BRIEF COMMUNICATION

Ecologically Relevant Cooling Early in Life Alters Prefledging Adrenocortical Response in Free-Living Songbirds*

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ABSTRACT

In vertebrates, exposure to stressful stimuli early in development may alter the activity of the hypothalamo-pituitary-adrenal (HPA) axis, with the potential for fitness consequences later in life. For altricial species, whose young rely on their parents for food, warmth, and protection from predators, adult behavior can modify the impact of some stressors on their offspring after birth or hatching. We have shown that single bouts of cooling that normally occur when brooding females leave the nest elevate corticosterone secretion in very young free-living eastern bluebird (*Sialia sialis*) chicks. Thus, natural variation in maternal brooding patterns can result in differential exposure of offspring to cooling, and also to glucocorticoids, very early in development. We tested the hypothesis that exposure to repeated bouts of cooling (mimicking those that occur normally when females leave the nest) would alter the activity of the chicks' HPA axis later in life. We exposed free-living chicks to either four 18-min bouts of cooling or brooding temperatures (control) during the first week after hatching. Then, just before fledging (i.e., at least 7 d after the cooling treatments had ceased), we assessed their corticosterone responses to restraint. Repeatedly cooled chicks had a significantly lower corticosterone response to restraint than did control chicks but did not differ from controls in other measures of growth and development. Our data suggest that natural variation in maternal brooding patterns, and hence natural variation in the chicks' body temperature, can alter the activity of the HPA axis well beyond the brooding period.

Keywords: glucocorticoid, hypothalamo-pituitary-adrenal axis, temperature, altricial, postnatal stress, eastern bluebird (*Sialia sialis*), maternal programming.

Introduction

The activity of the hypothalamo-pituitary-adrenal (HPA) axis, an important mediator of the vertebrate response to unpredictable and noxious stimuli, can be both initiated and modified by stressors encountered early in life. For example, blood levels of glucocorticoids, a primary output of this axis, often rise substantially after young mammals and birds are exposed to stressful stimuli (Hennessy and Weinberg 1990; Pravosudov and Kitaysky 2006; Rensel et al. 2010; Schoech et al. 2011; Lynn and Kern 2014). Moreover, if exposure occurs early in development, the glucocorticoids released at that time can alter the HPA axis itself later in life (Rots et al. 1996; van Oers et al. 1997, 1998; Spencer et al. 2009; Banerjee et al. 2012; ter Wolbeek et al. 2015).

Because an individual's glucocorticoid response to environmental stimuli may have important fitness consequences (Blas et al. 2007; Breuner et al. 2008; Bonier et al. 2009), the potential impacts of early-life events that can modify HPA function have garnered a great deal of attention. Much of this attention has focused on prenatal stress (Henriksen et al. 2011; Love et al. 2013). However, postnatal environments are also characterized by an array of stressors that can directly affect developing offspring after birth or hatching. This is particularly true for altricial young, which complete much of their development after birth or hatching and are hence highly dependent on one or both parents for food, warmth, and protection. Consequently, parenting behaviors may exacerbate or minimize exposure of young to stressors throughout postnatal development.

In altricial young, access to parental care clearly influences development of the HPA axis. For example, periods of maternal separation have profound and lasting impacts on HPA function in both mammals and birds (Rots et al. 1996; van Oers et al. 1997, 1998; Rentesi et al. 2010; Banerjee et al. 2012), and recent studies have demonstrated that even subtle variation in parental care can affect the HPA activity of offspring. A well-studied example of the latter occurs in rodents, where levels of maternal licking and grooming, as well as arched-back nursing, modify the glucocorticoid secretion of pups in response to stressors later in life (Liu et al. 1997; Meaney 2001). In birds, reducing

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the provisioning rate or the food intake of chicks, as well as maternal absence from the nest, elevated the corticosterone secretion of the nestlings in an immediate time frame and also altered their physiology, morphology, and behavior later in life (Kitaysky et al. 2006; Pravosudov and Kitaysky 2006; Sears and Hatch 2008; Rensel et al. 2010; Schoech et al. 2011).

