1   Environmental unpredictability shapes glucocorticoid regulation across populations of tree swallows

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3   Cedric Zimmer[1], Conor C. Taff[1,2], Daniel R. Ardia[3], Alexandra P. Rose[4], David A. Aborn[5], L. Scott Johnson[6]

4   and Maren N. Vitousek[1,2]

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6   [1] Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853, USA

7   [2] Cornell Lab of Ornithology, Ithaca, NY 14850, USA

8   [3] Department of Biology, Franklin and Marshall College, Lancaster, PA 17604, USA

9   [4] Institute of Arctic and Alpine Research, University of Colorado, Boulder, CO 80303, USA

10   [5] Biology, Geology and Environmental Science, The University of Tennessee Chattanooga, Chattanooga,

11   TN 37403, USA

12   [6] Department of Biological Sciences, Towson University, Towson, MD 21252, USA

13

14   Correspondence: Cedric Zimmer, cgg.zimmer@gmail.com, 813-974-4105

15 **Abstract**

16 Responding appropriately to challenges is an important contributor to fitness. Variation in the regulation

17 of glucocorticoid hormones, which mediate the phenotypic response to challenges, can therefore

18 influence the ability to persist in a given environment. We compared stress responsiveness in four

19 populations of tree swallows *(Tachycineta bicolor)* along an environmental gradient to evaluate support

20 for different selective pressures in driving the evolution of glucocorticoid regulation. In accordance with

21 the environmental unpredictability hypothesis, stronger stress responses were seen in more

22 unpredictable environments. Contrary to the reproductive value hypothesis, the stress response was not

23 lower in populations engaging in more valuable reproductive attempts. Populations with stronger stress

24 responses also had stronger negative feedback, which supports a "mitigating" rather than a

25 "magnifying" effect of negative feedback on stress responses. These results suggest that combining a

26 robust stress response with strong negative feedback may be important for persisting in unpredictable

27 or rapidly changing environments.

28

29 **Introduction**

30 Global environmental changes are altering the habitats of many species (Peñuelas et al., 2013). For

31 species with wide geographic ranges, intra-specific variation in life-history strategies resulting from

32 historical selection might predispose some populations to be more or less susceptible to increasing

33 environmental changes (Candolin, 2019; Debecker and Stoks, 2019; Harding et al., 2019). Environmental

34 variation favors individuals that differentially allocate time and energy to reproduction and self-

35 maintenance in order to maximize lifetime fitness (Stearns, 1989; Stearns, 1992; Ricklefs and Wikelski,

36 2002). Thus, characterizing differences in the regulation of this trade-off across environments is critical

37 for understanding the mechanisms that have shaped phenotypic responses and that may allow

38 successful adaptation and population persistence under rapid global changes. While the ultimate

39 reasons for variation in life-history traits across environments and latitudes have been well studied, we

40 have a limited understanding of the proximate mechanisms that underlie this variation (Eikenaar et al.

41 2012, Atwell et al. 2014). Because environmental factors may influence the evolution of life-history

42 traits by acting on physiological systems that integrate external conditions, hormones have been

43 proposed to play a crucial role as mediators of life-history trade-offs (Zera and Harshman, 2001; Ricklefs

44 and Wikelski, 2002; Eikenaar et al., 2012).

45

46   The hypothalamic-pituitary-adrenal (HPA) axis is a fundamental component of the endocrine system

47   that forms an interface between an animal and its environment (Wingfield and Sapolsky, 2003; Breuner,

48   2011; Hau et al., 2016). The HPA axis coordinates the response to energetic and other challenges mainly

49   by regulating the production and release of glucocorticoids (Wingfield et al., 1998; Sapolsky et al., 2000).

50   In non-chronically or acutely stressed individuals, glucocorticoids are usually maintained at low levels to

51   regulate energy balance and mediate foraging and other locomotor activities (Sapolsky et al., 2000;

52   Landys et al., 2006). When facing unpredictable challenges, circulating glucocorticoids increase

53   dramatically, promoting a suite of processes that facilitate responding to and recovering from these

54   challenges (Wingfield et al., 1998; Sapolsky et al., 2000). When sustained, this stress response can

55   trigger an emergency life-history stage in which breeding activities are usually reduced, and energy is

56   redirected toward survival (Wingfield et al., 1998). Thus, glucocorticoids – particularly in the presence of

57   a stressor – have been widely predicted to mediate life history trade-offs between current and future

58   reproduction.

59

60   While models of the stress response make clear predictions about redirecting effort, empirical studies

61   have proved equivocal in linking environmental conditions to appropriate responses. One limitation of

62   much empirical work to date is that it has focused on the relationship between glucocorticoids and

63   fitness in a single context (i.e., a single population, year, or environment; Hau et al., 2016; Guindre-

64   Parker, 2018). The factors that shape these relationships are best understood by measuring

65   glucocorticoids across different environmental and life-history contexts (Apfelbeck et al., 2017; Schoenle

66   et al., 2018; Vitousek et al., 2019a). Because the costs and benefits of mounting a robust glucocorticoid

67   stress response are likely to differ across environments and species, populations are predicted to differ

68   in how they regulate glucocorticoids (Schoenle et al., 2018; Vitousek et al., 2019b). For instance, a

69   comparative study in birds found a positive association between latitude and the glucocorticoid

70   response to a standardized retraint stressor (acute challenge that activates the HPA axis resulting in

71   stress-induced glucocorticoids increase) during breeding (Bókony et al., 2009). However, willow warblers

72   (*Phylloscopus trochilus*) breeding in northern Sweden—where the breeding season is shorter and

73   environmental conditions less predictable—show lower stress-induced glucocorticoid levels than those

74   breeding in southern Sweden, where conditions are more predictable (Silverin et al., 1997). Breuner and

75   colleagues (2003) compared males from three populations of white-crowned sparrows (*Zonotrichia*

76   *leucophrys*) breeding at different latitudes from California to Alaska. They found that males had similar

77   stress-induced glucocorticoid levels, but differed in their corticosteroid-binding globulin and intracellular

78   receptor affinity, suggesting that HPA axis regulation varied across the populations. Thus, while the

79   stress response appears to vary with latitude, additional research is needed to determine which of the

80   many potential selective pressures that covary with latitude are driving this variation.

81

82   Two factors thought to be particularly important in shaping the costs and benefits of stress

83   responsiveness are the frequency with which organisms face major unpredictable changes in their

84   environment (the "environmental unpredictability" hypothesis), and how valuable each breeding

85   attempt is to lifetime reproductive effort (the "brood value" or "reproductive value" hypothesis). The

86   "environmental unpredictability" hypothesis predicts that species or populations in less predictable

87   environments will have higher stress-induced glucocorticoid levels to facilitate responding effectively to

88   challenges (Wingfield, 2013; Vitousek et al., 2019a). The reproductive value hypothesis predicts that the

89   stress response differs based on the proportion of lifetime reproductive effort represented by a single

90   breeding attempt. According to this hypothesis, because high glucocorticoid levels can be deleterious to

91   reproduction by diverting energy away from breeding activities, organisms engaging in more valuable

92   reproductive attempts should have lower stress-induced glucocorticoids to avoid jeopardizing the

93   current breeding attempt (Bókony et al., 2009). The relative value of a single reproductive event

94   depends on both the length of the breeding season, which influences the number of potential

95   reproductive attempts an individual can engage in per season, and its lifespan. A shorter breeding

96   season results in less opportunity to engage in multiple successive reproductive attempts or to restart a

97   breeding attempt in case of failure of the initial one, which increases the relative value of each

98   reproductive attempt (Breuner et al., 2003; Wingfield and Sapolsky, 2003; Hau et al., 2016). Therefore,

99   the reproductive value hypothesis predicts that populations living in environments with shorter

100   breeding seasons will show lower stress-induced glucocorticoid levels during breeding (Bókony et al.,

101   2009; Breuner, 2011). This desensitization of the HPA axis to challenges facilitates not interrupting

102   valuable breeding attempts (Wingfield and Sapolsky, 2003; Breuner, 2011).

