) was the response variable in a general linear model (GLM), with CORTinc (or CORTnest), treatment, year, age, site, and number of nestlings fledged as main effects. In analyses of CORTnest, “year” was not included in the model because CORTnest was only measured in a single year (2016). We also used a chi square test to determine whether there was a difference in return rate by treatment or year.

RESULTS

Morphology and hormonal measures of adult females

We implanted 51 females with corticosterone-filled implants (CORT), and 48 with sham implants (Sham); an additional 25 females were captured for the first time during incubation

were allocated to the Control treatment (Table 1). There was no difference in pre-egg laying body mass between females allocated to the CORT and Sham groups (treatment: $p=0.108$; Table S1); females in the Control group were not captured prior to egg laying, so there was no pre-egg laying mass. Focussing on individuals that retained their implants, wing length and clutch initiation date did not differ significantly among treatments (Wing length, treatment: $p = 0.238$; Clutch ignition date, treatment: $p=0.859$; Table S1). There was no significant difference between the Sham and CORT treatments in the percentage of females that retained their implants and subsequently laid eggs (Sham: 40% (19 of 48), CORT: 31% (16 of 51); $\chi^2=0.287$, $df=1$, $p>0.05$; Table 1).

There were no significant differences by year or by treatment in the number of implanted females that were recaptured during incubation (GLM: year $F_{1,96} = 3.884$, $p=0.052$, $N_{2015}=26$ recapture and 33 non-recapture, $N_{2016}=35$ recapture and 5 non-recapture; treatment $F_{1,96} = 3.832$, $p=0.053$, $N_{\text{Sham}}=34$ recapture and 14 non-recapture, $N_{\text{CORT}}=27$ recapture and 24 non-recapture). Control birds were not included in the recaptured/not recaptured analysis because they were caught for the first time during incubation.

Implants failed to raise long-term maternal corticosterone levels

During incubation, females were recaptured on average 16.42 days (± 0.11) after implantation (range 11 to 26 days). Contrary to expectations, when females were recaptured there was no difference in CORT levels among the 3 treatments (Treatment: $p=0.145$; Table 2; Fig. 1A). Lay date (i.e., clutch initiation date) was also not a significant predictor of CORT_{inc} ($p = 0.629$, Table 2). However, older mothers (ASY) had higher CORT_{inc} levels than SY mothers (Age: $p=0.022$) and levels differed between years (Year: $p = 0.045$, Table 2). During nestling

provisioning, maternal baseline CORT (CORTnest) did not differ among treatments (Treatment: $p=0.118$; Table 2, Fig. 1B), nor with any other fixed effects (all, $p > 0.20$, Table 2).

Maternal corticosterone levels did not predict reproductive investment

Mean clutch size (\pm SE) of females was 5.3 eggs \pm 0.1 (range = 3 to 7 eggs per nest, $N=57$ nests). Reproductive investment during laying, measured as clutch mass, did not correlate with maternal CORT levels during incubation nor with maternal treatment (CORTinc: $p=0.348$; Treatment $p=0.259$, Table 3). Similarly, during the nestling stage, there was no relationship between either maternal CORT or treatment on the number of female nest box visits (CORTnest: $p=0.831$; Treatment: $p=0.843$; Table 3). Although maternal treatment did not influence nestling growth rate between days 3 and 7 ($p = 0.730$), there was a marginally significant negative relationship between maternal CORTnest and nestling growth rate ($p = 0.076$, Table 3). Maternal age influenced nestling growth rates, with nestlings from SY mothers having higher growth rates than nestlings from ASY mothers (age: $p<0.001$, Table 3).

Maternal corticosterone levels did not predict reproductive success

As indices of reproductive success, we measured hatching success, nestling mass at day 14 post-hatch, and fledging success. Hatching success was not significantly predicted by any of the factors we measured (Table 4). There was, however, a nonsignificant trend for mothers with higher CORT during incubation to have higher hatching success (CORTinc: $p=0.061$), and for older females to have higher hatching success than younger females (age: $p=0.093$; Table 4). Nestling mass at day 14 post-hatch was not predicted by either CORTnest, nor maternal

treatment (CORTnest: $p=0.123$; treatment $p=0.372$), although nestlings at the Nature Area site tended to be heavier (Site: $p = 0.092$; Table 4).

