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The timing of embryonic exposure to elevated temperature alters stress endocrinology in domestic chickens (*Gallus domesticus*)

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ABSTRACT

Patterns of glucocorticoid (GC) release in response to stimuli vary both among individuals and within individuals across their lifetime. While much work has focused on how the prenatal steroid environment can affect GC release, relatively little is known about how environmental parameters, such as incubation temperature affect GCs. We tested the hypothesis that variation and timing of elevated incubation temperature within the thermoneutral zone can alter the pattern of GC release. We incubated domestic chicken eggs (*Gallus domesticus*) at the optimal incubation temperature (37.5 °C) or at a slightly higher temperature (+1.1 °C) either early, late, or throughout incubation. At three weeks post-hatch, all birds were (i) exposed to a capture-restraint stress to measure stress-induced GC release (naïve). Three days following the naïve stressor, birds were (ii) exposed to a heat challenge, which was followed the next day by a second capture-restraint stress (post-heat challenge). Regardless of treatment, birds had similar patterns of GC release following the naïve stress series. However, during the post-heat challenge stress series, birds incubated at optimal temperatures increased their peak GC release. In contrast, birds exposed to slightly elevated temperatures for any period of development failed to increase peak GC release, and their specific response varied with timing of exposure to the elevated incubation temperature. Our results demonstrate that subtle variation in the embryonic environment, such as elevated incubation temperature within the thermoneutral zone, can impact the pattern of GC release of offspring. Further work is needed to understand the mechanisms underlying these changes and the relationship between fitness and environmentally-altered phenotypes.

1. Introduction

An organism's ability to respond to variable environmental conditions is directly related to its ability to survive and reproduce (Ellis et al., 2006; Han and Brooks, 2014). One way in which individuals respond to environmental stressors is through activation of the hypothalamic–pituitary–adrenal (HPA) axis, which results in an increase in circulating levels of glucocorticoids (GCs; Wingfield et al., 1998). GCs coordinate many essential physiological functions, from the maintenance of peripheral-clock synchronization to behavioral modification and energy mobilization in response to acute stress (Sapolsky et al., 2000; Schoech et al., 2011; Dickmeis, 2009). In the short-term, GCs modulate their own release and circulation through negative feedback at the level of the hypothalamus and pituitary. However, GC release can be adjusted in the long-term to facilitate acclimatization to chronic disturbance by decreasing GC responsiveness (Partecke et al., 2006). This type of habituation can prevent pathologies associated with long-term elevation of GCs, termed chronic stress, which includes impaired cognitive function, muscle wasting, and suppression of the immune system (Sapolsky et al., 2000; Romero, 2004; Schoech et al., 2011).

Because of the extensive and variable effects GCs have on vertebrate physiology, individual variation in the pattern of stress-induced GC release often correlates with altered survival and reproduction (Bonier et al., 2009). Given this, recent biological inquiry has focused on factors that contribute to variation in the pattern of stress-induced GC release (Haussmann et al., 2012; Henriksen et al., 2011), and the effect of prenatal environments on altered HPA axis function and offspring phenotype have received particular attention (Love and Williams, 2008). While the majority of this work has focused on the prenatal steroid environment (Henriksen et al., 2011), the role that prenatal abiotic environmental parameters, such as temperature, play in shaping the pattern of GC release is far less understood. Previous work has shown that incubation temperatures slightly below the

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optimum slow hatchling growth (DuRant et al., 2010; Rhen and Lang, 1995), reduce immunocompetence (DuRant et al., 2012), and decrease reproductive output and survival of offspring (Hepp and Kennamer, 2012). However, to our knowledge only a handful of studies have explored how variation within the optimal range of incubation temperatures affects GC release in response to acute stress (DuRant et al., 2010, 2012).

