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

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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; Wilcoxen 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 tridactlya* (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 0.007g ± 0.0007g 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 (±1mm), mass was measured with a Pesola spring scale (±0.25g). 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 (±0.01 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.

156 **Nestling measurements**

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

168

169 **Blood sampling procedure**

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

179 females from males during subsequent behavioural observations (Whittingham, Dunn &
180 Clotfelter, 2003; Bonier, Moore & Robertson, 2011). If the female had not been captured
181 previously (i.e., she was to become a Control female), upon first capture during incubation we
182 recorded her head-bill length and wing length, and banded her.
183 We collected nestling blood samples (50µl) from the brachial vein on days 7 or 8, and 13
184 post-hatch. Samples taken on day 7 or 8 post-hatch were for molecular sexing and were added to
185 1ml of lysis buffer in the field and subsequently stored at -20ºC. Samples collected on day 13
186 were centrifuged and plasma was stored at -80ºC (as part of a separate study).
187
188 **Adult behavioural observation**
189 On day 7 or 8 post-hatch between 0830 and 1400 hours, nest boxes were observed from a
190 distance of 10 m for 1 hr (Lendvai et al., 2015), during which we counted the number of visits
191 made by males and females to the nest box. This was the maximum distance at which it was still
192 possible to distinguish the sex of the adult entering the box through binoculars. Observations
193 made mid-day have been shown to provide the best estimates of feeding rate, although 1-hour
194 observations periods done at any time of day predict total daily feeding rates (Lendvai et al.,
195 2015)
196
197 **Lab procedures**
198 *Corticosterone radioimmunoassay*
199 Plasma samples were analyzed for total corticosterone in duplicate using a ^{125}I
200 radioimmunoassay (MP Biomedicals #07120103) following the manufacturer's instructions
201 (Washburn et al., 2002). Plasma was diluted 1:25 (10µl of plasma plus 240µl of assay buffer).

202 Samples that were not detectable were set to the lowest point on the standard curve (3.125
203 ng/ml), following Hogle and Burness (2014). We did not extract plasma because a serial dilution
204 of non-extracted plasma pooled from five individuals was parallel to the standard curve. A total
205 of 23 individual assays was performed. The average inter-assay variation was 7.99%; the intra-
206 assay variation was 10.7%.
207
208 *Molecular sexing protocol*
209 Nestling blood samples taken on day 7 or 8 post-hatch were used for genetic sexing using the
210 CHD1W and CHD1Z genes (Fridolfsson & Ellegren, 1999; Hogle & Burness, 2014). DNA
211 extraction was done using DNEasy blood and tissue kits (Qiagen 69506). A touchdown PCR
212 procedure was used with 10ul volumes consisting of 1.2ul 10X buffer, 0.4ul $MgCl_2$, 1.0ul dNTP,
213 0.25ul BSA, 0.2µl each of primers 2550 and 2718 (Fridolfsson and Ellegren, 1999), 1.0µl Taq
214 polymerase, 3.75µl H_2O, and 2µl DNA in an Eppendorf thermocycler. Initial denaturing began at
215 94°C for 5 min followed by a touchdown sequence where the annealing temperature was lowered
216 1°C per cycle from 94° to 50°C. A further 24 cycles were run with a denaturing temperature of
217 94°C for 30 s, annealing temperature of 40°C for 30 s and extension of 72°C for 30 s, followed
218 by a final extension at 72°C for 2 min after the last cycle. PCR products were separated in a 3%
219 agarose gel stained with ethidium bromide and run in 1X TBE buffer. Each gel was run with
220 known male and female adult samples for comparison (N=273 chicks).
221
222 **Statistical analyses**
223 All data will be deposited in the DataDryad data repository. We used R version 3.4.3 (2017) to
224 run all analyses, and statistical significance was claimed at $P < 0.05$. During field work we were