The probability of a nestling fledging did not vary with maternal corticosterone levels measured during either incubation (Fledging success (A), CORTinc: $p = 0.257$) nor the nestling phase (Fledging success (B), CORTnest: $p = 0.590$). Similarly, maternal treatment had no effect on fledging success (Fledging success (A), Treatment: $p=0.136$; Fledging success (B), Treatment: $p = 0.329$). Fledging success was significantly higher at the Sewage Lagoon site (Fledging success (A), Site: $p=0.042$; Table 4).

Maternal return rate was predicted by the number of fledglings but not maternal corticosterone levels

Nineteen of 62 (31%) of experimental females (Sham, CORT or Controls) returned in the year after they were initially caught, and all returning females returned to the same breeding site where initially caught. The number of females included in the analysis differs from totals in Table 1, because only females with corticosterone measurements were included. The probability that a female returned increased with her number of fledglings in the previous year (Return rate (A): $p=0.015$; Return rate (B): $p = 0.007$; Table 5). There was no significant effect of CORTinc, CORTnest or maternal treatment on the likelihood of a female returning to the nest sites the following year (each, $p > 0.50$, Table 5). There was no significant difference by treatment or year in the number of females that returned ($\chi^2=1.039$, $df=2$, $p>0.05$; 2015 returns: 2 Sham, 1 CORT, 3 Control; 2016 returns: 4 Sham, 5 CORT, 4 Control).

DISCUSSION

Our data do not convincingly support either the CORT-fitness or the CORT-adaptation hypothesis. During egg incubation, corticosterone levels of female tree swallows were positively related (albeit non-significantly) to one measure of reproductive success. However, during the nestling stage, there was no relationship between corticosterone and indices of either reproductive investment or reproductive success. During neither period did we detect a negative relationship between CORT and fitness, as predicted by the CORT-fitness hypothesis.

Maternal corticosterone levels during incubation

We tested for a relationship between maternal corticosterone levels during incubation (CORT_{inc}) and clutch mass (as a single measure of reproductive investment), and hatching success and survival to fledging as measures of reproductive success (Bonier et al., 2009b; Schoenle et al., 2017). We found no relation between CORT_{inc} and clutch mass, however, female tree swallows with higher CORT_{inc} levels had greater hatching success (albeit not significantly so, $P = 0.061$), but not higher fledging success. The positive relationship we detected with hatching success (if real) may be due to CORT mobilizing energy stores, and thus allowing for increased reproductive effort (Riechert, Becker & Chastel, 2014). However, positive (common terns *Sterna hirundo*) (Riechert, Becker & Chastel, 2014), negative (zebra finches) (Khan, Peters & Robert, 2016), and null relationships (red-winged blackbirds) (Schoenle et al., 2017) have all been reported between maternal CORT and hatching success. Differences in the directionality of the relationships are presumably due to various environmental factors, including weather conditions (Schoenle et al., 2017) and/or food availability (Riechert, Becker & Chastel, 2014).

A lack of relationship between CORTinc and fledging success contrasts with positive relationships reported in eastern bluebirds (Burtka et al., 2016) and blue tits (Henderson et al., 2017). However in tree swallows, both negative (Bonier et al., 2009b) and statistically non-significant (Madliger & Love, 2016a) relationships between CORTinc and number of fledglings have been reported. A positive relationship between maternal CORT during incubation and fledging success might be expected if the relationship were mediated through maternal transfer of CORT into the egg, leading to higher begging rates and body size in nestlings of mothers with higher CORT (Bowers et al., 2016). However, this would be a plausible mechanism only if CORT levels during incubation correlated with levels pre-laying, as has been found in other tree swallow populations (Ouyang, Hau & Bonier, 2011); something we did not evaluate in our study.