Incubation temperatures are an especially relevant environmental parameter for bird species. While parental behaviors usually buffer eggs from variable environments, under consistently suboptimal conditions (much warmer or cooler than usual), maintaining 'optimal' egg temperature can result in a complex trade-off for parents. Parents must budget time between incubation and foraging to maintain both their own body condition and egg temperature (Conway and Martin, 2000). For ground-nesting species, such as most species in the order Galliformes, it may be more likely for eggs to experience higher incubation temperatures than cooler incubation temperatures (With and Webb, 1993). The trade-off faced by parents is particularly problematic in hot conditions, where parents must spend more time foraging to sequester the extra energy needed to cool themselves and their eggs. The increased foraging time can leave eggs exposed to higher temperatures for greater periods of time. Moreover, hyperthermia reduces egg survival more than hypothermia (Webb, 1987), and the upper limit to the incubation temperature range is therefore a more critical temperature for hatching success. Interestingly, exposure to elevated incubation temperatures primes domestic chicken to handle elevated ambient temperature post-hatch better than unprimed-conspecifics (Pieston et al., 2011; Yahav et al., 2004), suggesting limited exposure to elevated temperatures may prepare offspring for specific post-hatch environmental challenges.

To explore the effects of variation in incubation temperature on offspring stress endocrinology we incubated domestic chicken eggs (*Gallus domesticus*) at either the optimal incubation temperature (T_0) or at a slightly elevated incubation temperature (T_+) that was within the thermoneutral zone for chicken embryos (Webb, 1987). We also investigated effects of the timing of elevated incubation temperature on post-hatch stress endocrinology by exposing birds to the elevated incubation temperature either early (T_{+0}), late (T_{0+}), or throughout incubation (T_{++}). To assess the effects of our treatment on stress endocrinology post-hatch, we measured stress-induced GC release following:

(a) exposure to an acute stressor (naïve), and then
(b) following exposure to an identical acute stressor after a heat challenge (post-heat challenge).

This design allowed us to determine (a) whether the timing of exposure to elevated incubation temperature affects stress-induced GC release post hatch (naïve), and (b) whether the magnitude of stress in the postnatal environment can manifest effects of prenatal manipulation on postnatal stress endocrinology (naïve compared to post-heat challenge). Furthermore, the choice of postnatal heat challenge was important because it attempted to directly link elevated temperatures experienced in the prenatal environment to a heat-challenge encountered in the postnatal environment. Our experiment therefore tested three primary hypotheses:

(1) Small elevations in incubation temperature alter postnatal stress-induced GC release.
(2) The timing of exposure to elevated incubation temperatures (early versus late prenatal development) differentially affects postnatal stress-induced GC release.
(3) The presence and timing of elevated incubation temperature differentially primes individuals to cope with variable and repeated stressors post-hatch through modulation of GC release.

While our specific choice of temperatures and study species are novel, hypothesis (1) is based on prior work reporting that altered incubation temperature in oviparous species can affect stress-induced corticosterone (DuRant et al., 2010, 2012). In addition, the elevated incubation temperature falls within the thermoneutral zone for chicken embryos (Webb, 1987), and therefore did not constitute a heat stress.

Hypothesis (2) follows findings that the timing of exposure to prenatal stressors can be just as important to altering offspring phenotype as the type of prenatal stress. While these timing effects have been clearly demonstrated in the steroid maternal effects literature (Kapoor et al., 2006; Smith and Waddel, 2000; Welberg et al., 2001), to our knowledge, they have yet to be tested for altered incubation temperature. We limited elevated incubation temperature exposure in one treatment group (T_{+0}) to the period of hypothalamic development (d0–5), while exposing another treatment group (T_{0+}) only during hypothalamic activation (d6–18; Jenkins and Porter, 2004). We exposed our third treatment group (T_{++}) to the slightly elevated incubation temperatures throughout development (d0–18). We were therefore able to diagnose timing-dependent effects of elevated incubation temperatures on post-natal manipulations in addition to additive effects.

Hypothesis (3) follows two lines of evidence. First, studies from the steroid maternal effects literature suggest that embryonic exposure to high maternal GCs can produce variation in HPA axis sensitivity that may affect fitness in ways that are contingent upon the postnatal environment (Sheriff and Love, 2013). Second, prenatal exposure to elevated incubation temperatures alters GCs and improves survival in birds post-hatch by conferring thermotolerance (Pieston et al., 2011; Yahav et al., 2004). Taken together, this research suggests that birds may respond to stressors differently depending on variation in the abiotic prenatal environment. By measuring stress-induced GC release following a heat challenge, we can determine whether birds exposed to elevated, but not stressful, prenatal incubation temperatures differentially alter GC reactivity following postnatal environmental challenges that are linked to the prenatal environment.

