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Are baseline and short-term corticosterone stress responses in free-living amphibians repeatable?

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ABSTRACT

Amphibians respond to environmental stressors by secreting corticosterone, a stress hormone which promotes physiological and behavioral responses. Capture handling can be used to stimulate physiological stress response in amphibians. The use of single blood sampling and presentation of mean data often limits the quantification of within and between individual variation in baseline and short-term corticosterone stress responses in amphibians. It is important for studies of amphibian physiological ecology to determine whether baseline and short-term corticosterone stress responses are consistent or not. We quantified repeatability (r), a statistical measure of consistency, in baseline and short-term corticosterone stress responses to a standard capture and handling stress protocol in free-living adult male cane toads (*Rhinella marina*). Corticosterone metabolite concentrations were measured entirely non-invasively in male toad urine samples via an enzyme-immunoassay. During the first sampling occasion, urine samples were collected manually from individual male toads ($n = 20$) immediately upon field capture. Toads were handled for 5 min then transferred to plastic bags (constituting a mild stressor), and urine samples were collected hourly over 8 h in the field. The toads were resampled for baseline (0 h) urine corticosterone with hourly urine sampling over 8 h (for quantification of the stress induced corticosterone) at 14 day intervals on three consecutive occasions. Within and between sample variations in urinary corticosterone metabolite concentrations were also quantified. All toads expressed a corticosterone stress response over 8 h to our standard capture and handling stress protocol. Variations both within and between toads was higher for corrected integrated corticosterone concentrations than corticosterone concentrations at baseline, 3 or 6 h. Baseline urinary corticosterone metabolite concentration of the male toads was highly repeatable ($r = 0.877$) together with high statistical repeatabilities for 3 h ($r = 0.695$), 6 h ($r = 0.428$) and 8 h ($r = 0.775$) corticosterone metabolite concentrations, and for the total and corrected integrated corticosterone responses ($r = 0.807$; $r = 0.743$ respectively). This study highlights that baseline and short-term corticosterone stress responses are repeatable in free-living amphibians. Future studies should utilize this non-invasive tool to explore repeatability among seasons and across years, and determine its functional significance in relation to behavioral ecology and reproduction in amphibians generally.

1. Introduction

Amphibians, like other animals, face challenges in their daily lives such as predation, weather variations and disease (Pounds et al., 2006; Cockrem, 2007). The hypothalamo-pituitary interrenal (HPI) axis, a major part of the neuroendocrine system, controls amphibian physiological responses to physical and psychological stressors. Corticosterone is the main stress hormone in amphibians. The non-invasive measurement of urinary corticosterone metabolites (mainly conjugated end-products of corticosterone metabolism) via enzyme-immunoassay (EIA) and radio-immunoassay (RIA) are valuable tools for amphibian conservation physiology and stress endocrinology research (Narayan et al., 2010b; Narayan et al., 2011c). Plasma corticosterone concentrations increase within minutes in response to short-term stressors, stimuli that activate the HPI axis (Cockrem, 2007). This increase in corticosterone is known as a corticosterone stress response, which adjusts the body's physiological and behavioral processes, such as through metabolic actions by increasing blood glucose levels and behavioral effects, such as increasing foraging activity and other necessary actions that help the animal to respond to and protect itself from the stressor (Hull et al., 2007). Earlier studies of corticosterone stress responses in amphibians used blood plasma samples and presented data as means and standard errors (Orchinik et al., 1988; Coddington and Cree, 1995; Homan et al., 2003), which also required large sample sizes for each mean data point.

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Repeated blood sampling in amphibians via more invasive techniques, such as aortic puncture, is also regarded as unethical (Narayan et al., 2010b). Such studies did not consider within individual variation in corticosterone concentrations hence it is important now to determine the amount of variation in baseline and short-term corticosterone stress responses within and between individual free-living amphibians. Most importantly, the studies on individual variation and repeatability in stress hormone responses will not just to advance empirical knowledge such as relating the findings to environmental factors, individual behaviors and reproductive condition, but understanding their functional relevance with respect to the fitness and survival of the target species.

