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Research paper

Time as tyrant: The minute, hour and day make a difference for corticosterone concentrations in wild nestlings

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ABSTRACT

The hypothalamic-pituitary-adrenal (HPA) axis has been studied extensively in adults, but the HPA axis in early life is not well characterized, and there is an immense amount of unexplained variation in glucocorticoid levels during early life, especially in wild animals. To characterize population-wide natural variation in early-life HPA axis function, we compared plasma corticosterone levels (at baseline and after 30 min acute restraint-stress) from seven-day-old nestlings (n = 123) from a free-living, marked population of Savannah sparrows (*Passerculus sandwichensis*). We found a surprising sensitivity of the HPA axis to timing of sample collection across time scales. Even within the accepted 3-min framework to collect baseline samples, time to collect blood had a significant effect on baseline corticosterone concentrations. Daily rhythms also influenced baseline levels, which increased significantly during the relatively short window of sample collection (1100 and 1600). On a broader timeframe, there was a strong effect of hatch date (over a 2 month period) on HPA axis responsiveness, where nestlings hatched later in the breeding season had lower stress-induced corticosterone levels than those hatched earlier. The ecophysiological mechanisms and implications of these patterns warrant future investigation; meanwhile this study highlights the critical need to consider, and potentially restrict, time across scales when collecting blood samples from wild birds to assess stress physiology.

1. Introduction

"'Repent, Harlequin!' said the Ticktockman. 'Get stuffed' the Harlequin replied sneering." – Harlan Ellison

Animals interact with the environment through a variety of physiological mechanisms that ultimately affect their survival and reproductive performance (Wingfield, 2005). One such mechanism, the hypothalamic-pituitary-adrenal (HPA) axis, is highly conserved across vertebrate taxa and, among other functions, regulates circulating glucocorticoid levels (Sapolsky et al., 2000). Experiences and conditions during early-life are known to have a lasting impact on HPA axis function (Monaghan and Haussmann, 2015; Schoech et al., 2011) and a key component of understanding these effects is being able to measure hormones indicative of HPA axis function, such as corticosterone, at an early age. The few studies that have measured corticosterone in nestling birds have reported an immense amount of inter-individual variability, even among nest-mates that experience similar developmental environments (Blas et al., 2007; Cockrem and Silverin, 2002; Evans et al., 2006; Pakkala et al., 2016; Rensel et al., 2011). However, before we attempt to link early-life HPA axis function to ecological and life-history traits, we must appreciate and accommodate the crucial influence of the most fundamental elements of research protocols.

Research protocols for studies on avian stress physiology typically account for two fundamental aspects of short-term timing. First, while there is some suggestion that the response is species or season specific, the widely-accepted timeframe to collect blood samples that represent "baseline" conditions, before plasma corticosterone levels rise in response to a stressor, is within 3 min of contact/disturbance (e.g. Cyr and Romero, 2007; Ouyang et al., 2011; but see: Baugh et al., 2013; Romero and Reed, 2005 and Small et al., 2017). Of the hundreds of studies that have followed the 3 min convention, over 80% have been conducted in adults, and many are conducted in the laboratory on captive individuals. Secondly, many studies set a restricted window of time during the day in which to collect blood samples for corticosterone analysis to reduce diel effects (e.g. 10:00–14:00 Rensel et al., 2011; 9:30–11:30 Schmidt et al., 2014; 11:00–15:00 Spencer et al., 2009). Presence of daily rhythms in plasma corticosterone concentrations across vertebrates has been well described, and in many adult birds, corticosterone levels peak just prior to the morning

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active period, within an hour of dawn (e.g. Breuner et al. (1999), and decline throughout the morning. Importantly, in two separate studies on captive adult North American passerine species, both Breuner et al. (1999) and Romero and Remage-Healy (2000) demonstrate that baseline corticosterone levels are not affected by time of day on a shorter timescale between late morning and late afternoon. However, there is a paucity of data around daily rhythms in early development.

In addition to short-term patterns, seasonal changes throughout the annual cycle in plasma corticosterone have been well documented, both at baseline and after a standard 30-min restraint stress (e.g. Landys et al., 2006; Newman and Soma, 2009; Romero et al., 1997). But, patterns within a season are variable across species and not well described save for a few studies that have tried to quantify within-season repeatability in adults (Ouyang et al., 2011), and an analysis of fluctuations in nestling corticosterone levels over time within a season has not been conducted.

Here, we characterized population-wide variation in early-life HPA axis function of 7-day old wild Savannah sparrow (*Passerculus sandwichensis*) nestlings to examine effects of temporal aspects of blood sample collection on corticosterone concentrations in a standard field protocol: 1) time to collect baseline sample (from 50 s to 3 min), 2) time of day (between 1100 and 1600 h, 3) day of sample collection (spanning ~2 months during the breeding season). By quantifying temporal effects on variation in corticosterone concentrations and subsequently refining research protocols to reduce intra and inter-individual variation in these endocrine measures, we can begin to more accurately illuminate the "eco-physiological blackbox" that is the relationship between the early-life environment and physiological development.