2. Materials and methods

2.1. Experimental design and rationale

To test our three hypotheses simultaneously, we designed a factorial experiment (Fig. 1) with two levels of incubation temperature (optimal [T_0], 37.5 ± 0.01 °C; and elevated [T_+], 38.6 ± 0.01 °C) and two periods of prenatal exposure (early, d0–5; and late, d5–18), resulting in three treatment groups (T_{++}, elevated incubation temperatures for the duration of incubation; T_{+0}, early elevated incubation temperatures only; T_{0+}, late elevated incubation temperatures only), and a control (T_{00}, optimal temperature for the duration of incubation). Optimal incubation temperature was defined as that which produces the highest hatch rate in domestic chickens (Lourens et al., 2005).

2.2. Hatching and care

All procedures were conducted under approval from the Bucknell University IACUC. Eighty Bovans brown domestic chicken (*Gallus domesticus*) eggs were obtained from Moyer's Chicks, Inc. (Quakertown, PA, USA), weighed, and randomly assigned to incubators set at the elevated (38.6 ± 0.01 °C) or optimal (37.5 ± 0.01 °C) temperature with approximately 52% humidity.

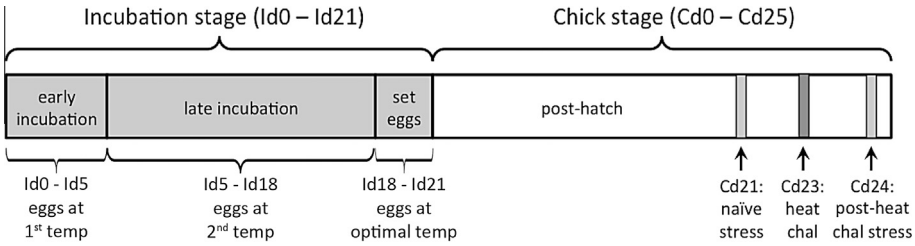

Fig. 1. Experimental timeline during the incubation and chick stage (incubation day, Id; chick day, Cd). Eggs were incubated at either an optimal incubation temperature (37.5 °C), or a slightly elevated incubation temperature (38.6 °C) between incubation day 0 and 5. At incubation day 5, eggs either switched between the two temperatures, or remained at the same temperature yielding four treatment groups (optimal temperature throughout, elevated temperature throughout, optimal temperature early-elevated temperature late, elevated temperature early-optimal temperature late). At incubation day 18, all eggs were placed at the optimal temperature to limit hatching mortality. On chick day 21, all birds were exposed to a capture-restraint stress to determine naïve reactivity to an acute stressor. On chick day 23, all birds were exposed to a heat challenge for 3 h. The following morning (chick day 24), all birds underwent a second acute restraint stress to determine stress reactivity post-heat challenge.

Mean egg mass did not differ among treatments ($F_{3,76} = 0.13$, $P = 0.942$). Two identical incubators (1550 Cabinet Model, GQF Manufacturing Co., Savannah, GA, USA) were used throughout the study, one at the elevated temperature, and the other at the optimal incubation temperature. On day 5, 40 eggs switched conditions from optimal to elevated temperature incubators or vice versa (T_{+0} and T_{0+} groups), while the remaining 40 continued incubation at the same temperature (T_{00} and T_{++} groups; Fig. 1).

We chose day 5 of incubation to change incubation temperature based on known milestones in the developmental pathway of the HPA axis. While chicken embryos show adrenal activity of 3-β-hydroxysteroid dehydrogenase (a key enzyme in the steroidogenesis pathway) by day 4, lipids and cholesterols are not present in the adrenal glands until day 5 (Jenkins and Porter, 2004). The hypothalamus and pituitary do not establish vascular connections required for exchange until day 6, and the hypothalamic-hypophyseal portal is not complete until day 12 (Jenkins and Porter, 2004). In concert, these milestones suggest that the HPA-axis is not functional until at least day 6 or later. By switching incubation temperatures prior to HPA-axis completion and functionality, we roughly isolated HPA axis formation (organization) from HPA axis activity (activation). Regardless of treatment, on day 18 all eggs were moved into a hatcher set to optimal conditions (37.5 ± 0.01 °C; H: 76.0 ± 1.4%) to limit mortality during hatching (Fig. 1). Similar precautions are common in heat-challenge literature (Loyau et al., 2012; Moraes et al., 2003).