103

104   Previous comparative studies investigating the role of environmental predictability in shaping

105   glucocorticoid concentrations have predominantly used latitude as a proxy for environmental

106   predictability or harshness (e.g., Bókony et al., 2009; Jessop et al., 2013). Variability in environmental

107   conditions, especially temperature, typically increases with increasing latitude and elevation (Breuner

108   2011); however, these patterns are not always consistent. Furthermore, as many factors (including the

109   duration of the breeding season) also commonly covary with latitude, analyses that use latitude alone

110   could conflate the selective pressures imposed by living in an unpredictable environment with other

111   selective pressures. We are aware of only one previous study that has directly quantified and tested the

112   role of environmental variation in predicting variation in stress-induced glucocorticoids. A phylogenetic

113   comparative analysis of the relative support for these and other factors in shaping glucocorticoid

114   variation across vertebrates found that reproductive value better predicted large-scale variation in

115   stress-induced glucocorticoids than environmental variability (Vitousek et al., 2019a). However, the

116   relative roles of different selective pressures in shaping glucocorticoid variation are expected to vary

117   across species. Disentangling the relative roles of reproductive value and environmental unpredictability

118   in shaping glucocorticoid regulation will require comparing populations of the same species inhabiting

119   different environments.

120

121   Central to the hormonal mediation of life history is an understanding of the potential cost of the stress

122   response. The costs of the stress response likely depend not only on maximum glucocorticoid levels but

123   also on the duration of exposure to high levels, which is influenced by the strength of negative feedback.

124   Negative feedback is triggered after activation of the HPA axis and is coordinated by glucocorticoids

125   binding to receptors in the brain inducing a decrease in circulating glucocorticoids (de Kloet et al., 1998;

126   Breuner and Orchinik, 2001; Romero, 2004). Despite increasing evidence that differences in the strength

127   of negative feedback affect aspects of health and performance (Sapolsky, 1983; Dallman et al., 1992;

128   Weaver et al., 2004), its functional effects have been largely neglected in free-living organisms (but see

129   (Romero and Wikelski, 2010; Zimmer et al., 2019). We are not aware of any previous studies that have

130   assessed how negative feedback varies across environments. We hypothesized that differences in

131   negative feedback efficacy could either mitigate or magnify the costs of a stress response. Strong

132   negative feedback could serve to mitigate the costs of mounting a robust stress response by inducing a

133   fast decrease in circulating glucocorticoids. This could be particularly important when a strong stress

134   response is required to cope effectively with frequent unpredictable short-term challenges. In this case,

135   strong negative feedback may make it possible to avoid the negative effects of sustained glucocorticoid

136   elevation and hence recover quickly and resume critical activities such as breeding. A recent study

137   within a single population of tree swallows (*Tachycineta bicolor*) supported the mitigating hypothesis:

138   incubating females that exhibited both a robust stress response and strong negative feedback were less

139   likely to abandon reproductive attempts when facing stressors (Zimmer et al., 2019). Alternatively,

140   coupling an elevated stress response with weak negative feedback could serve to magnify the effects of

141   the stress response. This could be adaptive if mounting a longer stress response facilitates avoiding or

142   alleviating severe challenges; for example, if a more robust response enhances sensitivity to

143   environmental cues (Wingfield, 2013; Vitousek et al., 2019b). Accordingly, individuals breeding in highly

144   variable environments are predicted to show elevated stress repsonses followed by strong negative

145   feedback.

146

147   Here, we compared the support for two sets of predictions about how HPA axis regulation differs across

148   populations and environments in breeding tree swallows. First, we asked whether variation in the

149   glucocorticoid stress response (peak stress-induced glucocorticoids) across populations is better

150   predicted by the reproductive value hypothesis or the environmental unpredictability hypothesis.

151   Second, we assessed whether negative feedback varies across populations, and if so, whether the

152   patterns suggested a magnifying or mitigating effect on the stress response. The tree swallow, a

153   common passerine bird that breeds across much of North America, is an ideal species in which to test

154   these predictions as it breeds along an expansive latitudinal as well as elevational gradient within the

155   temperate zone. As such, different populations face different amounts of time available for

156   reproduction as well as differing levels of environmental unpredictability. We compared populations

157   breeding in Tennessee, New York, Wyoming and Alaska. We assessed females' HPA axis activity by

158   measuring baseline glucocorticoids, stress responses, and negative feedback during two life history

159   stages: incubation and nestling rearing. In order to test whether between-population differences in the

160   stress response are better predicted by differences in the time available for reproduction or

161   environmental unpredictability, we characterized both parameters in each population. We first

162   determined breeding synchrony and the length of the breeding season in each population. Breeding

163   season length is a good proxy for differences in reproductive value across populations breeding at

164   different latitudes (Breuner, 2011). Because increasing reproductive value is generally associated with

165   higher parental investment (Williams, 1966; Ardia, 2005; Ardia and Clotfelter, 2007) and thus potentially

166   with higher reproductive success, we also determined breeding effort and success. To characterize

167   differences in environmental predictability, we calculated an index of unpredictability for different

168   weather variables using historical weather data for each site. We predicted that if variation in the

169   magnitude of the glucocorticoid stress response is predominantly shaped by reproductive value, females

170   breeding at the two sites with a relatively short breeding season (Alaska and Wyoming) would mount a

171   lower acute stress reponse (Bókony et al., 2009; Vitousek et al., 2019a). Conversely, if environmental

172   unpredictability plays a greater role, we expected the opposite pattern: females breeding in the more

173   unpredictable environments of Alaska and Wyoming would have higher stress-induced corticosterone

174   levels. Concerning negative feedback, the mitigation hypothesis predicts strong negative feedback in

175   populations with greater stress responses. The magnifying hypothesis, in contrast, predicts weaker

176   feedback in the face of greater stress responses.

177

178   Differences in HPA axis regulation across populations could also be associated with downstream

179   physiological costs. Glucocorticoids affect both redox balance and glucose metabolism (Sapolsky et al.,

180   2000; Costantini et al., 2011). We also tested whether oxdidative stress, another potential physiological

181   mediator of the trade-off between reproduction and survival (Casagrande and Hau, 2018), or glucose

182   levels varied across populations. We predicted that oxidative stress and circulating glucose would be

183   higher in populations breeding in more unpredictable environments and/or with higher reporductive

184   value, and in those with higher baseline glucocorticoid levels.

185

186   **Results**

187   *Environmental unpredictability*

188   The unpredictability of temperature during the breeding season was the lowest in Tennessee and the

189   highest in Wyoming and Alaska (Table 1, Fig 1). Temperature unpredictability in New York was

190   intermediate, but closer to Alaska and Wyoming than to Tennessee (Table 1).