225 generally blind to the experimental treatment, but not during statistical analysis. Sample sizes
226 were determined by the number of breeding individuals in our study population that could be
227 captured. To improve normality, all CORT values were $\log_e$ transformed; all other metrics were
228 untransformed. Raw means are reported ± SE. Sample sizes varied among analyses because we
229 were not always able to collect all measurements from all individuals. We included 'year' as a
230 factor only in analyses of maternal CORT during incubation (CORTinc), because during the
231 nesting phase (CORTnest) we measured CORT in one year only (2016).
232 We constructed our statistical models including only main effects that were of likely
233 biological importance; as such, not all two-way interactions were included. We report outputs
234 from global statistical models. Because we had explicit hypotheses, and because none of our
235 response variables was correlated, we did not to use a post-hoc correction for the number of tests
236 performed (Perneger, 1998; Streiner, 2015).
237
238 *Morphological and hormonal measures of adult females*
239 We ran preliminary tests to determine whether females that had been assigned to CORT or Sham
240 treatment groups differed in pre-implant body mass (measured at time of implant; females in the
241 Control group were not captured prior to incubation and thus there was no pre-implant mass
242 measurement). To test for possible differences in body size among treatments, we compared a
243 female's wing length (measured pre-laying in the CORT and Sham treatments, and during early
244 incubation in the Control females). Finally, we tested for differences in clutch initiation date in
245 Julian days among treatments. Separate linear models (LM) were run with female pre-laying
246 mass, wing length, and clutch initiation date as the response variable, and treatment (CORT,
247 Sham, Control), site (Nature areas, Sewage Lagoon), year (2015, 2016), and age (Second year,

248 SY; After second year, ASY) as the predictors. We did not include any interactions terms as they
249 were not of *a priori* interest.
250 Implanted females recaptured during incubation may have differed phenotypically from
251 individuals that abandoned their nests (and were never recaptured). To test this, we ran a
252 generalized linear model (GLM) with binomial errors, with recapture status (recaptured/non-
253 recaptured) as the dependent variable, and treatment and year as the fixed effects. To test
254 whether the total number of individuals that subsequently laid eggs differed between the CORT
255 and Sham maternal treatment groups, we used a chi-square test (because Control females were
256 only captured post-egg laying, they were not included in this analysis).
257
258 *Maternal baseline corticosterone during incubation and nestling stages*
259 To test whether treatment affected maternal CORT levels within each breeding stage (incubation
260 and nestling), we used linear models (LM) with either CORT during incubation (from hereafter
261 CORTinc) or CORT during the nestling stage (CORTnest) as the response variable and maternal
262 treatment, age, site, sample time (time from initial contact with bird to end of blood sample), and
263 clutch initiation date (in Julian days) as fixed effects. We had no *a priori* predictions regarding
264 interactions, so none was included in the models.
265 We analyzed CORTinc and CORTnest separately because CORTnest was only measured
266 in 2016. Baseline CORTinc measurements (N=56) had one suspected outlier (121.22 ng/ml)
267 removed prior to analysis. This value was > 3 standard deviations from the mean; considerably
268 higher than the 0.5 to 14 ng/ml range reported for previously (Franceschini et al., 2008; Ouyang
269 et al., 2011; Patterson, Winkler & Breuner, 2011; Madliger et al., 2015). Preliminary analyses

270 were run with and without this outlier, and although no difference was found in the pattern of
271 significance of parameters, we chose to exclude it.
272
273 *Measures of female reproductive investment*
274 As indices of maternal investment during incubation we used clutch mass (summed mass of
275 individual eggs at laying), and during the nestling phase we used maternal nest box visitation rate
276 and nestling growth rate. To test whether a female's clutch mass correlated with her CORT
277 levels, we used a linear model (LM) with clutch mass as the response variable and CORTinc,
278 maternal treatment, age, site, and year as main effects. To explore investment during the nestling
279 stage, we used a LM with the number of nest box visits per chick per hour (by the female) as the
280 response variable and CORTnest, treatment, maternal age, site, and male nest box visits per
281 chick per hour as fixed effects. Finally, we calculated nestling growth rate per day during the
282 liner growth phase (Burness, Ydenberg & Hochachka, 2001) as the difference in individual mass
283 between days 3 and 7 post-hatch, divided by 4 days. We used a linear mixed model (LMM, lmer
284 in R package lme4) with individual chick mass gain per day as the response variable, and nest
285 ID. Fixed effects were CORTnest, maternal treatment, maternal age, site, nestling sex.
286 Additionally, we included an interaction between nestling sex and CORTnest.
287
288 *Measures of female reproductive success*
289 To test for a relationship between CORTinc and indices of reproductive success, we used a
290 generalized linear mixed model (GLMM; glmer in R package lme4) with binomial errors, with
291 either hatching or fledging success as the response variable (0 or 1 for each chick) and CORTinc,
292 maternal treatment, age, site, and year as fixed effects, and nest ID as a random effect. To