All birds hatched within a 12 h window and upon hatch, chicks were culled at random to 9–11 birds per group, balanced for sex. Chicks were housed in mixed treatment and sex groups (Model CQB20 Brooder, Brower Equipment, Houghton, IA, USA) at 33 °C with 20 birds per brooder ($n = 5$ per treatment). Following standard chick husbandry protocols, the brooder temperature was reduced by 3 °C per week until it reached 25.5 °C. Brooders remained within ±1.67 °C of each other throughout the study. Chicks were provided Purina Game Bird Startena and water ad lib under 14:10 LD conditions.

2.3. Morphometrics

Following hatch, birds were wing-banded, weighed, and sexed using primary feather growth. Throughout the duration of the experiment, masses and tarsus length were measured from all individuals. Feather sexing was validated by comb sexing at 4 weeks of age.

2.4. Acute stress exposure and sample collection

On day 21, to determine naïve reactivity to an acute stressor, we used the well-established capture-restraint protocol (Wingfield et al., 1992) and measured plasma corticosterone, the primary GC in birds (Fig. 1). Briefly, baseline plasma corticosterone sample was taken within 3 min of entering each brooder. The birds were placed in a cotton bag for 20 min, followed by a second plasma sample ($t = 20$ min; peak). Following the second blood sample, birds were returned to their brooder to recover. A third blood sample was taken at 45 min ($t = 45$ min; recovery, <3 min) following the initial brooder entry. Whole blood was collected from the alar vein using 27G needles and immediately placed on ice. Blood was centrifuged and plasma was stored at −20 °C until corticosterone analysis.

To determine how elevated incubation temperature impacts how animals modulate the acute stress response following a heat challenge all birds were exposed to a heat challenge on day 23 post-hatch (Fig. 1). During the heat challenge, birds were exposed to a high temperature (35–37.7 °C) for 3 consecutive hours (Hao et al., 2012). These temperatures are well-above that of the thermoneutral zone for 3-week old chicks (29–33 °C; Meltzer et al., 1982), and all birds exhibited behavioral signs of heat stress including gular fluttering and postural changes. This temperature range and period of exposure induces the highest expression of heat shock protein production in chicken tissues (Hao et al., 2012), suggesting that the heat challenge elicits a physiological response while not risking mortality due to heat exposure. The following morning (day 24), chicks underwent a second acute restraint stress (post-heat challenge), identical to the one performed on day 21 (Fig. 1). Both acute restraint stresses were performed at the onset of the photophase. Therefore, all birds in the study were exposed to identical stress regimes post-hatch, experiencing both the same number and order of stressors.

2.5. Corticosterone assay

Blood samples were assayed using commercially available corticosterone EIA kits (Corticosterone ELISA Kit, ENZO Life Sciences Inc., Farmingdale, NY, USA). The assay was validated in our lab prior to use by recovering spiked corticosterone in plasma samples. We optimized the assay based on plasma dilution and % Steroid Displacement Reagent (SDR) following Wada et al. (2007). In addition, the EIA was cross-validated with a standard RIA for corticosterone (Wingfield et al., 1992) of which the specifics are discussed in Haussmann et al. (2012).

In brief, room-temperature plasma was prepared with 1 part SDR to 25 parts plasma and diluted 1:4 in assay buffer. Small-volume samples were run at 1:7, 1:7.5, and 1:15 dilutions, as volume allowed. Samples were run in duplicate as plasma volume allowed. Pooled plasma and pooled spiked plasma were run in duplicate on each plate as inter-assay controls. Sex, treatment, and brooder were distributed equally across plates. Steroid concentrations were calculated using a standard curve that ranged from 32 to 20,000 pg mL^{-1}. Inter- and intra-assay coefficient of variation were

Table 1
Mixed effects model of log-transformed corticosterone release following an acute stressor prior to and following a heat challenge. Stress series refers to naïve or post-heat challenge stress series, which occurred 3 days apart. Time refers to the time point within a single stress series (t = 0 min, baseline; t = 20 min, peak; t = 45 min, recovery). Treatment groups differed in exposure to slightly elevated incubation temperature during development.