191

192   Table 1: Unpredictability of daily average temperature, daily average active time (between 0600 and 2000)

193   temperature, daily maximum temperature and daily total precipitation at the field sites in Tennessee, New York,

194   Wyoming and Alaska. Higher SD_{res} value indicates greater unpredictability.

	Tennessee	New York	Wyoming	Alaska
SD_{res} average daily temperature	0.18	0.33	0.41	0.38
SD_{res} average daytime temperature	0.21	0.42	0.53	0.50
SD_{res} maximun daily temperature	0.21	0.38	0.51	0.47

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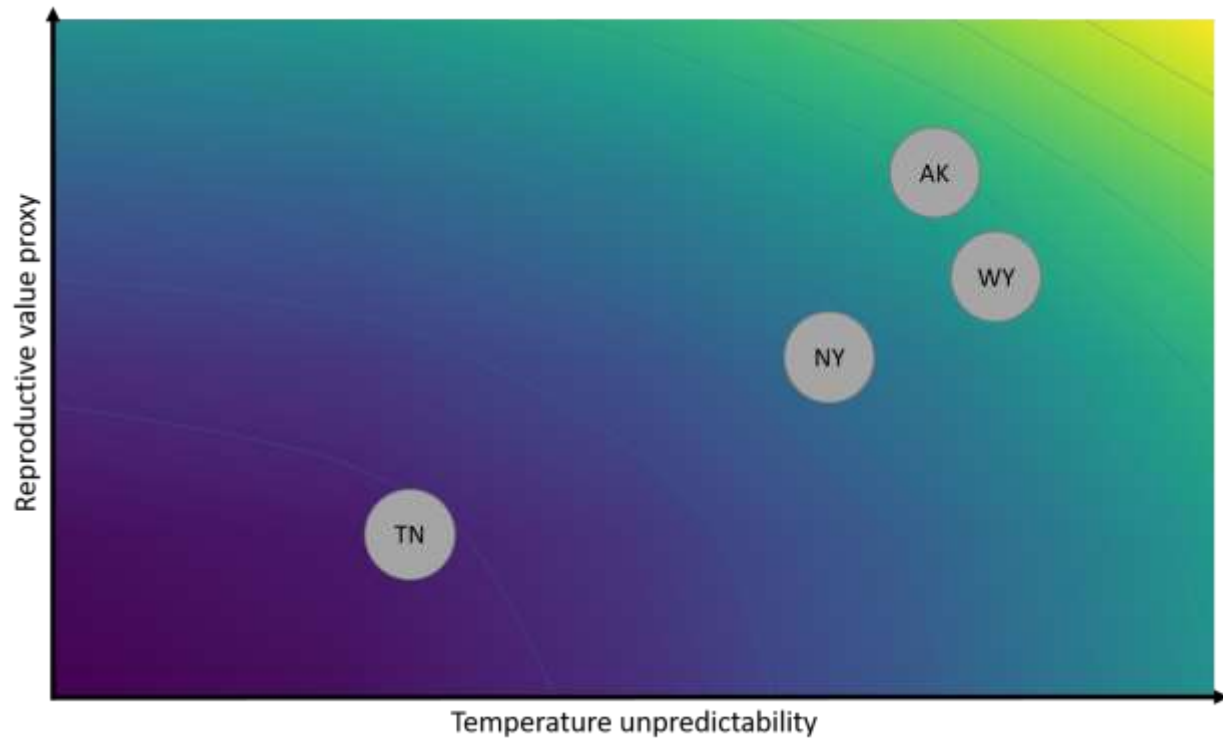
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197 Figure 1: Plot illustrating the difference between the four populations (Tennessee (TN), New York (NY), Wyoming

198 (WY) and Alaska (AK) along a gradient of increasing temperature unpredictability (x axis) and a proxy of

199 reproductive value based on breeding season length (y axis). Warmer color indicates higher environmental

200 unpredictability and higher reproductive value.

201

202 *Reproductive value*

203 The synchrony with which females laid first clutches differed between populations ($\chi^2_{3,250}$ = 89.51, p <

204 0.0001) with females in TN (17.7 ± 1 days) showing a more extended clutch initiation period than

205 females in the three other populations (NY: 8.9 ± 1.1 days, WY: 9.2 ± 0.7 days, AK: 8.8 ± 0.6 days; z ≥

206 6.68, p < 0.0001). Total length of the breeding season also differed between populations (χ^2_3 = 8.49, p =

207 0.037) with a longer breeding season in TN (99 days) and the shortest in AK (66 days). Breeding season

208 lengths in NY (74 days) and WY (70 days) were intermediate, but more similar to AK than TN. Overall,

209 these patterns suggest that reproductive value was the lowest in TN and relatively similar across the

210 other three populations (Fig 1).

211

212 *Corticosterone regulation*

213 Females' corticosterone phenotypes differed between populations. This difference was influenced by

214 the life history substage during which the female was captured and corticosterone sample type

215   (baseline, stress-induced, post-dexamthasone (post-dex); population x life history substage x sample:

216    $F_{12,1263} = 3.92$, p < 0.0001; Fig 2).

217

218   Across sampling periods, baseline corticosterone levels tended to be lower in Tennessee than in the

219   other three populations (Fig. 2, see supplementary material for statistical analyses).
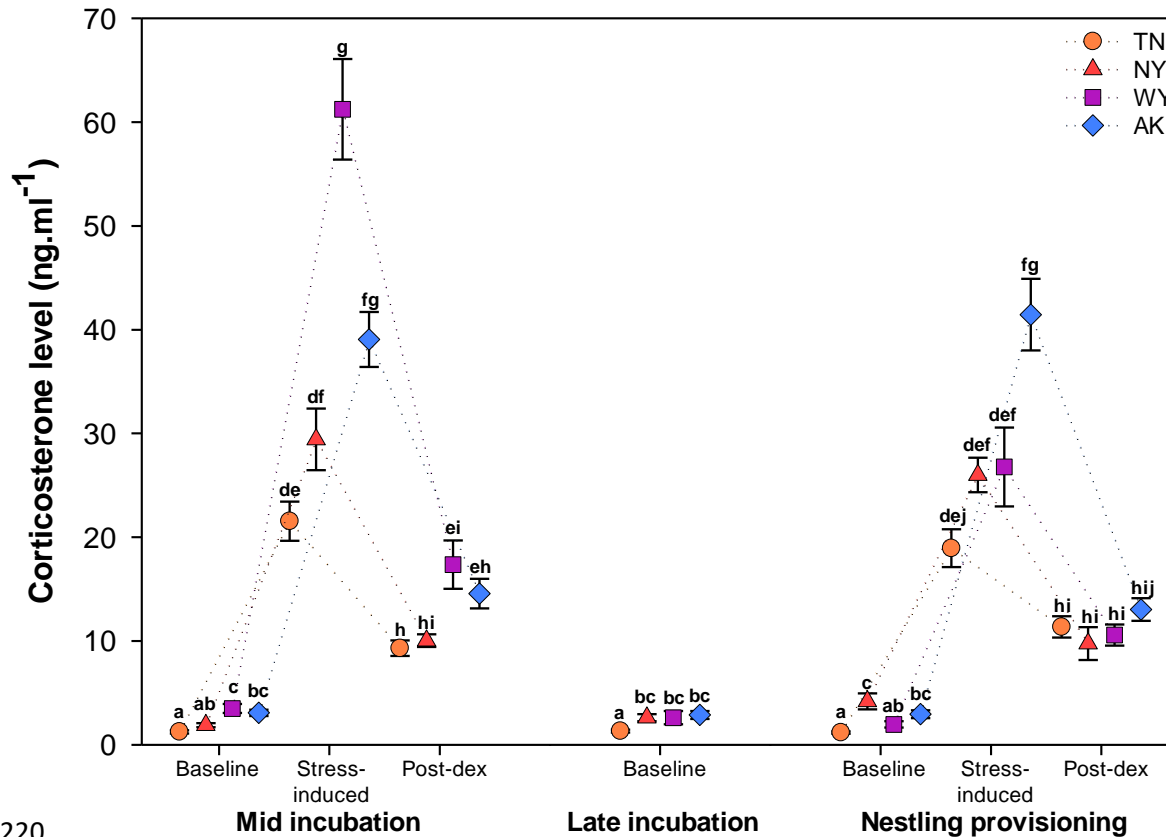
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221   Figure 2: Corticosterone levels (mean ± SE) of females when captured at three points during the reproductive

222   cycle (mid incubation: day 6-7 of incubation, late incubation: day 12-13 of incubation, and nestling provisioning:

223   day 6 post-hatch) in Tennessee (TN), New York (NY), Wyoming (WY), and Alaska (AK). At all captures baseline

224   corticosterone levels were measured. During the first and third captures, stress-induced corticosterone after 30

225   minutes of restraint and 30 minutes after injection of dexamethasone (post-dex) were also measured. Different

226   letters indicate significant differences.