We have recently shown that brief decreases in body temperature of less than 10°C (which commonly occur when females leave the nest) elevate the corticosterone levels in young eastern bluebirds (*Sialia sialis*) at a younger age than does handling (a processive stressor; Lynn et al. 2013; Lynn and Kern 2014). This suggests that cooling is an ecologically relevant, systemic stressor to which chicks are commonly exposed in the field. In addition, chicks in some nests are more likely to experience repeated, cooling-induced elevations of corticosterone than those in others, because maternal attentiveness during brooding varies in free-living bluebirds (Lynn and Kern 2014). We suggest that natural variation in maternal brooding behavior may hence generate variation in the exposure of chicks to glucocorticoids during development. Such variation may thereby modify a bird's ability to respond to noxious stimuli later in life.

Here, we describe a study in which we exposed very young, free-living eastern bluebird chicks to repeated bouts of cooling in order to produce changes in nestling temperature similar to those that occur naturally when females are away from the nest (Lynn and Kern 2014). Then, shortly before the chicks fledged, we compared their corticosterone response to restraint (a novel stressor) with that of control chicks. We hypothesized that such brief, repeated cooling episodes early in life would alter the chicks' later responses to a novel stressor.

Methods

Birds and Study Area

We studied eastern bluebird chicks in 15 nests in Wayne (40° 45′N, 81°W) and Ashland (40°55′N, 82°W) Counties, Ohio, during June–August 2014. We monitored nest boxes with sufficient regularity to predict hatch date, which was confirmed whenever possible by visiting the nest box on the expected day. We also verified the nestlings' ages by comparing their morphological measurements to previously established growth curves for body mass (±0.01 g), wing chord (±0.5 mm), and tarsus (±0.1 mm) length that were developed for nestlings in this population (see Lynn and Kern 2014 for details).

Nestling Temperature Treatments

To investigate how repeated bouts of cooling affected later HPA responses to restraint, we exposed chicks to one of two temperature treatments, following methods described in detail in Lynn and Kern (2014). Hereafter, these temperature treatments are referred to as "Cooled" and "Control." In the Cooled treatment, the chick was placed in a weighing boat within a 7.6-L-capacity cooler (Coleman) containing two ice blocks, one on each side of the weighing boat. In the Control treatment, which maintained chicks at brooding temperatures (Lynn and Kern 2014), the nestling was placed in a weighing boat within a 5.7-L-capacity cooler (Igloo) near a single activated hand warmer (HeatMax). Both chambers were set up 40–60 min before testing to ensure that temperatures within them had equilibrated at the time of testing. We have shown that the equilibrium temperatures of chambers in this design were as follows: Cooled: 9.08° ± 0.04°C, Control: 23.3° ± 0.06°C (Lynn and Kern 2014).

To control for the possibility that nestlings within a brood might show similar responses to temperature treatments, we used a balanced design in which two chicks of the same age were randomly selected from each brood, with one being assigned to the Cooled treatment and the other to the Control treatment for the duration of the study. Individual toenails were clipped on the chicks so that we could identify them on repeated visits to the nest. On the days that temperature treatments were administered, both chicks were removed from the nest and placed in their designated chambers at the same time. They remained in their chambers, and were thus exposed to their assigned temperature treatments, for 18 min. This 18-min exposure is shorter than the average cooling-bout length that occurs when females leave the nest, but it is well within the range of naturally occurring cooling bouts in this study population (Lynn and Kern 2014). Chicks were exposed to these assigned temperatures on each of 4 days, beginning on the day of hatching (day 0) or the following day (day 1). Temperature treatments for individual chicks occurred 24–48 h apart, and in all cases the treatments were completed on or before day 7 after hatching. We have established that eastern bluebird chicks do not exhibit a corticosterone response to restraint until they are 9 d of age, nor does handling at this age alter later responses to restraint (Lynn et al. 2013). After the final temperature treatment, the chicks were not handled again until their corticosterone responses to restraint were assessed at 14 or 15 d of age (see below). On each day of treatment, we measured the surface temperature of the chicks, using an infrared thermometer (Cole Parmer, model 39755–00, Vernon Hills, IL), just before they were put in their assigned chambers and again after 18 min of exposure.