Effect	DF_{Num}	DF_{Den}	F ratio	p-Value
Brooder	1	29.73	0.20	0.65
Sex	1	29.75	2.19	0.15
Treatment	3	29.84	0.63	0.60
Stress series	1	142.7	15.49	0.0001
Time	2	142.4	52.21	<0.0001
Date × time	2	142.6	0.31	0.73
Treatment × time	6	142.4	1.31	0.25
Treatment × date	3	142.6	2.44	0.07
Treatment × date × time	6	142.5	2.32	0.03

9.6% and 4.5%, respectively, as determined by running standards in each assay.

2.6. Statistical analysis

All statistical analyses were performed in JMP Pro 10.0 (SAS Institute Inc., 2012). Egg mass across treatment groups was assessed using an ANOVA with egg mass as the response and treatment as a categorical predictor. Differences in hatch rates were assessed using Fisher's exact test of independence to account for small counts in several columns. Differences in mass at hatch between treatment groups were assessed using a one-way ANCOVA including egg mass as a covariate.

To model growth rates, we used a REML Mixed Model to predict log-transformed mass from days 2 through 23. Predictors included age (continuous variable), sex, and treatment group. ID and brooder level were included as random effects. Peig and Green's scaled mass was calculated and modeled in an identical model to assess differences in body condition (Peig and Green, 2009). However, there were no differences in body condition or change in body condition among groups (data not shown).

We modeled log-transformed plasma corticosterone in a mixed effects model with brooder, sex, treatment, time, and date as effects, along with all possible interactions between treatment, time and date (Table 1). ID was included as a random effect. We then used a one-way ANOVA to assess differences in peak corticosterone release among groups within a single stress series (naïve, post-heat challenge). We used matched-pair t-tests to specifically assess changes in corticosterone release within groups because we were interested in only a few comparisons within the significant effect of the three-way interaction (see Table 1). Corticosterone was normalized using log-transformation prior to matched-pairs analyses. All values are expressed as mean ± s.e.m.

3. Results

3.1. Hatch success and early growth

Mean hatch rate among all groups was 87 ± 3%, and there were no differences in hatch rates among groups, with all embryo death occurring late in development and at low rates (Fisher's Test for Exact Differences; P_{table} = 0.011, p = 0.649). While egg mass was strongly correlated with mass of chicks at hatch ($F_{1,63}$ = 85.59, p < 0.001), mean hatch weight did not differ among treatments ($F_{1,64}$ = 0.21, p = 0.646).

There were no significant differences among groups in overall mass or growth rate prior to the heat challenge (d0–d23) post-hatch (treatment: $F_{3,34}$ = 1.69, p = 0.19; treatment × age: $F_{3,312}$ = 1.43, p = 0.23), nor in the week following the heat challenge (d23–d29) (treatment: $F_{3,28}$ = 1.51, p = 0.39; treatment × age: $F_{3,3,103}$ = 0.15, p = 0.93).

3.2. Acute stress response

In response to the naïve stress, all birds exposed to the elevated incubation temperature (T_+) significantly elevated circulating corticosterone (T_{++}: t_5 = 2.70, p = 0.02; T_{+0}: t_8 = 2.93, p = 0.01; T_{0+}: t_6 = 1.96, p = 0.05), while the control (T_{00}) birds neared significance (T_{00}: t_4 = 1.73, p = 0.08). There were no differences among groups for baseline, peak, or recovery corticosterone levels during the naïve stress (p > 0.25 for all; Fig. 2).