227

228   Circulating stress-induced corticosterone levels generally increased with temperature unpredictability

229   (Fig 2). The exception to this pattern was in WY during mid incubation, when stress-induced

230   corticosterone levels were significantly higher than all other populations except for AK (see

231   supplementary material for statistical analyses). Corticosterone stress responses (the difference

232   between stress-induced and baseline corticosterone) showed a similar pattern. Overall, stress responses

233   differed between populations and life history substages (population x life history substage: $F_{3,220.1} = 9.45$,

234   p < 0.0001; Fig 3a). During mid-incubation, stress responses were highest in WY (t ≥ 5.08, p < 0.0001; Fig

235   3a) and intermediate in AK (t ≥ 2.71, p ≤ 0.044; Fig 2a). During nestling provisioning, females in AK had a

236   stronger stress response than females in all other populations (t ≥ 3.01, p ≤ 0.048; Fig 3a).

237

238   Circulating corticosterone levels after dexamethasone injection did not differ between the four

239   populations (t ≤ 1.73, p ≥ 0.99; Fig 2). However, negative feedback, i.e., the decrease in corticosterone

240   following dexamethasone injections, differed between populations and life history substages

241   (population x life history substage: $F_{3,236.3} = 8.30$, p < 0.0001; Fig 3b). During mid-incubation, negative

242   feedback was stronger in WY than in all other populations (t ≥ 5.36, p < 0.0001; Fig 3b). In the three

243   other populations, negative feedback was highest in AK, intermediate in NY, and weakest in TN (pairwise

244   comparisons: AK vs. TN: t = 3.60, p = 0.016, AK vs. NY: t = 0.69, p = 0.99, TN vs. NY: t = 1.55, p = 0.77; Fig

245   3b). During the nestling provisioning period, females in AK had stronger negative feedback than females

246   in all other populations (t ≥ 3.16, p ≤ 0.04; Fig 3b); negative feedback did not differ among the other

247   populations (t ≤ 1.24, p ≥ 0.91; Fig 3b). Overall, negative feedback was stronger in populations that

248   mounted a strong stress response.

249

250   Within populations, the magnitude of the stress response did not change between substages of the

251   reproductive period (t ≤ 1.49, p ≥ 0.81; Fig 3a), except in WY where it decreased between incubation and

252   nestling provisioning (t = 7.09, p < 0.0001; Fig 3a). Negative feedback efficacy also did not change

253   between life history substages within populations (t ≤ 1.28, p ≥ 0.90; Fig 3b), except in WY where it

254   decreased between mid-incubation and nestling provisioning (t = 6.08, p < 0.0001; Fig 3b). See

255   supplementary material for detailed information about within population corticosterone changes across

256   the breeding season.

257

258   Correlations between the different measures of the HPA axis differed between populations and life

259   history substages. Overall, the different aspects of the HPA axis are not correlated or weakly to

260   moderately correlated(Table 2). However, the strength of the stress response and the efficacy of

261   negative feedback are positively correlated in all populations at both life history substages (Table 2).

262

263   Table 2: Correlations between baseline, stress-induced and post-dexamethasone (post-dex) glucorticoid levels and

264   between the strength of the stress response and the efficacy of negative feedback within each population. Data

265   are R^2, p-value. Bold indicates significant correlations.

		Mid incubation			Nestling provisioning		
		stress-induced (R^2, p-value)	post-dex (R^2, p-value)	negative feedback (R^2, p-value)	stress-induced (R^2, p-value)	post-dex (R^2, p-value)	negative feedback (R^2, p-value)
Tennessee	baseline	0.001, 0.94	0.005, 0.54		**0.09, 0.03**	**0.18, 0.002**	
	stress-induced		**0.08, 0.02**			**0.27, 0.0001**	
	stress response			**0.84, <0.0001**			**0.70, <0.0001**
New York	baseline	0.02, 0.43	0.02, 0.41		0.001, 0.89	0.03, 0.43	
	stress-induced		**0.15, 0.01**			0.01, 0.62	
	stress response			**0.96, <0.0001**			**0.50, 0.0008**
Wyoming	baseline	**0.07, 0.02**	0.005, 0.55		**0.11, 0.07**	0.01, 0.60	
	stress-induced		0.001, 0.91			0.08, 0.12	
	stress response			**0.91, <0.0001**			**0.92, <0.0001**
Alaska	baseline	0.001, 0.78	0.003, 0.66		0.01, 0.43	0.007, 0.54	
	stress-induced		0.03, 0.15			**0.25, 0.0001**	
	stress response			**0.69, <0.0001**			**0.90, <0.0001**

266

267

268   Resistance to oxidative stress and reactive oxygen metabolite levels did not differ between populations

269   (Supplementary material). Baseline and stress-induced glucose did not differ between females in TN, NY

270   and AK, but baseline glucose was lower in WY than in the other populations (Supplementary material).