A positive relationship between maternal CORT and fitness (CORT-adaptation hypothesis) should emerge when CORT levels are increased to meet higher energetic demands associated with reproduction (Bonier et al., 2009a; Crossin et al., 2013; Rivers et al., 2017). During incubation, individuals may experience more unpredictable stressors than during the nestling stage (Romero, 2002). For example, challenging environmental conditions such as lower temperatures and scarcer food resources in early spring can cause a negative relationship between both temperature and foraging success and baseline CORT levels, depending on the fitness and environmental measure used (Angelier et al., 2007; Wingfield, Weimerskirch & Chastel, 2010; Ouyang et al., 2015). Because higher baseline levels may prime the body to perform better under stress, females with higher baseline CORT during incubation in our study may have been better able to meet these challenges (Romero, 2002).

Maternal corticosterone levels during chick rearing

We predicted that if there were a relationship between CORT and reproductive investment and success, it would most likely emerge post-hatch, given the higher maternal energy expenditure required during chick rearing than during incubation (Nilsson & Raberg, 2001; Humphreys, Wanless & Bryant, 2006; Sakaluk, Thompson & Bowers, 2018) but see (Williams, 2018) . However, female CORT levels during chick rearing were unrelated to any measure of reproductive investment (nest box visits and and nestling growth rate) nor any measure of reproductive success (nestling mass at 14 and fledging success). Despite our inability to detect relationships, others have reported that individuals with higher baseline CORT levels during chick rearing had higher parental foraging effort, provisioning rates, and energy transfer to the nestlings, e.g. macaroni penguins *Eudyptes chrysolophus* (Crossin et al., 2012); tree swallows (Wingfield & Sapolsky, 2003; Bonier, Moore & Robertson, 2011); mourning doves (Miller, Vleck & Otis, 2009). Across studies, differences in the relationship between CORT and reproductive success may be due to various fitness measures used, the relative importance of paternal investment, or environmental variation.

While female tree swallows are solely responsible for egg incubation, nestling provisioning is shared with the male (Winkler et al., 2011). As a result, variation in paternal quality may obscure relationships between maternal CORT and investment during the nestling stage. A lack of relationship between CORT_{nest} and female nest box visits has been found in bluebirds (Davis & Guinan, 2014) and other populations of tree swallows (Patterson, Winkler & Breuner, 2011), suggesting variation among females in their glucocorticoid levels may not directly reflect maternal behaviour. In contrast, Madliger & Love (2016b) found that higher

baseline CORT_{nest} in female tree swallows correlated with lower rates of maternal provisioning; however, males compensated for the females' low rates by increasing their own provisioning rates such that nestlings were not affected. Similarly, Patterson et al. (2011) suggested male tree swallows could compensate for decreased provisioning of their mates, although no male compensation for reduced maternal performance has also been found (Hogle & Burness, 2014). Given the importance of male provisioning to nestling mass gain (Lendvai & Chastel, 2010; Madliger & Love, 2016b; Nomi, 2018), male nest box visits and paternal quality need to be considered when predicting a pairs' reproductive investment in a nest. Future studies should include male CORT levels, and their relationship with male feeding rates and reproductive success, as in (Ouyang et al., 2011).

The directionality of the relationship between maternal CORT and fitness varies among life stages, populations and species (e.g., Bonier et al., 2009a). Some of this variation is presumably due to the context-dependency of the CORT-fitness relationship and variation in environmental conditions (Burtka, Lovern & Grindstaff, 2016; Madliger & Love, 2016b). For example, experimental elevation of maternal CORT levels increased brood mortality in tree swallows, but only when weather conditions were benign (Ouyang et al 2015). Our inability to detect relationships between maternal hormone levels and reproductive success could be due to the influence of such factors as food availability or weather, both of which could affect body condition and reproductive success of the mother (Schoech et al., 2007; Madliger & Love, 2016a). Maternal baseline CORT may also depend on the habitat type in which female tree swallows were breeding (Madliger & Love, 2016b). While we found no difference in reproductive investment or success between the two study sites, we did find that CORT_{inc} was higher at one of our sites (Sewage Lagoon). This suggests that while habitat may affect maternal

CORT, this does not necessarily translate into differences in reproductive investment or reproductive success. Reproductive success may perhaps be mediated by a relationship between CORT and foraging conditions (Henderson et al., 2017), which could change from incubation to the nestling stage.