However, when comparing the naïve and the post-heat challenge stress there were significant differences in corticosterone release between the naïve and post-heat challenge stress series, among sampling times (baseline, peak, recovery), among treatment groups, and multiple interactions of these predictors (Table 1). In comparison to peak corticosterone levels during the naïve stress series, peak levels in the post-heat challenge stress series were higher in T_{00} birds (t_8 = 2.51, p = 0.018), but did not increase in any of the T_+ birds (Fig. 2). However, the specific response of the T_+ birds depended on the timing of elevated incubation temperature, and birds exposed to elevated incubation temperature early had significantly lower peak corticosterone compared to their naïve stress series (T_{++}: t_8 = −4.23, p = 0.001; T_{+0}: t_8 = −3.03, p = 0.008), while birds exposed to elevated incubation temperatures only during late development had unchanged peak corticosterone compared to their naïve stress series (T_{0+}: t_8 = 0.04, p = 0.97; Fig. 2). In addition, in the post-heat challenge stress series, peak corticosterone did not differ from baseline corticosterone in birds exposed to the elevated incubation temperature throughout development (T_{++}) (t_7 = 0.136, p = 0.89), though it did in all other groups (T_{+0}: t_5 = 2.67, p = 0.02; T_{0+}: t_5 = 2.22; p = 0.04; T_{00}: t_6 = 3.73, p = 0.005). Regardless of treatment group, all birds significantly reduced their circulating corticosterone between the peak and recovery sample during the post-heat challenge stress series (T_{++}: t_8 = −2.77, p = 0.01; T_{+0}: t_8 = −2.81, p = 0.01; T_{0+}: t_8 = −5.12, p = 0.0005; T_{00}: t_8 = −6.54, p < 0.0001), suggesting they all exhibited negative feedback (Fig. 2).

4. Discussion

4.1. Summary

Our results suggest that exposure to slightly elevated incubation temperatures within the thermoneutral zone differentially program the HPA axis in ways that affect an individual's GC release during acute stressors later in life. Following the post-heat challenge stress series, all treatment birds inhibited the increase in corticosterone release that control birds exhibited, however birds with exposure early in development differed from birds that were exposed only during late development. While we cannot determine whether these changes are the result of exposure to the heat challenge specifically or to repeated acute stressors, these results suggest that both the timing of exposure and absolute exposure to elevated incubation temperature shape the pattern of GC release, and that the effects are not simply additive.

4.2. Effects of early versus late exposure

Our results suggest that the HPA axis is programmed differently depending on the timing of exposure to elevated incubation temperatures. These results are consistent with prenatal steroid hormone exposure research in which the effects of prenatal stress