271

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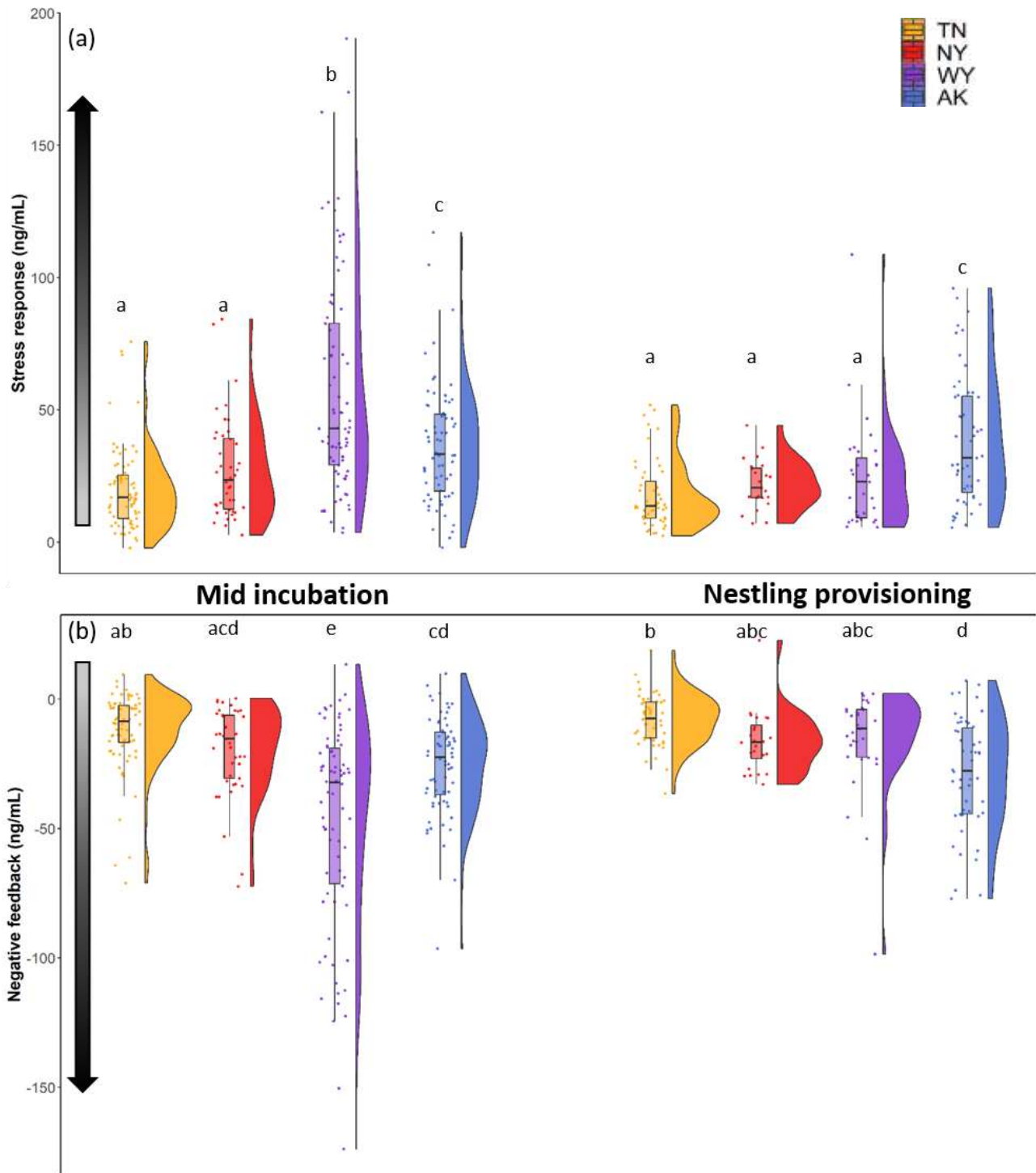
Figure 3: Females' glucocorticoid stress responses (a) and the strength of negative feedback (b) in Tennessee (orange), New York (red), Wyoming (purple) and Alaska (blue) during mid incubation and nestling provisioning. Points overlaying the boxplot show raw data and the half-split violin shows the probability density function. Shaded arrows along the y-axis denote increasing strength of the stress response and negative feedback with increasing darkness. Different letters indicate significant differences.

278

279 *Reproductive effort and success*

280 There were no differences in clutch size or brood size at hatching between populations ($\chi^2 \leq 5.90$, p $\geq$

281 0.12, see supplementary material). Because of an extended period of cold, wet weather that occurred

282 during the incubation stages of most females, hatching success ($\chi^2_{3,250}$ = 29.24, p < 0.0001) was lower in

283 WY (50.7 %, 38 of 75 nests) than in the other three populations (TN: 82.2 %, 60 of 73 nets; NY: 79.5 %,

284 31 of 39 nests; AK: 84.4 %, 54 of 64 nests; z $\geq$ 3.58, p $\leq$ 0.002). Provisioning effort differed across

285 populations ($F_{3,155.4}$ = 6.00, p = 0.0007). Females in AK and WY provisioned at higher rates than those in

286 NY and TN (Fig 4).

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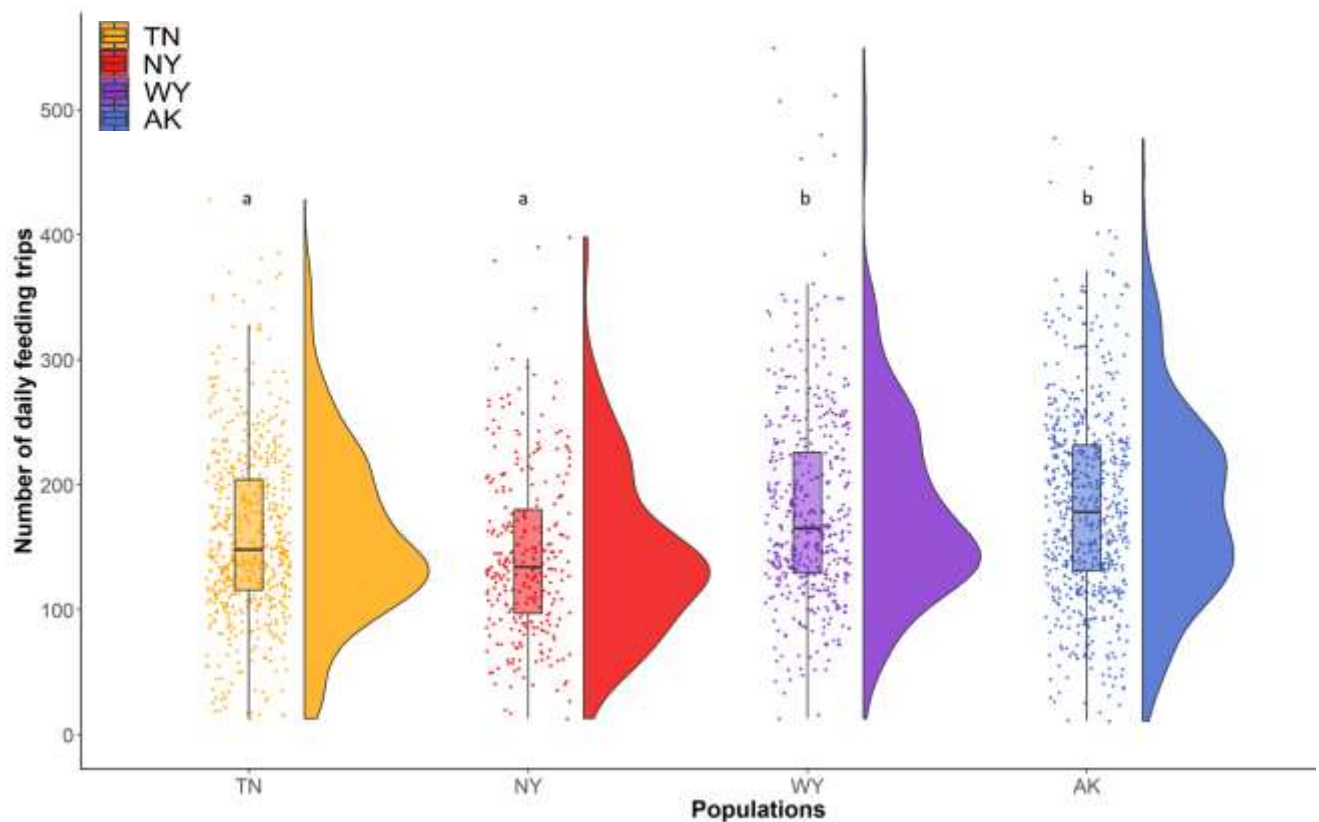
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289 Figure 4: Number of daily feeding trips for females in Tennessee (orange), New York (red), Wyoming (purple), and

290 Alaska (blue), calculated from the total number of raw visits recorded (329,047). Points overlaying the boxplot

291 show raw data and the half-split violin shows the probability density function. Different letters indicate significant

292 differences between populations.