No relationship between corticosterone and return rates

We found no relationship between either CORT_{inc} or CORT_{nest} level and the probability of whether a female returned to the breeding sites the following year, as reported previously (Patterson, Winkler & Breuner, 2011; Rivers et al., 2017). One explanation is that relationship between CORT levels and return rates may be non-linear. For example, in cliff swallows (*Petrochelidon pyrrhonata*), highest return rates were seen in individuals with intermediate baseline CORT levels, which could be due to stabilizing selection on CORT levels acting against the detrimental effects of very high or low CORT (Brown et al., 2005; Bonier et al., 2009a). Additionally, environmental variables (Clark et al., 2018) or an individual's reproductive success may better predict return rates than baseline CORT: the positive effect of fledgling number on maternal return rates that we detected suggests that females with higher reproductive success are more likely to return to a certain area to breed (Bonier et al., 2009b). Thus, CORT may affect return rates and survival only indirectly, by affecting fledging success (Shitikov et al., 2017; Weegman et al., 2017).

Efficacy of silastic implants to raise plasma glucocorticoids

We implanted pre-egg laying females with corticosterone-filled silastic implants, but when females were recaptured during early-to mid-incubation (mean \pm SE: 17.1 days \pm 0.6 after

implantation), the baseline CORT levels of implanted birds did not differ from unmanipulated birds. Across species, silastic implants have been successfully used to raise CORT levels for anywhere from a few days (Astheimer, Buttemer & Wingfield, 2000; Hayward & Wingfield, 2004; Criscuolo et al., 2005; Martin et al., 2005; Angelier et al., 2007) to three weeks post-implantation *in vivo* (Ouyang et al., 2013) and *in vitro* (Newman et al., 2010). However, the use of implants to raise CORT levels has not been consistently successful (Crossin et al., 2012; Ouyang et al., 2013; Hau & Goymann, 2015; Lattin, Breuner & Romero, 2016). Although the implants used in our study may have failed to release CORT, this seems unlikely given that *in vitro* studies have shown that CORT continues to be released across the membrane over 4 weeks (Newman et al., 2010). More likely, the implants resulted in decreased secretion of endogenous CORT via negative feedback, or increased clearance of CORT from the blood via increased excretory activity (Newman et al., 2010; Henriksen, Groothuis & Rettenbacher, 2011; Robertson, Newman & MacDougall-Shackleton, 2015).

Rather than experimentally manipulate CORT levels via implants, an alternative approach may be to manipulate maternal condition, such as with feather clipping (Rivers et al., 2017), predator experiments (Clinchy et al., 2011; Pitk et al., 2012), or density manipulations (Bentz, Navara & Siefferman, 2013). Such an approach would encompass how maternal CORT levels change based on how each female perceives her condition/ environment, how that is reflected in blood CORT levels, and how those levels might influence the next generation (Madliger & Love, 2016a; Rivers et al., 2017).

Conclusions

The differing directionality of relationship between CORT and fitness among studies and species raises the simple question: is there is a consistent relationship to be found among individuals

within a population? Many factors can affect both CORT and fitness, including condition (Love et al., 2005), life-history stage (Romero, 2002), weather (Pakkala et al., 2016) and habitat variability (Madliger & Love, 2016b). If it can be reasonably assumed that these will always differ among individuals, then perhaps there is no consistent relationship, and any relationship detected will always be context-dependent (Madliger & Love, 2016a). A formal meta-analysis of passerine birds, such as was done recently in seabirds (Sorenson et al., 2017), may help clarify patterns.

