Research paper

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


Department of Integrative Biology, University of Guelph, Guelph, ON N1G 2W1, Canada

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.

© 2017 Elsevier Inc. All rights reserved.

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, 2005and 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
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 nesting 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 aluminium 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 dates). 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 aluminium 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 1535 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 nesting 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 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 ≤ 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...
collect the baseline blood sample and the effect of restraint stress, and ii) hatch date and the effect of restraint stress. Investigating these significant effects separately for baseline vs. 30 min samples, we revealed that baseline corticosterone concentrations were significantly affected by the amount of time (between 51 and 180 s, mean = 111 ± 40.5 s) required to collect the blood sample (Fig 1B: \( F_{1,111} = 24.46, p < 0.0001 \)) as well as the time of day (between 1115 and 1555) the blood sample was collected (Fig 1C: \( F_{1,111} = 7.33, p = 0.01 \)). In contrast, plasma corticosterone after 30 min acute restraint stress was not influenced by the time required to collect the initial baseline sample (\( F_{1,122} = 0.03, p = 0.85 \)), nor by the time of day the sample was collected (\( F_{1,122} = 1.22, p = 0.28 \)). We also confirmed that the order in which the nestlings were banded and measured did not influence corticosterone concentrations measured after the 30 min restraint (\( R^2 = 0.00, F_{1,122} = 0.04, p = 0.96 \)). However, plasma corticosterone concentrations collected after 30 min acute restraint stress significantly decreased over the course of the breeding period (hatch dates between June 3rd and July 23rd; Fig 1D: \( F_{1,122} = 7.24, p = 0.01 \)), but baseline corticosterone concentrations were not

### Table 1

Results from a GLM to explain plasma corticosterone concentrations in 7-day old nestling Savannah sparrows. “Nestling ID” and “Nest ID” were included as random effects, \( n = 123 \).

<table>
<thead>
<tr>
<th>Term</th>
<th>Beta Coefficient</th>
<th>Standard Error</th>
<th>95% CI</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.004</td>
<td>0.09</td>
<td>-0.19, 0.18</td>
<td>-0.05</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Baseline vs. 30 min</td>
<td>-0.35</td>
<td>0.05</td>
<td>-0.45, -0.25</td>
<td>-7.18</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Time to Collect Baseline</td>
<td>0.15</td>
<td>0.05</td>
<td>0.04, 0.26</td>
<td>2.72</td>
<td>0.008</td>
</tr>
<tr>
<td>Time of Day</td>
<td>0.22</td>
<td>0.09</td>
<td>0.02, 0.41</td>
<td>2.29</td>
<td>0.03</td>
</tr>
<tr>
<td>Hatch Date</td>
<td>-0.17</td>
<td>0.09</td>
<td>-0.36, 0.02</td>
<td>-1.85</td>
<td>0.07</td>
</tr>
<tr>
<td>Time to Collect Baseline × Baseline vs. 30 min</td>
<td>0.12</td>
<td>0.05</td>
<td>0.01, 0.22</td>
<td>2.22</td>
<td>0.03</td>
</tr>
<tr>
<td>Time of Day × Baseline vs. 30 min</td>
<td>0.007</td>
<td>0.05</td>
<td>-0.09, 0.10</td>
<td>0.14</td>
<td>0.89</td>
</tr>
<tr>
<td>Hatch Date × Baseline vs. 30 min</td>
<td>0.21</td>
<td>0.05</td>
<td>0.11, 0.31</td>
<td>4.28</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Percent of total variation explained by: 1) Nestling ID: 0% (Ratio of variance component: -0.09); 2) Nest ID: 34% (Ratio of variance component: 0.52).
affected by hatch date \((F_{1,111} = 0.43, p = 0.52)\). The seasonal change in 30 min corticosterone levels were not attributable to a seasonal change in nestling body condition (residuals of mass given tarsus length) as body condition did not change over the course of the breeding season \((R^2 = 0.00, F_{1,222} = 0.12, p = 0.73)\) and nestling body condition was not a significant factor in explaining plasma corticosterone concentrations \((F_{233} = 0.43, p = 0.52)\). Nor was parental age (second year as a breeder vs. after second year: experienced breeder) related to either nestling body condition \((F_{233} = 1.79, p = 0.15)\) or nestling corticosterone concentrations \((F_{233} = 0.96, p = 0.41)\). Across the population of nestlings, there was a weak but positive correlation between baseline and 30 min corticosterone concentrations \((R^2 = 0.11, F_{1,122} = 13.74, p = 0.0003)\).

4. Discussion

There is an immense amount of variation in stress physiology among young animals, particularly in wild individuals. Even in young that are experimentally exposed to identical stressors, there remains impressive individual variation in corticosterone levels (e.g. Pakkala et al., 2016). Using a comprehensive population-wide study, our results uncover important aspects of standard field protocols when collecting blood samples for plasma corticosterone analysis in nestlings. By fine-tuning field protocols, a substantial amount of among-individual variation may be reduced and true ecophysiological linkages revealed.