293

294 Nestling body mass differed across populations, mirroring the observed differences in female

295 provisioning rates ($F_{3,128.5}$ = 4.02, p = 0.009). On day 12 post-hatching, nestlings were heaviest in AK (21.2

296   ± 0.2 g) and WY (20.6 ± 0.2 g), and lightest in NY (18.7 ± 0.5 g) and TN (19.6 ± 0.2 g). Among nests in

297   which one or more eggs hatched, the number of nests in which one or more nestlings fledged also

298   differed among populations ($\chi^2_{3,250}$ = 22.49, p < 0.0001); fledging success was lower in NY (35.5 %, 11 of

299   31 nests) than in the other populations (TN: 76.7 %, 46 of 60 nests, AK: 80.4 %, 41 of 51 nests, WY: 80.0

300   %, 28 of 35 nests; t ≥ 3.30, p ≤ 0.005).

301

302   **Discussion**

303   In the current context of global changes, understanding how organisms have evolved in response to

304   environmental challenges is critical. Our results demonstrate that glucocorticoid regulation across

305   different environmental and life-history contexts may underlie successful adaptation and population

306   persistence. First, we showed that female tree swallows breeding in environments with higher

307   temperature unpredictability secrete more glucocorticoids in response to acute challenges during

308   breeding. This pattern was strong even though at these sites birds also experienced shorter breeding

309   seasons and would normally be predicted to skew investment towards offspring. Thus, if elevated

310   glucocorticoids triggered reproductive failure or delay, birds at those sites would have less time to

311   complete reproduction and re-nest if necessary. This seemingly contrary result reveals environmental

312   unpredictability may be a stronger force than the value of the current breeding attempt in determining

313   stress-induced glucocorticoid levels in female tree swallows. Second, we found that populations with

314   higher stress responses also have stronger negative feedback, which likely functions to limit potential

315   damage caused by high glucocorticoid levels. The combination of high stress-induced glucocorticoid

316   levels followed by the induction of strong negative feedback may allow females to cope effectively with

317   frequent unpredictable challenges, and to recover faster in order to continue breeding activities. This

318   phenotype may be most strongly favored in environments with both high unpredictability and greater

319   time constraints on reproduction.

320

321   *Life history and environmental unpredictability*

322   Across the four study populations we found differences in key selective pressures. Birds breeding in

323   Alaska and in the mountains of Wyoming faced more unpredictable temperatures than birds in

324   Tennessee; New York conditions were intermediate. The time constraints on reproduction differed

325   similarly across populations, resulting in higher apparent reproductive value in Alaska and Wyoming and

326   lowest apparent reproductive value in Tennessee. As predicted by life history theory (and consistent

327   with previous comparative work in tree swallows: Ardia, 2006; Ardia, 2007; Rose and Lyon, 2013; Akçay

328   et al., 2016), birds in Alaska and Wyoming showed higher parental investment (measured as offspring

329   feeding rate) and reared larger offspring than those in New York and Tennessee.

330

331   *Glucocorticoid regulation*

332   At our two sites with stronger time constraints on reproduction and high temperature unpredictability

333   (AK and WY) we found that the magnitude of the glucocorticoid stress response was greatest. This result

334   provides the first empirical support for the idea that environmental unpredictability could be a stronger

335   force shaping the peak glucocorticoid response to challenges than brood value, which should select for

336   suppressed stress responses in the face of low residual reproductive value. In contrast with this finding,

337   a recent large scale phylogenetic comparative analysis found greater support for reproductive value

338   shaping glucocorticoid stress responses across vertebrates than environmental variation (Vitousek et al.

339   2019b). Although other studies have not directly compared these effects, a number of analyses have

340   supported reproductive value as a driver of variation in the strength of glucocorticoid stress responses

341   within and across species (Silverin et al., 1997; Bókony et al., 2009; Breuner, 2011; Hau et al., 2016, but

342   see Breuner et al., 2003; Krause et al., 2016). However, previous studies have also found that latitude

343   (which is often used as a proxy for environmental variation or harshness: e.g., Bókony et al., 2009;

344   Jessop et al., 2013) covaries positively with stress-induced glucocorticoids. We predict that differences in

345   the relative importance of environmental unpredictability and reproductive value in shaping stress

346   responsiveness is likely to vary across species. Specifically, we predict that the stress responsiveness of

347   income breeders will be more strongly influenced by environmental unpredictability than that of capital

348   breeders. Similarly, we predict that stress responsiveness will be particularly closely tied to

349   environmental unpredictability in species that rely on critical food sources impacted by short-term

350   environmental fluctuations (e.g., aerial insectivores).

351

352   A robust stress response could allow appropriate responses to challenges, but might also result in

353   associated reproductive costs (Wingfield and Sapolsky, 2003). However, we found no evidence that

354   maintaining a robust stress response had negative effects on reproduction. Reproductive success did not

355   vary consistently across populations; instead, the differences that we saw in hatching success (lowest in

356   WY) and fledging success (lowest in NY) mirrored temporary periods of inclement weather in those

357   populations. Consistent with life history theory, females in Alaska and Wyoming showed a higher

358   investment in nestlings. Nevertheless, it is possible that the costs of maintaining an elevated stress

359   response would manifest under more prolonged stressful conditions, or that it imposes longer-term

360   costs (e.g., accelerated telomere shortening or senescence).

361

362   We found support for a mitigating effect of negative feedback on stress responsiveness: female tree

363   swallows breeding in Alaska and Wyoming, where both time constraints on reproduction and

364   temperature unpredictability were the highest, showed both a high stress response and strong negative

365   feedback. Stronger negative feedback allows for a faster decrease in circulating glucocorticoids,

366   potentially reducing the costs associated with sustained glucocorticoid elevation. Thus, strong negative

367   feedback should be particularly beneficial for individuals with a strong stress response (Zimmer et al.,

368   2019). We recently demonstrated that variation in negative feedback efficacy is related to the speed of

369   recovery of the HPA axis from repeated transient stressors, stress resilience, and reproductive success in

370   tree swallows (Taff et al., 2018; Vitousek et al., 2019b; Zimmer et al., 2019). These results indicate that

371   regulation of negative feedback is crucial for coping with perturbances, particularly in more challenging

372   environments.

373

374   Taken together, our results suggest that when environmental conditions become more variable female

375   tree swallows couple high magnitude stress responses and strong negative feedback, instead of

376   decreasing the hormonal response to challenges. Through strong negative feedback individuals could

377   limit the reproductive costs of glucocorticoid exposure, and resume reproductive activities as soon as

378   the stressor has passed. Thus, this combination of glucocorticoid regulatory traits may enable individuals

379   living in harsher environments to balance the challenges of high environmental unpredictability and a

380   brief reproductive period in order to maximize survival and reproductive success.

381

382   Our findings are also consistent with the idea that interactions among glucocorticoid regulatory

383   elements may be important for appropriately responding to challenges, and for fitness (Vitousek et al.,

384   2018b; Vitousek et al., 2019b; Zimmer et al., 2019). In all four populations the strength of the stress

385   response and the efficacy of negative feedback positively covary; however, there were weakly positive

386   or no phenotypic correlations between stress-induced and post-dexamethasone corticosterone levels.

387   Covariation in components of glucocorticoid regulation could result from similar regulatory pathways, as

388   negative feedback is regulated by glucocorticoids binding to glucocorticoid receptors (de Kloet et al.,

389   1998; Romero, 2004). As such, higher glucocorticoid levels could activate more receptors, inducing

390   faster negative feedback (Breuner and Orchinik, 2001; Romero, 2004). However, it has been suggested

391   that different components of the HPA axis are modulated independently (e.g., Romero, 2004).