293 explore the relationship between CORTnest and post-hatching reproductive success, we
294 examined individual nestling mass at day 14 post-hatch and fledging success as indices of
295 reproductive success. To test whether nestling mass differed with maternal CORT or treatment,
296 we used a LMM with nestling mass at day 14 as the response variable and CORTnest, maternal
297 treatment, maternal age, and site as fixed effects (year was not included because CORTnest was
298 measured in 2016 only), and Nest ID as a random effect. Finally, to test whether fledging success
299 differed with maternal CORT or treatment, fledging success (0 or 1 for each chick) was used as
300 the response variable in a GLMM with binomial errors with maternal treatment, maternal age,
301 site, and CORTnest as fixed effects, and Nest ID as a random effect. No interaction terms were
302 included in these analyses.
303
304 *Measures of female survival*
305 We estimated female survival by using the return rates of adult females to the study sites the
306 following spring and comparing this with CORTinc or CORTnest during the previous year in
307 separate models. Return rate (either 0 or 1) was the response variable in a general linear model
308 (GLM), with CORTinc (or CORTnest), treatment, year, age, site, and number of nestlings
309 fledged as main effects. In analyses of CORTnest, "year" was not included in the model because
310 CORTnest was only measured in a single year (2016). We also used a chi square test to
311 determine whether there was a difference in return rate by treatment or year.
312
313 **RESULTS**
314 **Morphology and hormonal measures of adult females**
315 We implanted 51 females with corticosterone-filled implants (CORT), and 48 with sham
316 implants (Sham); an additional 25 females were captured for the first time during incubation

317 were allocated to the Control treatment (Table 1). There was no difference in pre-egg laying
318 body mass between females allocated to the CORT and Sham groups (treatment: p=0.108; Table
319 S1); females in the Control group were not captured prior to egg laying, so there was no pre-egg
320 laying mass. Focussing on individuals that retained their implants, wing length and clutch
321 initiation date did not differ significantly among treatments (Wing length, treatment: p = 0.238;
322 Clutch ignition date, treatment: p=0.859; Table S1). There was no significant difference between
323 the Sham and CORT treatments in the percentage of females that retained their implants and
324 subsequently laid eggs (Sham: 40% (19 of 48), CORT: 31% (16 of 51); χ^2=0.287, df=1, p>0.05;
325 Table 1).
326 There were no significant differences by year or by treatment in the number of implanted
327 females that were recaptured during incubation (GLM: year $F_{1,96}$ = 3.884, p=0.052, N_{2015}=26
328 recapture and 33 non-recapture, N_{2016}=35 recapture and 5 non-recapture; treatment $F_{1,96}$ = 3.832,
329 p=0.053, N_{Sham}=34 recapture and 14 non-recapture, N_{CORT}=27 recapture and 24 non-recapture).
330 Control birds were not included in the recaptured/not recaptured analysis because they were
331 caught for the first time during incubation.
332
333 **Implants failed to raise long-term maternal corticosterone levels**
334 During incubation, females were recaptured on average 16.42 days (±0.11) after implantation
335 (range 11 to 26 days). Contrary to expectations, when females were recaptured there was no
336 difference in CORT levels among the 3 treatments (Treatment: p=0.145; Table 2; Fig. 1A). Lay
337 date (i.e., clutch initiation date) was also not a significant predictor of CORTinc (p = 0.629,
338 Table 2). However, older mothers (ASY) had higher CORTinc levels than SY mothers (Age:
339 p=0.022) and levels differed between years (Year: p = 0.045, Table 2). During nestling

340 provisioning, maternal baseline CORT (CORTnest) did not differ among treatments (Treatment:
341 p=0.118; Table 2, Fig. 1B), nor with any other fixed effects (all, p > 0.20, Table 2).
342
343 **Maternal corticosterone levels did not predict reproductive investment**
344 Mean clutch size (±SE) of females was 5.3 eggs ± 0.1 (range = 3 to 7 eggs per nest, N=57 nests).
345 Reproductive investment during laying, measured as clutch mass, did not correlate with maternal
346 CORT levels during incubation nor with maternal treatment (CORTinc: p=0.348; Treatment
347 p=0.259, Table 3). Similarly, during the nestling stage, there was no relationship between either
348 maternal CORT or treatment on the number of female nest box visits (CORTnest: p=0.831;
349 Treatment: p=0.843; Table 3). Although maternal treatment did not influence nestling growth
350 rate between days 3 and 7 (p = 0.730), there was a marginally significant negative relationship
351 between maternal CORTnest and nestling growth rate (p = 0.076, Table 3). Maternal age
352 influenced nestling growth rates, with nestlings from SY mothers having higher growth rates
353 than nestlings from ASY mothers (age: p<0.001, Table 3).
354
355 **Maternal corticosterone levels did not predict reproductive success**
356 As indices of reproductive success, we measured hatching success, nestling mass at day 14 post-
357 hatch, and fledging success. Hatching success was not significantly predicted by any of the
358 factors we measured (Table 4). There was, however, a nonsignificant trend for mothers with
359 higher CORT during incubation to have higher hatching success (CORTinc: p=0.061), and for
360 older females to have higher hatching success that younger females (age: p=0.093; Table 4).
361 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 (χ^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