Corticosterone Responses to Restraint at 14–15 Days after Hatching

When the chicks were 14–15 d of age (i.e., just before fledging), we measured their blood levels of corticosterone in response to a 1-h period of restraint. We again used a paired design in which nestmates that had received the Cooled and Control treatments earlier were tested on the same day and were hence the same age. We have established, in previous work with this study population, that the corticosterone response of chicks to restraint does not differ between days 12 and 15 after hatching (Lynn et al. 2013; S. E. Lynn and M. M. Phillips, unpublished data). Chicks that had been subjected to the Cooled and Control treatments during the first third of the nestling phase were

removed from the nest at the same time, and a first blood sample (<50 μL) was collected within 3 min. Subsequent blood samples were collected from each chick 15, 30, and 60 min later. The young birds were kept in individual cloth bags between sampling times. After the final sample was collected, the chicks were weighed (±0.01 g) with a digital microbalance and then returned to the nest.

Blood samples were obtained by puncturing a wing vein with a 26-gauge hypodermic needle and collecting blood in heparinized microhematocrit capillary tubes, which were kept on ice until we returned to the laboratory. At this time, we centrifuged the samples, determined the hematocrit of the initial (time < 3 min) samples, and then removed the plasma and stored it at −20°C until it was assayed for corticosterone. All work was conducted within the guidelines of federal and local banding permits and the College of Wooster's Institutional Animal Care and Use Committee.

Corticosterone Assay

Corticosterone levels in the plasma were determined with enzyme immunoassay kits (Enzo Life Sciences, Ann Arbor, MI) that we optimized for eastern bluebird plasma (for a detailed description of the optimization procedure, see Lynn et al. 2007, Wada et al. 2007, and Lynn and Porter 2008). Before each assay, plasma was diluted 1 : 40 with assay buffer. We assayed 100 μL of diluted plasma in triplicate. Samples were assayed for corticosterone across a total of seven 96-well plates. Samples from both chicks in a given nest (Cooled or Control) were always analyzed on the same plates, but samples were randomized within a plate.

Corticosterone standards of known concentration were assayed on each plate. When a sample's concentration was below the limits of detectability on a given plate, we assigned the lowest measured corticosterone value for the plate. We calculated intra-assay variability as the average percent coefficient of variation (%CV) among replicates for each assay and interassay variability as %CV among multiple measurements of a corticosterone standard of known concentration that was run in each assay. Intra-assay variability averaged 6.78% and interassay variability 7.10%. Cross reactivity of the corticosterone antibody was as follows: testosterone: 0.13%, progesterone: 1.7%, and estradiol: <0.03% (Enzo Life Sciences).

Data Analysis

All data met the assumptions of normality for parametric testing. For corticosterone responses to restraint, we generated an integrated measure of corticosterone secretion over time by calculating the area under the curve generated by graphing corticosterone versus time (Breuner et al. 1999; Cockrem and Silverin 2002). We then compared these integrated corticosterone values between Cooled and Control chicks, using a paired *t*-test. In addition, because changes in baseline corticosterone can be related to chronic stress (Rich and Romero 2005; Cyr and Romero 2007), we also used a paired *t*-test to determine whether the baseline corticosterone levels of the chicks differed between treatments. To avoid the potential for type I error due to baseline corticosterone levels being incorporated into two different analyses, we conservatively set our alpha at 0.025 for all tests.

To confirm our previous findings that an 18-min exposure in the Cooled chamber reduced the chicks' surface temperatures (T_s) whereas exposure in the Control chamber did not, we calculated the difference between the initial and final T_s's of each chick for each of its four temperature exposures. We then calculated an average change in T_s for each chick and compared these values between Cooled and Control chicks, using a paired *t*-test. We also used paired *t*-tests to compare body mass and hematocrit of the two groups on the day of blood sampling. For all analyses, final sample sizes reflect samples collected from chicks in 15 broods, with the exception of hematocrit, for which we obtained data from chicks in only 14 broods. Values in this article are means ± 1 SEM.