392   Therefore, the phenotypic correlations seen here could also result from selection favoring combinations

393   of these traits. Determining whether stress-induced and post-dexamethasone corticosterone levels are

394   genetically correlated and whether glucocorticoid profiles covary with receptor expression could help to

395   illuminate the flexibility and physiological underpinnings of these traits and their potential to respond to

396   selection.

397

398   Baseline glucocorticoid levels also differed across populations. However, as both the reproductive value

399   hypothesis and the environmental unpredictability hypothesis predict higher baseline glucocorticoid

400   levels in Alaska and Wyoming, where the season is short and weather unpredictable, these patterns do

401   not allow us to differentiate between the role of these forces in shaping glucocorticoid evolution.

402   Elevated baseline glucocorticoid levels have been shown to support energetically demanding activities,

403   and are associated with more challenging conditions and increased investment in reproduction within

404   and across species (Hau et al., 2010; Bonier et al., 2011; Jessop et al., 2013; Apfelbeck et al., 2017;

405   Vitousek et al., 2019a). The particularly high baseline (and stress-induced) corticosterone levels in

406   Wyoming during incubation may also have reflected a temporary upregulation in HPA axis activation

407   because of a period of unusually cold and wet weather that occurred during this time. In contrast, in

408   Tennessee, in which tree swallows experience a long breeding season and more predicatable conditions

409   baseline glucocorticoid levels were consistently low throughout the reproductive period.

410

411   Contrary to our predictions that elevated stress levels would incur physiological costs, we did not detect

412   differences between populations in measures of oxidative damage, antioxidant capacity, or glucose

413   metabolism. Investment in reproduction is not always associated with higher oxidative stress (Garratt et

414   al., 2011; Speakman and Garratt, 2014). However, within individuals both oxidative stress and glucose

415   have been shown to increase with increasing glucocorticoids levels or workload (Sapolsky et al., 2000;

416   Costantini et al., 2011; Metcalfe and Monaghan, 2013). The lack of these relationships at the population

417   level could reflect different trade-offs operating across populations and within individuals.

418

419   *Conclusion*

420   These findings provide support for the hypotheses that environmental unpredictability may be a critical

421   factor in shaping glucocorticoid stress responses, and that selection favoring strong negative feedback in

422   more stress responsive individuals could serve as a mechanism to mitigate the costs of mounting a

423   strong stress response. Our results are also in accordance with the hypothesis that negative feedback

424   and the dynamic regulation of glucocorticoids are important for coping with challenging conditions

425   (Romero and Wikelski, 2010; Taff and Vitousek, 2016; Vitousek et al., 2019b). In the current context of

426   global changes, intraspecific differences in the response to stressors may be particularly important for

427   survival or for the ability to adapt to new conditions (Angelier and Wingfield, 2013; Harding et al., 2019).

428   Our results suggest that this phenotype (elevated stress response and strong negative feedback) has

429   been selected for in unpredictable environments and might therefore be expected to become

430   increasingly common over time, assuming genetic variation exists. However, as climate change affects

431   both the length of the breeding season (Dunn and Winkler, 2010) and environmental predictability

432   (Thornton et al., 2014), populations may face rapidly changing regimes of selection on glucocorticoid

433   regulation outside the bounds of evolutionary history. Confirming that selection is occurring in these

434   populations will require testing whether among individual differences in glucocorticoid phenotype affect

435   fitness. Ultimately, determining the evolutionary causes and consequences of differences in

436   glucocorticoid levels within and among populations will help to reveal how selection drives HPA axis

437   regulation and whether the history of selection on hormonal regulation influences the ability to cope

438   with unpredictable or changing environments.

439

440   **Methods**

441   *Populations*

442   Field data were collected from 2016 to 2018 in four different populations of tree swallows breeding in

443   nest-boxes. Populations were located in Chattanooga, Tennessee (TN) (35.1°N, 85.2°W, 206m

444   elevation), Ithaca, New York (NY) (42.5°N, 76.5°W, 340m elevation), Burgess Junction, Wyoming (WY)

445   (44.5°N, 107.3°W, 2451m elevation) and in McCarthy, Alaska (AK) (61.4°N, 143.3°W, 445m elevation).

446   Tree swallows are widely distributed across North America and breed in a variety of environments.

447   These populations were chosen to allow for comparisons among populations breeding in environments

448   with different degrees of environmental predictability and reproductive value. Populations at higher

449   latitude (Alaska) or elevation (Wyoming) are expected to experience cooler and more unpredictable

450   weather conditions and a shorter breeding season starting later in the year (late May). In Tennessee,

451   near the Southern edge of the breeding distribution, tree swallows are expected to experience warmer

452   and more predictable weather conditions and a long breeding season with the first egg usually laid

453   earlier in the season (early to mid-April). In New York, environmental conditions and breeding season

454   length are expected to be intermediate, with the first egg usually laid in early May.

455

456   *General field methods and stress manipulation*

457   In the four populations, nests were monitored every 1-2 days throughout the breeding season from the

458   initiation of activity at each site to fledging, except for the last week of nestlings' development (to avoid

459   inducing premature fledging). For every active nest, we recorded clutch initiation date and completion

460   dates, clutch size, hatch date, brood size, and the number of nestlings fledged. Nestling fates were

461   determined by checking boxes 22-24 days after hatching. We installed radio frequency identification

462   (RFID) units on each box on the fourth day of incubation (see below). Birds were captured at their nest

463   boxes by hand or using a manually activated trap. All birds were captured and sampled on specific days

464   of life history substages, and during a set time of day, to reduce the variation in circulating

465   glucocorticoid hormones resulting from circadian rhythms. Adult females were captured between 0700

466   and 1000h in NY, TN and WY and between or 0600 and 0900h in AK to compensate for the earlier start

467   of activity due to the increased day length compared to the other populations.

468

469   Females were initially captured 6 or 7 days after clutch completion (capture number 1). At this capture,

470   we took a first blood sample within 3 min of initial disturbance to measure baseline circulating

471   corticosterone levels. A second blood sample was taken after 30 min of restraint in a cloth bag to

472   measure stress-induced corticosterone levels. Immediately after this sample was taken, females were

473   injected with dexamethasone (dex) (0.5 μl.g^{-1}, Dexamethasone Sodium Phosphate, Mylan Institutional

474   LLC), a synthetic glucocorticoid that binds to receptors within the HPA axis, in order to induce negative

475   feedback (Zimmer et al., 2019). A final blood sample was taken 30 min after dex injection to measure

476   the degree of down-regulation in circulating corticosterone (a measure of negative feedback). Between

477   samples, we weighed the females, and measured the length of their skull from the back of the head to

478   the bill tip (head-bill) and flattened wing length. Non-banded individuals received USGS leg bands and a

479   celluloid color band with attached passive integrated transponder (PIT) tag encoding a 10-digit

480   hexadecimal string (Cyntag, Cynthiana, KY). Female age was determined based on plumage coloration

481   and characterized as second year (SY) or after second year (ASY) (Hussell, 1983).

482

483   As part of a separate study, at their first capture, adult females were allocated to one of the

484   experimental groups: control, feather restraint (in which three primaries were reversibly attached to

485   alter flight ability and thereby increasing the cost of foraging), or predator exposure (see Zimmer et al.,

486   2019 for details on both experimental treatments). Treatments started after the first capture and lasted

487   for 5-6 days.

488

489   Females were then recaptured 5-6 days later (on incubation day 12 or 13; capture 2), at the end of the

490   experimental treatments (see above). At this capture, we only took a baseline blood sample and

491   weighed the bird before release. Finally, we recaptured females again 6-8 days after eggs hatched

492   (capture 3). We followed the same procedure as in capture 1, taking a baseline, restraint stress-induced,

493   and post-dex blood samples, and again weighed each female.