385 Our data do not convincingly support either the CORT-fitness or the CORT-adaptation
386 hypothesis. During egg incubation, corticosterone levels of female tree swallows were positively
387 related (albeit non-significantly) to one measure of reproductive success. However, during the
388 nestling stage, there was no relationship between corticosterone and indices of either
389 reproductive investment or reproductive success. During neither period did we detect a negative
390 relationship between CORT and fitness, as predicted by the CORT-fitness hypothesis.
391

392 **Maternal corticosterone levels during incubation**

393 We tested for a relationship between maternal corticosterone levels during incubation
394 (CORTinc) and clutch mass (as a single measure of reproductive investment), and hatching
395 success and survival to fledging as measures or reproductive success (Bonier et al., 2009b;
396 Schoenle et al., 2017). We found no relation between CORTinc and clutch mass, however,
397 female tree swallows with higher CORTinc levels had greater hatching success (albeit not
398 significantly so, $P = 0.061$), but not higher fledging success. The positive relationship we
399 detected with hatching success (if real) may be due to CORT mobilizing energy stores, and thus
400 allowing for increased reproductive effort (Riechert, Becker & Chastel, 2014). However, positive
401 (common terns *Sterna hirundo)* (Riechert, Becker & Chastel, 2014), negative (zebra finches)
402 (Khan, Peters & Robert, 2016), and null relationships (red-winged blackbirds) (Schoenle et al.,
403 2017) have all been reported between maternal CORT and hatching success. Differences in the
404 directionality of the relationships are presumably due to various environmental factors, including
405 weather conditions (Schoenle et al., 2017) and/or food availability (Riechert, Becker & Chastel,
406 2014).

407 A lack of relationship between CORTinc and fledging success contrasts with positive
408 relationships reported in eastern bluebirds (Burtka et al., 2016) and blue tits (Henderson et al.,
409 2017). However in tree swallows, both negative (Bonier et al., 2009b) and statistically non-
410 significant (Madliger & Love, 2016a) relationships between CORTinc and number of fledglings
411 have been reported. A positive relationship between maternal CORT during incubation and
412 fledging success might be expected if the relationship were mediated through maternal transfer
413 of CORT into the egg, leading to higher begging rates and body size in nestlings of mothers with
414 higher CORT (Bowers et al., 2016). However, this would be a plausible mechanism only if
415 CORT levels during incubation correlated with levels pre-laying, as has been found in other tree
416 swallow populations (Ouyang, Hau & Bonier, 2011); something we did not evaluate in our
417 study.
418 A positive relationship between maternal CORT and fitness (CORT-adaptation
419 hypothesis) should emerge when CORT levels are increased to meet higher energetic demands
420 associated with reproduction (Bonier et al., 2009a; Crossin et al., 2013; Rivers et al., 2017).
421 During incubation, individuals may experience more unpredictable stressors than during the
422 nestling stage (Romero, 2002). For example, challenging environmental conditions such as lower
423 temperatures and scarcer food resources in early spring can cause a negative relationship
424 between both temperature and foraging success and baseline CORT levels, depending on the
425 fitness and environmental measure used (Angelier et al., 2007; Wingfield, Weimerskirch &
426 Chastel, 2010; Ouyang et al., 2015). Because higher baseline levels may prime the body to
427 perform better under stress, females with higher baseline CORT during incubation in our study
428 may have been better able to meet these challenges (Romero, 2002).
429