Results

Cooling produced drops in the T_s's of very young chicks relative to controls: the average change in temperature for the Control chicks was −0.998° ± 0.284°C, whereas that of the Cooled chicks was −12.471° ± 0.255°C ($t = -32.104$, df = 14, $P < 0.001$; fig. 1). The T_s's of the nestlings in this study were within the range of those that had elevated corticosterone secretion in eastern bluebird chicks previously and that occurred naturally at undisturbed nests in our study population (Lynn and Kern 2014). The integrated corticosterone secretion in response to restraint on days 14–15 was also lower in the Cooled chicks than in the Control ones ($t = 2.774$, df = 14, $P = 0.015$; fig. 2), but baseline corticosterone levels were similar between the two groups (Control: 3.33 ± 0.72 ng/mL, Cooled: 2.59 ± 0.55 ng/mL; $t = -0.892$, df = 14, $P = 0.387$).

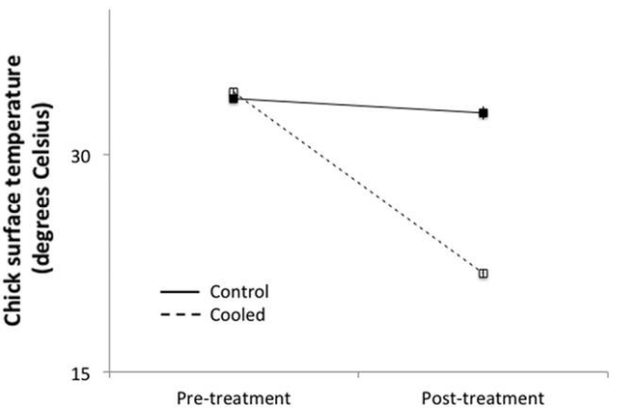

Figure 1. Surface temperature (T_s) of eastern bluebird chicks before and after 18-min exposure to temperature chambers. Cooled chicks had a significantly greater drop in T_s over the course of temperature treatments than did Control chicks ($P < 0.001$).

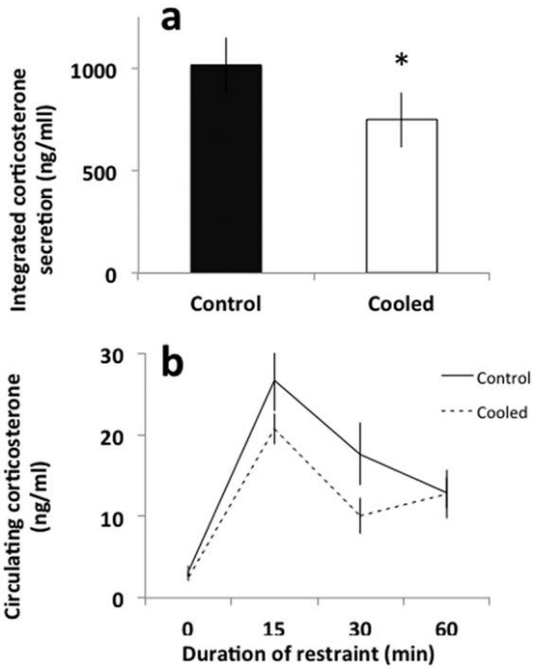

Figure 2. Corticosterone responses to 1 h of restraint in eastern bluebird chicks were lower on day 14–15 after hatching for chicks that had been exposed to four cooling episodes early in life (Cooled) than for chicks that had been held at brooding temperatures for the same time periods (Control). *a*, Integrated corticosterone response over 1 h of restraint; $^*P < 0.05$. *b*, Average corticosterone values at <3, 15, 30, and 60 min after initiation of restraint. Values shown are means ± SEM.

Cooled and Control chicks did not differ in body mass (Control: 27.04 ± 0.36 g, Cooled: 27.71 ± 0.47 g; $t = -1.632$, df = 14, $P = 0.125$) or hematocrit (Control: 37.84% ± 2.2%, Cooled: 38.40% ± 2.7%; $t = 0.477$, df = 13, $P = 0.641$) at the time of blood sampling.

Discussion

We found that brief, repeated bouts of ecologically relevant cooling significantly reduced the corticosterone response of wild eastern bluebird chicks to a novel stressor at the time of fledging. This effect occurred despite there being no differences in baseline corticosterone, body mass, or hematocrit between Cooled and Control chicks, suggesting that the repeatedly cooled chicks were neither chronically stressed (Cyr and Romero 2007) nor growing at slower-than-normal rates. Our study did not specifically address whether the pace of development of the HPA axis was in fact slower in Cooled chicks than in Control chicks; however, this is a likely explanation for our results, given that the timing of cold exposure coincided with early development of the HPA axis (Lynn et al. 2013).