494

495   Twelve days after eggs hatched, each nestling received an USGS leg band, was weighed and had head-

496   bill and flat wing length measured.

497

498   All blood samples were collected from the alar vein, in heparinized microhematocrit capillary tubes.

499   Glucose levels in baseline and stress-induced blood samples were determined in the field (see

500   supplementary). Blood samples were then transferred to microcentrifuge tubes, and kept on ice until

501   centrifugation (within 4h). After separation, the plasma was stored at -20^0C in the field and then at -80

502   ^{0}C in the lab until analysis. All methods were approved by Cornell IACUC and conducted with

503   appropriate state and federal permits.

504

505   *Provisioning behavior*

506   Number of feeding trips for females from nestling ages 1-18 was recorded using radio-frequency

507   identification (RFID) devices (Cellular Tracking Technologies; Rio Grande, NJ, USA) (Bridge and Bonter,

508   2011). RFID units were installed on each active box on day 4 of incubation. Antennae were fastened

509   around each entrance hole so that birds had to pass directly through an antenna to enter or exit the

510   box. We programmed our RFID units to sample for PIT tags every second between 0500 and 2200h each

511   day as tree swallows are not very active at night. Poll time was set at 500, and cycle time at 1000. The

512   delay time (minimum period of time between successive tag recordings) was set to 1s. RFID boards were

513   powered by 12V 5Ah (PS-1250, PowerSonic, San Diego, CA) batteries that were replaced every five days.

514   At the first capture, each bird was fitted with a PIT tag attached to a color band. Each PIT tag encoded a

515   unique 10-digit hexadecimal string that was recorded, along with a time stamp, when birds passed

516   through or perched on the antenna (see Vitousek et al., 2018a for more details). From the raw RFID

517   records, we determined the number of daily feeding trips for each female through 18 days of age for the

518   brood using an algorithm validated in the New York population (Vitousek et al., 2018a).

519

520   *Corticosterone assay*

521   Steroids were extracted from plasma samples using a triple ethyl acetate extraction and then

522   corticosterone levels were determined using an enzyme immunoassay kit (DetectX Corticosterone,

523   Arbor Assays: K014-H5) previously validated for tree swallows (Taff et al., 2019). Samples were run in

524   duplicate and all samples from an individual were run on the same plate. In total we ran 47 assays with

525   an average extraction efficiency of 92.8 % and a detection limit of 0.47 ng.ml^{-1}. The intra-assay variation

526   was 8.88 % and the inter-assay variation was 11.1 %.

527

528   *Data analysis*

529   To characterize the degree of environmental unpredictability at the different field sites we calculated

530   the unpredictability of temperature variables. We obtained historical weather data for each site over as

531   long a yearly range as possible. For New York we obtained data from the North East Climate Center

532   (http://www.nrcc.cornell.edu/) for the Game Farm road weather station (from 1983, located about 7km

533   from field sites) and from the Western Regional Climate Center (https://wrcc.dri.edu/) for the Prentice

534   Cooper State Forest station in Tennessee (from 2003, located about 16 km from field sites), the May

535   Creek station in Alaska (from 1990 located about 19 km from field sites) and the Burgess station in

536   Wyoming (from 1992, located about 5 km from field sites). From these data, we extracted the average

537   daily temperature, the daytime average daily temperature (between 0600 and 2200) which is the period

538   when the swallows are the most active and the daily maximum temperature, which is known to affect

539   flying insects' activity and therefore food availability (Winkler et al., 2013).

540

541   We quantified the unpredictability of these temperatures variables during the breeding season: from

542   April to June in Tennessee and New York and from May to July in Alaska and Wyoming. We calculated

543   unpredictability using a general additive model (GAM) following the methods described in Franch-Gras

544   et al. (2017). For each site, these variables were divided by their mean to normalize them before

545   analysis (Franch-Gras et al., 2017). This model considers the dispersion of the data in the time series

546   around a typical curve of the normalized variable. For each weather variable, the typical curve was fitted

547   in a GAM model in relation to the day of the year using the gam function in the mgcv package in R 3.5.3

548   (R Core Team, 2019). As suggested by Franch-Gras (2017), we fitted the GAMs using cubic splines as

549 smoothing function to not *a priori* constrain the shape of the curve. The standard deviation of the

550 residuals of the fitted model (SD_{res}) represents an index of unpredictability for each variable (Franch-

551 Gras et al., 2017). This index gives an overall measure of unpredictability using the historical records and

552 is not intended to indicate variation in weather in the particular years of study at each site.

553

554 We compared females' corticosterone levels by fitting a generalized linear mixed model (GLMM) with a

555 gamma distribution that included population, capture number, sample (baseline, stress-induced and

556 post-dex), female age and their interactions as fixed factors, relative clutch initiation date as a covariate

557 and female identity as random factor. We did not include treatment upon first capture (i.e., feather

558 restraint, predator exposure, or control) as an independent variable in the above analyses as it did not

559 affect HPA axis regulation, i.e., corticosterone levels (treatment x sample: $F_{4,1202}$ = 0.60, p = 0.66;

560 treatment x capture number x sample: $F_{8,1254}$ = 1.51, p = 0.15). We further characterized females'

561 corticosterone regulation by calculating their glucocorticoid stress response as the difference between

562 stress-induced and baseline corticosterone levels and their negative feedback as the difference between

563 post-dex and stress-induced corticosterone levels. We compared the magnitude of females' acute stress

564 response and negative feedback strength using GLMMs fit with a normal distribution including

565 population, capture number, female age and their interactions as fixed factors, relative clutch initiation

566 date as a covariate and female identity as a random factor. Within each population, we determined

567 whether corticosterone level at each time point, and stress response and negative feedback were

568 correlated using Pearson correlations.

569

570 In order to compare the populations in terms of breeding synchronization, we calculated the relative

571 clutch initiation date of the first clutch as the number of days after the first laying female in the

572 population. Then, we compared relative clutch initiation date between populations using GLMs with

573 population, female age and their interaction as fixed factors. We also calculated the total breeding

574 season length as the number of days between the first clutch initiation and the last day nestlings fledged

575 at each site. We compared populations usimg a GLM fitted with a Poisson distribution. We also

576 compared clutch size, brood size, hatching success and fledging success between populations.

577 Population, female age and their interactions were added as fixed factors, and relative clutch initiation

578 day as a covariate. The model for relative clutch initiation date was fitted with a normal distribution,

579 models for clutch size and brood size with a Poisson distribution and models for hatching success and

580 fledging success with a binomial distribution.

581

582   We compared the number of daily feeding trips females made using a generalized linear mixed model

583   GLMM fitted with a Poisson distribution. Population, female age, their interactions and nestling age

584   were added as fixed factors and brood size at each nestling age as a covariate. We also added relative

585   clutch initiation date and brood size as covariates and nest identity as random factor. We used GLMMs

586   to compare nestlings' body mass with population, female age and their interaction as fixed factors.

587   Female identity was added as a random factor.

588

589   GLMs were run using the GENMOD procedure and GLMMs the GLIMMIX procedure in SAS University

590   Edition (SAS Institute Inc., Cary, NC). Post-hoc comparisons were performed using Tukey-Kramer

591   multiple comparison adjustment to obtain corrected p-values. Probability levels <0.05 were considered

592   significant. Data are presented as mean ± SE.

593

594   **Acknowledgments**

597

598   **Competing interests**

599   No competing interests.

600

601   **References**

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