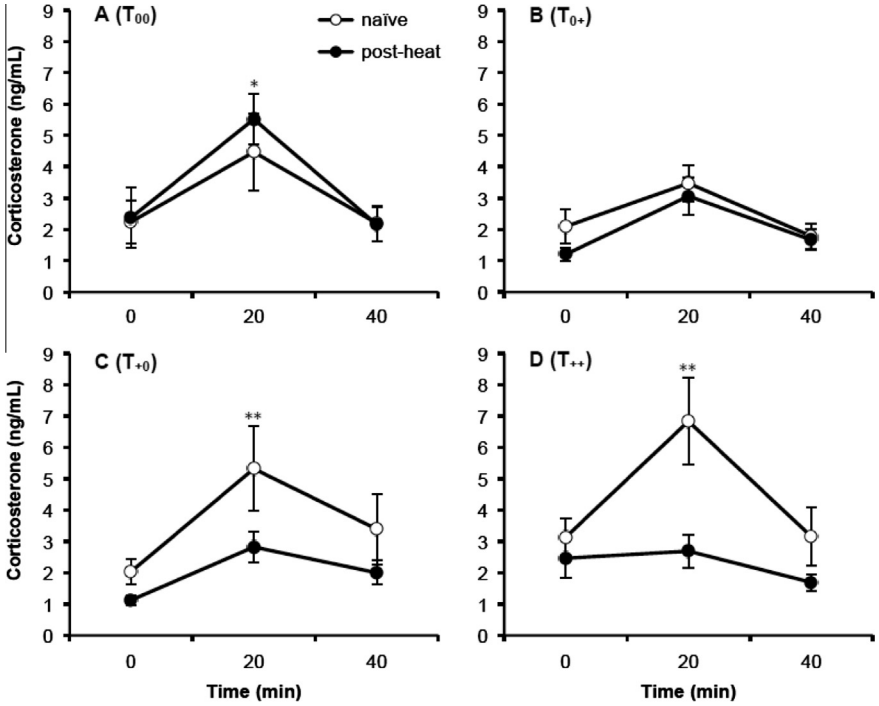

Fig. 2. Corticosterone release following an acute stressor. Plasma corticosterone was measured at baseline (t = 0 min, prior to the stressor), during the stressor (t = 20 min following initial exposure), and during recovery (t = 45 min) in birds experiencing (A) optimal temperatures for the duration of incubation, T_{00}; (B) late elevated incubation temperatures only, T_{0+}; (C) early elevated incubation temperatures only, T_{+0}; and (D) elevated incubation temperatures for the duration of incubation (T_{++}). Within in each group, corticosterone was measured during a naïve stress (open circles) and during the post- heat challenge stress (closed circles). There were no differences in corticosterone release among treatments prior to the heat challenge (naïve stress series; $p > 0.05$). In comparison to peak corticosterone levels during the naïve stress series, peak levels in the post-heat challenge stress series were higher in control birds (T_{00}), unchanged in birds exposed to elevated incubation temperatures only during late development (T_{0+}), and lower in the birds exposed to elevated incubation temperatures early in development (T_{++} and T_{+0}; N = 9 per treatment group). $^{*}p < 0.05$; $^{**}p < 0.01$ compared to naïve stress series within treatment group and sampling time.

on the pattern of GC release are specific to the embryonic developmental stage at which individuals are exposed (Kapoor et al., 2006).

Because birds exposed to the elevated incubation temperature throughout development exhibited corticosterone release almost identical to that of birds incubated at the elevated temperature only during early development, we argue that exposure to elevated incubation temperatures in early development is physiologically more potent than exposure in late development, These effects could reflect that early exposure modifies the organization of hypothalamus (Jenkins and Porter, 2004). In accordance with this, effects of prenatal steroid hormone exposure on GC release in mammals appears to be dependent not only on the timing of hormone exposure, but also on the duration and magnitude of exposure (Kapoor et al., 2006). Our findings suggest that timing crucially controls how a prenatal disturbance shapes offspring phenotype. These results also highlight the complex nature of prenatal HPA programming.

Interestingly, birds incubated throughout development at the elevated temperature (T_{++}) did not exhibit any change in corticosterone release relative to their baseline in the post-heat challenge acute stress series. In comparison, both treatment groups that switched incubation temperatures (T_{+0} and T_{0+}) did exhibit an increase between baseline and peak corticosterone levels following the heat challenge (albeit a reduced increase to peak corticosterone as compared to their naïve stress response). This suggests that the effects of early and late incubation temperature exposure on the pattern of stress-induced GC release are complex and are not simply additive in combination. Future work needs to explore how the timing, duration, and magnitude of altered incubation temperature can affect programing of the HPA axis, specifically in order to better understand how timing-dependent effects interact with one another.

4.3. Dampened and attenuated corticosterone release

In our experiment, we noted two cases in which birds exhibited low peak corticosterone release in response to acute stressors: control birds showed dampened corticosterone release following the naïve stress, while a subset of treated birds (T_{+0} and T_{++}) attenuated their corticosterone release following the post-heat challenge stress. In the case of the former, it is common for mammals and birds to show a stress hyporesponsiveness period (SHRP) during the first weeks following birth or hatch, which is hallmarked by a reduction in stress-induced GC release (Levine, 1994; Wada et al., 2007; Quillfeldt et al., 2009). Thus, it is possible that the birds incubated at the optimal temperature (T_{00}) in our study were still in the SHRP during the naïve stress series. In contrast, birds exposed to elevated incubation temperatures (T_{+}) showed a more typical stress-induced rise in corticosterone during the naïve stress. Other studies have reported that heat exposure during development decreases incubation time without affecting hatchling mass or size, suggesting that increased incubation temperatures can accelerate development (DuRant et al., 2010; Hepp and Kennamer, 2012). Therefore, the T_{+} birds may have experienced a similar acceleration in development that led to a more mature pattern of GC release during the naïve stress.

However, accelerated development of the HPA axis in the T_{+} birds does not explain the later attenuation of corticosterone release during the post-heat challenge stress, and we offer a few non-mutually exclusive interpretations. Attenuated corticosterone release in T_{+} birds could be an adaptive change in the pattern of GC

release related to energy conservation or habituation, or it could be a result of dysregulation or adrenal failure. Attributing adaptive or maladaptive potential to the attenuated corticosterone release is difficult because birds were maintained in conditions with ad lib resources and relative fitness was not quantitatively evaluated. To better assess adaptive potential of altered corticosterone release would require that birds be monitored long-term and/or placed in an environment in which fitness, through reproductive success or survival, could be assessed. However, we outline here three possible interpretations based on our results to help guide future work.