2. Methods

2.1. Study site and field protocol

From May 25th to August 1st 2015, we studied a marked population of wild, free-living, migratory Savannah sparrows on Kent Island, New Brunswick (44°35′N, 66°45′W), an isolated 80-ha island in the Bay of Fundy. The main study site measures ~10 ha and is divided by pathways into 50 × 50 m quadrats to facilitate mapping of territories and nest locations (Pakkala et al., 2016). We observed all breeding adults (n = 78) within the study area and used mist nets to capture any new individuals that were unmarked. All adults (>1 yr) are marked using a unique combination of three plastic colour leg bands and single aluminum leg band. This population has been monitored since 1987 (Wheelwright and Mauck, 1998).

All nests in the study site were located during laying or incubation and monitored for hatch date. Social parents were determined by shared territories, mate guarding, copulation, and confirmed by observing both the male and the female feeding the nestlings. All nests were monitored every second day to confirm timing of hatching. Eggs hatch over a 24–36 h period (Wheelwright and Rising, 2008), thus if on a monitoring day less than the full clutch had hatched, hatch day was assigned as that date, and if all eggs were found hatched, hatch day was assigned as the day prior (the intervening day between monitoring days). Dates were adjusted back one day if not all of the eggs eventually hatched. Seven days after hatching (d7: June 10-July 30; Julian date: 161–211), nestlings were fitted with leg bands (one colour band, one registered aluminum identification band) and morphological data (mass, tarsus length) were recorded. On d7, blood was collected (by two researchers) from up to three nestlings from each nest (n = 44 nests; n = 123 nestlings) between 1115 h and 1555 h. In accordance with the capture-restraint protocol, one blood sample (~50 μL) was collected from the brachial vein within three minutes of disturbing the nest to obtain a measure of baseline plasma corticosterone (Romero and Reed, 2005) and a second blood sample was collected after a 30 min restraint stress in a loose cotton bag. Banding and measurements were completed during the period of handling and restraint stress and completed for all nestlings within 10 min of initial nest disturbance (20 min prior to the second blood sample collection). Blood was extracted by brachial venipuncture using a sterile 26-gauge needle, drawn into a heparinized microhematocrit capillary tube and transferred to a microcentrifuge vial that was stored on ice in a cooler until returning to the lab. Samples were centrifuged at 10,000 rpm for 12 min, and plasma was stored at −20 °C until analysis.

2.2. Plasma corticosterone analysis

Baseline and stress-induced plasma corticosterone concentrations were quantified using a radioimmunoassay (ImmunoChem 07-120103; MP Biomedicals, Orangeburg, NY). This assay has been modified and validated for a number of songbird species (Newman et al., 2008; Washburn et al., 2002), including nestling Savannah sparrows (Pakkala et al., 2016). Following a validation with serially diluted plasma to confirm parallelism with the standard curve and optimal plasma volume per sample, 3–4 μL of nestling plasma were analyzed in duplicate. Samples (n = 5) that exceeded the maximum corticosterone detection limit were set at the maximum of 250 pg.

2.3. Statistical analysis

To characterize temporal effects on plasma corticosterone concentration in nestlings, we fitted a linear mixed-effects model (GLM) that included the effect of restraint stress as a factor (sample type: baseline vs. 30 min) and three continuous variables: 1) time (in seconds) to collect the baseline blood sample, 2) time of day for blood sample collection, and 3) day within the season for sample collection. Prior to analysis, we confirmed that none of the three continuous variables were correlated (time to collect baseline vs. time of day: $R^2 = 0.00$, $p = 0.94$; time to collect baseline vs. day in the season: $R^2 = 0.004$, $p = 0.52$; time of day vs. day in the season: $R^2 = 0.02$, $p = 0.10$).

The model also included interactions between time at each of the three scales and sample type. Continuous variables, including corticosterone concentrations, were standardized prior to analysis. To account for within-individual measurements before and after acute restraint stress, and common effects of parents and nest environment on nestlings raised in the same nest, the model included individual ID and nest ID as random effects. Significant effects from the GLM were subsequently examined using linear regressions on baseline and 30 min corticosterone with Nest ID included as a random effect.

Analyses were performed in JMP PRO (ver. 11.2, SAS, Cary, NC, USA). Any means are reported as (mean ± SE). To reduce heteroscedasticity, corticosterone concentrations were log transformed prior to statistical analyses. *P*-values were two-tailed and considered significant at $p \leq 0.05$.

3. Results

As expected, plasma corticosterone concentrations increased in response to a 30 min acute restraint stress in 7-day old Savannah sparrow nestlings (Table 1, Fig 1A). In addition, there was a significant effect of time of day on plasma corticosterone concentrations as well as significant interactions between i) the time required to