4.1. Time to collect baseline

There is a well-entrenched acceptance of the 3 min timeframe to collect baseline corticosterone samples in birds, before plasma corticosterone levels rise in response to a stressor (Cyr and Romero, 2007; Romero and Reed, 2005; Ouyang et al., 2011). We extend these results to include wild, free-living nestlings, and found evidence of surprising sensitivity in plasma corticosterone concentration to the time (within 3 min) that it took to collect a baseline blood sample (Fig 1B). Though mean baseline corticosterone levels were significantly lower than stress-induced levels, there was substantial variation among individuals in the time required to collect a baseline blood sample and the longer sample collection times were associated with higher corticosterone concentrations. These data are similar to Rensel et al. (2010) who also note a trend of increasing plasma corticosterone levels with the time required to collect a baseline sample (in under 3 min) from nestling scrub jays. Restricting sample collection to shorter time frames (e.g. 120 s as suggested by Romero and Reed, 2005), and including the precise time required for collection in statistical models, may yield more accurate inter-individual comparisons among baseline corticosterone levels. This is particularly important when the research questions focus on baseline glucocorticoid levels and inter-individual variation, or when the effect size is predicted to be small (e.g. in altricial young when the response to acute stress is not as pronounced as in adults). These restrictions may not be as crucial when working with large sample sizes or with adults when the variation in baseline glucocorticoid concentrations is relatively small. The rapid response to acute stress may represent a species-specific pattern for Savannah sparrows, or a more general pattern where HPA axis activation may occur more rapidly in nestlings than in adults. Evidence from fetal sheep demonstrates marked developmental changes in adrenal sensitivity throughout the perinatal period (Torres-Farfan et al., 2008), and while it is known that altricial birds develop rapidly from a state where the HPA axis is hypo-responsive to acute stress (Wada et al., 2007), whether there are fluctuations in the time course for HPA axis activation over development in birds is unknown. Interestingly, the rates of increase in glucocorticoid levels may also depend on the personality and stress phenotype of each individual (Baugh et al., 2013; Small et al., 2017) and whether these are established early in life is also an interesting question for future research. Regardless, these results highlight the importance of methodology optimized not only for each species, but also the age of the individual.

4.2. Time of day

Daily patterns in glucocorticoids are well described among vertebrates, and it has been suggested that, at least for adult passerines, plasma concentrations peak at arousal (near-dawn), decline for several hours thereafter, and are stable throughout the day from morning to late afternoon (Breuner et al., 1999; Romero and Remage-Healy, 2000). It is worth noting that these studies were conducted on wild-caught birds in captivity, and that captivity can alter the diel rhythms of endocrine patterns (Romero and Wingfield, 1999), but Schwabl et al. (2016) report similar patterns in several species of wild tropical forest birds. Like most field studies, we restricted blood sample collection to a relatively small window (between 1115 and 1600), but nevertheless found that baseline corticosterone in nestlings increased over the sample collection timeframe (Fig 1C). If the developing HPA axis is more finely tuned to acute activation during stress and to circadian rhythms during the day, perhaps due to an age-specific adrenal action of melatonin during early development (e.g. Torres-Farfan et al., 2008), this suggests that a narrower timeframe within each day is required for nestling sample collection to ensure meaningful comparison of plasma corticosterone levels among individuals in a population. Future studies focused on early-life plasma corticosterone must closely consider the acute timeframes of sample collection and not merely rely on methodology standards set for adults.

4.3. Seasonality of HPA axis responsivity

Beyond the variation in corticosterone levels within relatively small-scale timeframes of seconds to hours, there was also a significant effect of hatch date on 30 min stress-induced corticosterone concentrations in 7-day old nestlings over the course of the breeding season. To our knowledge, this study provides the first evidence of a within-season decline of early-life HPA axis responsiveness. Nestlings hatched later in the season had significantly lower 30 min corticosterone levels (Fig 1D), despite having similar baseline levels to their earlier hatched peers. The seasonal decrease in 30 min acute stress-induced corticosterone levels may reflect parental physiology (i.e. maternal effects), a shift in the temporal activation of the HPA axis, or a suite of ecological variables that are shaping the developing stress axis, all ecophysiological possibilities that require further investigation. One particularly obvious candidate for future studies is the influence of maternal glucocorticoids. While we did not measure parental corticosterone concentrations, it is known that maternal glucocorticoids can be transferred to the egg in birds (e.g. Almasi et al., 2012) and be associated with offspring physiology in a range of taxa (e.g. Dantzler et al., 2013; Hayward and Wingfield, 2004; Liu et al., 2001; Sopinka et al., 2017). Whether the dynamics of maternal glucocorticoid regulation, and subsequent offspring programming, shift over the course of the breeding season is not yet known. Brood size and hatchability remain constant over the breeding season, however, in this population, Savannah sparrows can raise two broods within a season if the first is successful (Woodworth et al., in press). In this study brood number and Julian date are highly correlated as second broods, by definition, are hatched later in the season, however understanding the relationship of previous
reproductive effort on offspring stress physiology will be interesting. Meanwhile, it is crucial to note that for researchers interested in studying the effects of experimental treatments or ecological variables on corticosterone in free-living nestlings, using a smaller subset of individuals hatched during a relatively short window in the breeding season, may yield more reliable comparisons.

4.4. Conclusions

To conclude, this is the first population-wide study to tease apart the multi-scale temporal effects of standard field protocols that influence the measurement and interpretation of plasma corticosterone from nesting birds. We found significant effects of time, scaling up from the number of seconds required to collect a baseline blood sample to the day of hatch across the breeding season. Hedging the tyranny of time and restricting sample collection timeframes across these scales is not without challenges, collecting samples from multiple individuals within a nest within 120 s requires several skilled people to collect blood simultaneously, although accessing nests during a truncated circadian timeframe within a day may be more straightforward. Finally, increasing the sample size of focal nests within a narrow number of days during the breeding season may balance the effect of hatch date on stress physiology. Overall, these slight modifications may reduce imposed variation among individuals and yield more accurate comparative results in a range of physiological and ecological studies.

Acknowledgments

We thank Ines Moran for assistance in the field and Mark Murray, Damon Gannon and Russell Ingalls for logistical support. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Undergraduate Student Research Award to HH and by NSERC Discovery grants to AEMN and DRN. This is contribution number 260 from the Bowdoin Scientific Station.

References