The use of integrative measures of CORT may be an alternative way to improve our understanding of the relationship between CORT and fitness. By measuring CORT deposited in feathers during growth, or metabolites excreted in feces, it may be possible to infer CORT levels over multiple days of the incubation or nestling stage (Lucas et al., 2006; Bortolotti et al., 2008; Romero & Fairhurst, 2016). For example, giant petrels that successfully bred had higher feather CORT levels than failed breeders, but were less likely to breed the following year, a pattern which was not observed using plasma CORT from these same individuals (Crossin et al., 2013). Ideally, studies could be extended over the winter, as has been done recently in adult tree swallows (Vitousek et al., 2018). This would help elucidate the longer-term effects of maternal CORT on offspring and maternal and fitness.

FUNDING

Funding was provided by the Natural Sciences and Engineering Research Council (NSERC) Canada, the Canadian Foundation for Innovation and the Ontario Innovation Trust.

ACKNOWLEDGEMENTS

546 We thank Noah Ben-Ezra, Chantelle Penney and Aleesa Manax for their many hours of help in
547 the field. Erica Nol, Joe Nocera, and Jeff Bowman provide numerous for suggestions for
548 improving the clarity of an earlier draft of the manuscript.

549

550 **COMPETING INTERESTS**

551 The authors declare no competing or financial interests.

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Table 1. Sample sizes of adult female tree swallows allocated to each maternal treatment group across two years.

	Sham		CORT		Control	
	SY	ASY	SY	ASY	SY	ASY
Number of females implanted	15	33	17	34	-	-
Number of females that laid	3 (8)	16 (26)	4 (6)	12 (21)	11	14
Number of females that laid eggs that hatched	2 (7)	16 (25)	4 (5)	12 (18)	9	14

Sham: females had an empty silastic implant; CORT: females had a silastic implant filled with crystalline corticosterone; Control: females had no implant.

Female age: SY (second-year); ASY (after second-year).

Bracketed values represent total number of individuals handled/implanted; non-bracketed values indicate sample sizes of birds with implants that were still present when the bird was recaptured during incubation.

Table 2. Factors contributing to variation in corticosterone levels in female tree swallows during incubation (CORTinc) and the nestling stage (CORTnest).

Response variable	Fixed effects	β	SE	df	F	P	R ²
CORTinc (ng/ml)	Intercept	4.541	3.172	1, 43	2.049	0.160	0.225
	Treatment (CORT)	-0.068	0.268	2, 43	2.021	0.145	
	Treatment (Control)	0.452	0.270	2, 43	2.021	0.145	
	Age	0.773	0.326	1, 43	5.611	0.022	
	Lay date	-0.011	0.023	1, 43	0.237	0.629	
	Site (Nature Area)	-0.487	0.251	1, 43	3.765	0.054	
	Sample time	-0.004	0.004	1, 43	0.865	0.357	
	Year	-0.598	0.289	1, 43	4.274	0.045	
CORTnest (ng/ml)	Intercept	6.526	3.632	1, 23	3.229	0.085	0.086
	Treatment (CORT)	0.304	0.333	2, 23	2.354	0.118	
	Treatment (Control)	0.304	0.333	2, 23	2.354	0.118	
	Age	-0.287	0.542	1, 23	0.280	0.602	
	Lay date	-0.032	0.025	1, 23	1.585	0.211	
	Site (Nature Area)	0.224	0.365	1, 23	0.375	0.546	
	Sample time	0.005	0.004	1, 23	1.596	0.219	

Year was not included for CORTnest, because data were collected in a single year.
Statistically significant main effects are in bold.

Table 3. Factors contributing to variation in reproductive investment in female tree swallows.