430

431 **Maternal corticosterone levels during chick rearing**

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

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

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

463 The directionality of the relationship between maternal CORT and fitness varies among
464 life stages, populations and species (e.g., Bonier et al., 2009a). Some of this variation is
465 presumably due to the context-dependency of the CORT-fitness relationship and variation in
466 environmental conditions (Burtka, Lovern & Grindstaff, 2016; Madliger & Love, 2016b). For
467 example, experimental elevation of maternal CORT levels increased brood mortality in tree
468 swallows, but only when weather conditions were benign (Ouyang et al 2015). Our inability to
469 detect relationships between maternal hormone levels and reproductive success could be due to
470 the influence of such factors as food availability or weather, both of which could affect body
471 condition and reproductive success of the mother (Schoech et al., 2007; Madliger & Love,
472 2016a). Maternal baseline CORT may also depend on the habitat type in which female tree
473 swallows were breeding (Madliger & Love, 2016b). While we found no difference in
474 reproductive investment or success between the two study sites, we did find that CORTinc was
475 higher at one of our sites (Sewage Lagoon). This suggests that while habitat may affect maternal

476 CORT, this does not necessarily translate into differences in reproductive investment or
477 reproductive success. Reproductive success may perhaps be mediated by a relationship between
478 CORT and foraging conditions (Henderson et al., 2017), which could change from incubation to
479 the nestling stage.
480
481 **No relationship between corticosterone and return rates**
482 We found no relationship between either CORTinc or CORTnest level and the probability of
483 whether a female returned to the breeding sites the following year, as reported previously
484 (Patterson, Winkler & Breuner, 2011; Rivers et al., 2017). One explanation is that relationship
485 between CORT levels and return rates may be non-linear. For example, in cliff swallows
486 (*Petrochelidon pyrrhonata*), highest return rates were seen in individuals with intermediate
487 baseline CORT levels, which could be due to stabilizing selection on CORT levels acting against
488 the detrimental effects of very high or low CORT (Brown et al., 2005; Bonier et al., 2009a).
489 Additionally, environmental variables (Clark et al., 2018) or an individual's reproductive success
490 may better predict return rates than baseline CORT: the positive effect of fledgling number on
491 maternal return rates that we detected suggests that females with higher reproductive success are
492 more likely to return to a certain area to breed (Bonier et al., 2009b). Thus, CORT may affect
493 return rates and survival only indirectly, by affecting fledging success (Shitikov et al., 2017;
494 Weegman et al., 2017).
495
496 **Efficacy of silastic implants to raise plasma glucocorticoids**
497 We implanted pre-egg laying females with corticosterone-filled silastic implants, but when
498 females were recaptured during early-to mid-incubation (mean ± SE: 17.1 days ± 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

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

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

540

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544

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COMPETING INTERESTS

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
	CORTnest	-0.244	0.137	1, 113	3.144	0.076	(C) 0.599
	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 R^2 values are provided for models without random effects; marginal (M) and conditional (C) R^2 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) R^2 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.

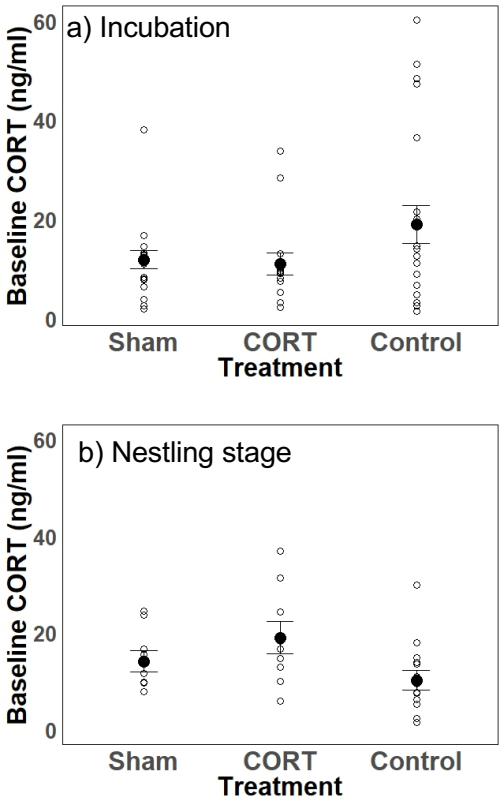

Figure 1. There was no significant difference among treatments in plasma corticosterone levels of female tree swallows when measured during (A) incubation and (B) nestling stage. Individuals in the CORT treatment had a single silastic implant containing crystalline corticosterone, those in the Sham treatment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