One potential mechanism explaining the attenuated corticosterone response to handling in Cooled chicks may involve the stress hormone corticosterone itself. We have previously shown that a single cooling episode of the type to which the chicks were exposed in this study is sufficient to elevate blood levels of corticosterone when the nestlings are only 5 d old. We also know that such cooling increases the corticosterone levels of chicks as young as 3 d of age (S. E. Lynn and M. D. Kern, unpublished data). The repeated cooling treatments in this study may therefore have produced several spikes in corticosterone secretion and in this manner may have affected the development of the HPA axis.

When very young bluebird chicks are subjected to cold, they are true poikilotherms, with no means of compensating for drops in body temperature: they cool at rates that are dependent on ambient temperature, and they do not shiver (Lynn and Kern 2014). Given their poikilothermic nature, the increase in corticosterone when chicks are cooled is somewhat surprising, although such increases are not without precedent in poikilothermic vertebrates (Chen et al. 2002; Dupoué et al. 2013). Thus, corticosterone secretion may be less sensitive to thermal conditions than other physiological processes, or cooling-induced elevations in corticosterone may reflect the temperature dependence of corticosteroid-binding globulin activity or enzymes associated with metabolic clearance of the hormone (Dupoué et al. 2013). Regardless of the mechanism, the repeated elevations of corticosterone that accompany repeated bouts of cooling may affect the function of the HPA axis later in life. For example, prior work demonstrates that multiple aspects of physiology and behavior, including development of the HPA axis, are sensitive to early, periodic corticosterone exposure in birds (Schoech et al. 2011; Love et al. 2013). However, the effects of such exposure on the subsequent function of the HPA axis are not always the same. In some species (e.g., zebra finches *Taeniopygia guttata* and chickens *Gallus domesticus*), corticosterone exposure increased the activity of the HPA axis later in life (Spencer et al. 2009; Haussmann et al. 2011), whereas in others (e.g., Japanese quail *Coturnix japonica*, European starlings *Sturnus vulgaris*, and eastern bluebirds), later HPA activity was attenuated (Hayward et al. 2006; Love and Williams 2008; Zimmer et al. 2013; this study). These findings undoubtedly reflect differences in experimental design and developmental time lines as well as species-specific differences. But collectively, they demonstrate that the development of the HPA axis is sensitive to glucocorticoid exposure early in life and thus that glucocorticoids represent a potential mechanism for developmental programming of the stress axis (reviewed in Love et al. 2013).

A second potential mechanism explaining our results is that the cooling itself may have directly altered development of the HPA axis. This has been found, for example, in wood ducks *Aix sponsa* and chickens *G. domesticus* (species that produce precocial chicks), by varying the temperature to which eggs are exposed before hatching (DuRant et al. 2010; Wilsterman et al. 2015). If temperature can effectively alter the development of the HPA axis in precocial species when applied during incubation, it may also affect altricial species when applied after hatching, when the chicks are at similar stages of development. However, further research comparing the effects of temperature fluctuations during incubation in precocial species and after hatching in altricial species is necessary to confirm this.

Regardless of whether the attenuation of HPA responsiveness that we report here was mediated indirectly by cooling-induced elevations of corticosterone, directly by cooling itself, by a com-

bination of direct and indirect effects, or by some other mechanism, we have documented developmental alterations in the stress axis that result from early-life experiences mimicking conditions to which free-living birds are exposed in this population (Lynn and Kern 2014). Several previous studies have shown that the effects of early-life experiences on the activity of the HPA axis can be long lasting (Rots et al. 1996; Liu et al. 1997; van Oers et al. 1997, 1998; Meaney 2001; Spencer et al. 2009; Rentesi et al. 2010; Banerjee et al. 2012) and may represent adaptive responses that prepare individuals to cope with stressful environments later in life (reviewed in Love et al. 2013). We do not know whether the effects we report here are transient or whether they persist throughout the birds' lives, since we are not able to follow our birds after they fledge. Future work on the persistence of cooling-induced effects on HPA function after fledging, and their potential consequences on fitness, is needed.

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