Measures of maternal investment	Fixed effects	β	SE	df	F or χ^2	P	R ²
Clutch mass (g)	Intercept	0.907	1.230	1, 44	54.385	<0.001	0.157
	CORTinc	0.407	0.429	1, 44	0.900	0.348	
	Treatment (CORT)	0.156	0.734	2, 44	1.392	0.259	
	Treatment (Control)	-0.075	0.774	2, 44	1.392	0.259	
	Maternal age	1.176	0.767	1, 44	2.530	0.132	
	Site (Nature Area)	-0.868	0.666	1, 44	1.701	0.199	
	Year	-1.058	0.674	1, 44	2.563	0.124	
Nest box visits (per chick per hour)	Intercept	1.624	1.082	1, 20	2.253	0.149	0.009
	CORTnest	0.076	0.350	1, 20	0.047	0.831	
	Treatment (CORT)	0.130	0.539	2, 20	0.172	0.843	
	Treatment (Control)	0.350	0.596	2, 20	0.172	0.843	
	Site (Nature Area)	0.668	0.501	1, 20	1.777	0.197	
	Male visits per chick per hour	0.376	0.273	1, 20	1.897	0.184	
	Maternal age	-0.181	0.578	1, 20	0.098	0.758	
Growth rate (mass gain per chick per day from day 3 to day 7 post-hatch)	Intercept	3.028	0.388	1, 113	60.891	<0.001	(M) 0.270 (C) 0.599
	CORTnest	-0.244	0.137	1, 113	3.144	0.076	
	Treatment (CORT)	0.048	0.190	2, 113	0.630	0.730	
	Treatment (Control)	-0.107	0.194	2, 113	0.630	0.730	
	Site (Nature Area)	0.154	0.180	1, 113	0.733	0.392	
	Maternal age	0.831	0.216	1, 113	14.849	<0.001	
	Nestling sex (male)	-0.196	0.316	1, 113	0.382	0.536	
	CORTnest x nestling sex	0.119	0.113	1, 113	0.945	0.331	

CORTinc and CORTnest refer to maternal CORT levels during incubation and the nestling stage, respectively.

Year was not included in analyses of Nest box visits or Growth rate, because CORTnest was measured in a single year only.

Adjusted R² values are provided for models without random effects; marginal (M) and conditional (C) R² values are provided for models with random effects.

Statistically significant main effects are in bold.

Table 4. Factors contributing to variation in reproductive success in female tree swallows.

Measures of maternal reproductive success	Fixed effects	β	SE	df	F or χ^2	P	R ²
Hatching success	Intercept	-0.073	1.191	1, 257	0.004	0.951	(M) 0.248
	CORTinc	0.779	0.416	1, 257	3.503	0.061	(C) 0.554
	Treatment (CORT)	0.952	0.799	2, 257	2.830	0.243	
	Treatment (Control)	-0.455	0.783	2, 257	2.830	0.243	
	Site (Nature Area)	-0.869	0.667	1, 257	1.696	0.193	
	Year	-0.172	0.701	1, 257	0.060	0.806	
	Age	1.338	0.796	1, 257	2.826	0.093	
Nestling mass at day 14 post-hatch	Intercept	22.823	1.051	1, 115	472.161	<0.001	(M) 0.081
	CORTnest	-0.546	0.354	1, 115	2.380	0.123	(C) 0.235
	Treatment (CORT)	0.781	0.555	2, 115	1.977	0.372	
	Treatment (Control)	0.400	0.579	2, 115	1.977	0.372	
	Site (Nature Area)	0.865	0.514	1, 115	2.834	0.092	
	Age	0.275	0.595	1, 115	0.214	0.643	
Fledging success A	Intercept	0.023	1.548	1, 223	0.002	0.988	(M) 0.311
	CORTinc	0.605	0.534	1, 223	1.285	0.257	(C) 0.692
	Treatment (CORT)	0.970	0.991	2, 223	3.995	0.136	
	Treatment (Control)	-1.245	1.032	2, 223	3.995	0.136	
	Site (Nature Area)	-1.924	0.947	1, 223	4.132	0.042	
	Age	1.765	1.104	1, 223	2.558	0.120	
	Year	-1.570	0.960	1, 223	2.678	0.102	
Fledging success B	Intercept	1.445	3.083	1, 151	0.220	0.639	(M) 0.136
	CORTnest	-0.517	0.961	1, 151	0.290	0.590	(C) 0.692
	Treatment (CORT)	1.668	1.513	2, 151	2.222	0.329	
	Treatment (Control)	-0.579	1.450	2, 151	2.222	0.329	
	Site (Nature Area)	-1.650	1.511	1, 151	1.193	0.275	
	Age	2.352	1.707	1, 151	1.897	0.168	