Attenuation of corticosterone release in response to acute stress could reduce energy expenditure or allow individuals to store energy, permitting treated birds to reactively shunt energy toward physiological recovery from the heat challenge or in response to the repeated acute stressors. Birds can dampen corticosterone release in preparation for energetically demanding events, such as migration (Long and Holberton, 2005). However, treated birds did not differ in weight gain from control birds following the heat challenge, so if energy shunting did occur, it did not result in increased growth rates compared to control birds. However, it is possible that other physiological parameters not measured here were affected. Alternatively, treated birds could be exhibiting attenuated corticosterone release as a result of habituation. While uncommon, single-exposure habituation to acute stressors such as capture-restraint has been shown in free-living Eastern bluebirds (*Sialia sialis*; Lynn et al., 2010). However, it is important to note that individual responses to repeated, non-chronic, restraint stressors over a short period are highly repeatable in several bird species, including chicken (Littin and Cockrem, 2001; Cockrem et al., 2009).

Lastly, the decrease in peak corticosterone levels may be indicative of an inability to respond to the stressor or dysregulation resulting in failure to release corticosterone (Cyr and Romero, 2009). Adrenal exhaustion, which results in the inability to maintain typical baseline GCs or to release GCs in response to stressors, can result from exposure to chronic stress (Rich and Romero, 2005). However, treated birds maintained baseline corticosterone levels comparable to controls, suggesting that treated birds were not exhibiting adrenal exhaustion. Because we see a significant decrease in corticosterone between peak and recovery samples, it suggests that the HPA axis was still responding to negative feedback signals upstream of corticosterone. If dysregulation is occurring, dexamethasone challenges or comparable techniques would help to pinpoint where. It is also worth noting that dysregulation could alter the temporal dynamics of corticosterone release, whereby birds reach peak corticosterone release more slowly or quickly than typical birds; however, we cannot diagnose such changes from our data.

4.4. Ecological implications

Our study uses elevated incubation temperatures within the thermoneutral zone of chicken embryos, and thus represents conservative expectations of variation in the environment a developing chick may experience. Our findings are in accordance with others that have shown that exposure to small alterations in incubation temperature has significant effects on offspring phenotype and, potentially, fitness (DuRant et al., 2010; DuRant et al. 2013; Hepp and Kennamer, 2012). Additionally, our results point to the importance of timing on the resulting phenotype, suggesting that altered environmental parameters do not necessarily need to be maintained throughout incubation to have important effects. Short heat waves or cold snaps could result in behavioral positive-feedback in which parents spend more time off of the nest foraging to counteract increased energetic expenditure related to their own thermoregulation and that of their egg. Increased time off the nest would further increase exposure of eggs to suboptimal temperatures and thus increase the likelihood that offspring exhibit altered phenotypes.

This work utilizes a domestic species and a constant-temperature incubation regime. To better and more rigorously investigate the ecological implications of these findings requires understanding the extent to which these phenotypes arise in wild species and under more natural incubation patterns. Future studies can build on these findings by using natural systems and incubation regimes to determine the extent to which these phenotypes can impact offspring viability in free-living systems. In addition, environmental context combined with long-term monitoring of individuals, will help inform our understanding about the fitness consequences of altered corticosterone release. Changes in GC release are likely to affect individual fitness because GCs are important for appropriately responding to the environment. Experimentation with birds in different post-hatch environments (increased predation pressure, increased ambient temperature, chronic psychological stressors) will help us to better understand how environmental exposure during incubation can alter phenotype in ways that may confer fitness advantages or costs.

4.5. Conclusions

The differences in the pattern of corticosterone release exhibited by birds exposed to elevated incubation temperatures are a novel demonstration of how environmental variation within the thermoneutral zone during development can alter modulation of GC release. These differences occurred after a series of postnatal acute stresses. While we cannot determine whether it was the number of stressors, type of stressors, or some combination of the two that resulted in the altered GC patterns of release, it is important to emphasize that these differences were revealed only after birds were exposed to several varied acute stressors. This suggests that it might take multiple challenges before the effects of an altered prenatal environmental translate into an altered phenotype. More importantly, our findings underscore the importance of considering both timing and type of exposure when investigating maternal and environmental effects on offspring phenotype.

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