Each model includes Nest ID as a random effect; marginal (M) and conditional (C) R² values are provided.

There were two analyses of Fledging success (A and B), with predictors including either CORTinc or CORTnest, respectively.

Year was not included in analyses of Nestling mass at day 14 or Fledging success B, because CORTnest was measured in a single year only. Statistically significant main effects are in bold.

Table 5. Factors predicting the return rate in female tree swallows in the following year.

Response variable	Fixed effects	β	SE	df	χ^2	P	R ²
Return rate A	CORTinc	0.334	0.483	1, 43	0.477	0.490	0.239
	Treatment (CORT)	-0.238	0.832	2, 43	0.615	0.735	
	Treatment (Control)	-0.693	0.900	2, 43	0.615	0.735	
	Number of fledglings	0.543	0.243	1, 43	5.906	0.015	
	Year	0.816	0.788	1, 43	1.123	0.289	
	Age	-1.211	0.921	1, 43	1.772	0.183	
	Site (Nature Area)	0.438	0.829	1, 43	0.283	0.595	
Return rate B	CORTnest	0.703	1.019	1, 21	0.503	0.478	0.445
	Treatment (CORT)	-0.686	1.255	2, 21	0.308	0.857	
	Treatment (Control)	-0.249	1.292	2, 21	0.308	0.587	
	Number of fledglings	0.970	0.429	1, 21	7.379	0.007	
	Age	-0.653	1.366	1, 21	0.232	0.631	
	Site (Nature Area)	2.191	1.607	1, 21	2.051	0.152	

There were two analyses of Return rate (A and B), with predictors including either CORTinc or CORTnest, respectively. Year was not included in analyses of Return rate B, because CORTnest was measure in one year only. Statistically significant main effects are in bold.

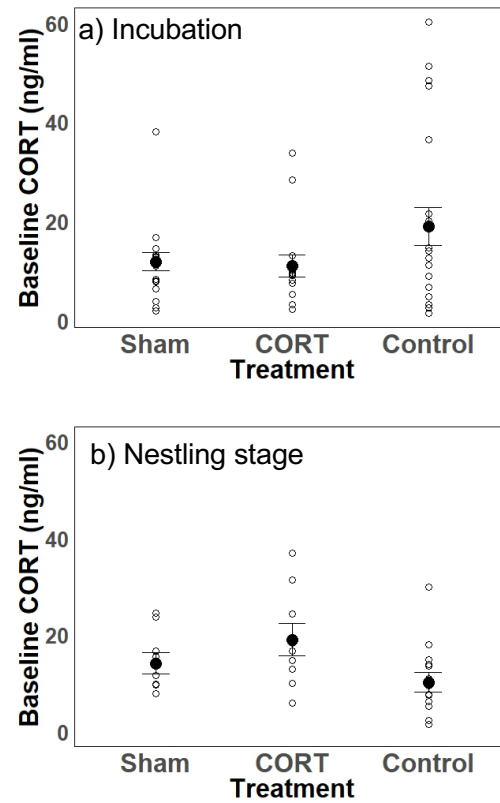


Figure 1. There was no significant difference among treatments in plasma corticosterone levels of female tree swallows when measured during (A) incubation and (B) nestling stage. Individuals in the CORT treatment had a single silastic implant containing crystalline corticosterone, those in the Sham treatment had an empty implant, while Control birds had no implant. The black circle indicates the mean \pm s.e.m. Individual data points are shown.