Variation within (intra) and between (inter) individuals can be quantified by calculating coefficients of variation (CVs) for corticosterone concentrations and integrated corticosterone responses (Littin and Cockrem, 2001; Cockrem and Silverin, 2002b; Cockrem et al., 2009; Narayan et al., 2012c). Consistency in glucocorticoid parameters (baseline and stress-induced levels of corticosterone) over time can be quantified using the established concept of statistical repeatability [*r*] (Bennett, 1987; Cockrem et al., 2009; Narayan et al., 2012c). Individual variation in baseline and short-term urinary corticosterone responses were recently demonstrated using cane toads (*Rhinella marina*) as model species under controlled laboratory conditions (Narayan et al., 2011c, 2012c). Variation in baseline and short-term urinary corticosterone responses was also recently demonstrated in two closely related free-living amphibian species of the *Platymantis* genus (Narayan et al., 2012b). Studies in other vertebrate groups such as birds (Wada et al., 2008; Littin and Cockrem, 2001; Cockrem and Silverin, 2002b; Cockrem et al., 2009; Rensel and Schoech, 2011), fish (Schjolden et al., 2005) and mammals (Guimont and Wynne-Edwards, 2006) have found consistent intra- and inter-individual variation in baseline and stress-induced plasma corticosterone. Several recent studies, especially in birds, have found support for the repeatability of corticosterone profiles within individuals (Beletsky et al., 1992; Vleck et al., 2000; Cockrem and Silverin, 2002b; Cockrem et al., 2009; Romero and Reed, 2008; Wada et al., 2008; Rensel and Schoech, 2011) again using plasma corticosterone measurements. While the majority of these studies were conducted under controlled laboratory conditions (see review by Ouyang et al. (2011)), a few recent studies have been conducted with free-living birds and reptiles. For example, corticosterone metabolites were measured non-invasively in faecal samples of graylag geese (*Anser anser*) in a free-living flock, which showed high repeatability across a roughly five-month span (Kralj-Fiser et al., 2007). Cockrem et al. (2009) demonstrated statistical repeatabilities in plasma corticosterone concentrations in free-living Adelie penguins (*Pygoscelis adeliae*) sampled on three occasions. Additionally, Rensel and Schoech (2011) showed that short-term corticosterone stress responses (assessed using plasma concentrations) were consistent in free-living Florida scrub-jay (*Aphelocoma coerulescens*) across life-history stages. Interestingly, there has been no report of repeatability in baseline and short-term corticosterone stress responses in free-living amphibians to date.

The aims of the present study were to describe and quantify variation in baseline and stress-induced urinary corticosterone metabolite concentration (referred to hereafter as urinary corticosterone) of adult male cane toads (*R. marina*) subjected to a standard capture and handling stressor under field conditions. We measured the repeatability of baseline urinary corticosterone and stress induced urinary corticosterone within individual male free-living cane toads on four repeated sampling occasions. We tested the null hypothesis that baseline and short-term corticosterone stress responses of adult free-living male cane toads to our standard capture and handling stressor will be statistically repeatable. The cane toad, an opportunistic breeder, is a large non-native terrestrial amphibian species abundant in some regions of Australia. Cane toads were introduced into Queensland, Australia in 1937 to control insect pests of sugarcane fields but they are now considered to be an invasive pest themselves (Shine, 2010). We selected adult male cane toads as a model species for this study because of their easy availability.

2. Materials and methods

2.1. Animals and urine sampling protocol

Baseline corticosterone and stress induced urinary corticosterone responses to our standard stressor (5 min manual handling and placement in plastic bags between hourly urine sampling) were measured over 8 h in free-living adult male cane toads ($n = 20$) on four consecutive occasions under field conditions. This sampling regime was selected based on our previous study (Narayan et al., 2012c) so that enough variation in urinary corticosterone with respect to standard capture and handling could be seen. The toads did not face dehydration or other adverse effects during sampling. Toads were captured from 1900 to 2100 h (on each sampling occasion), usually on wet nights (as this increased their activity and hence chances of re-capture) during the breeding season between August and September 2011 at the Parkwood International Golf Course in Southport, Queensland. Male toads were identified by the morphological characteristic of rough dorsal skin and categorized as mature adults according to Narayan et al. (2008), with mean (±S.E.) body mass (g) = 56.2 ± 2.50 g and snout vent length (mm) = 66.0 ± 2.31 mm. The first urine sample (0 h), representing baseline (also termed as "unstressed") urinary corticosterone was collected immediately upon capture of each toad in the field. Briefly, each toad was held above a 100 mm diameter sterile plastic cup and gently massaged to promote urination that usually occurred within 1 min, then held with both hands for 5 min before placing in a small resealable (Glad® snap-lock) measuring 15 cm × 15 cm. The resealable bags with the toads were placed individually in clear plastic containers (15 cm × 15 cm × 15 cm) and left on the ground for hourly urine collection (as part of the short-term stress response measurement).

For the short-term stress response measurement, same urine collection and stress protocol was repeated hourly for up to 8 h with placement in fresh bags after each sampling event. Our earlier studies on Fijian ground frogs (*Platymantis vitiana*) and cane toads have shown urinary corticosterone responses within 1 h after initial capture from the wild (Narayan et al., 2010b, 2011c). Afterwards, each toad was marked uniquely using the established amphibian marking technique (toe-clipping) and released *in-situ* (Hero, 1989). Urine samples were kept cold on ice packs in an eskey for up to 9 h before transfer to a freezer (−20 °C). Urine sampling on the following three occasions took place at intervals of 14 days at the same times each night as the first sampling occasion. The same individuals (or as many as could be re-captured) were resampled. This 14 day time-interval between repeated capture and urine sampling allowed the corticosterone metabolite to return to baseline before the next sampling occasion (Narayan et al., 2010b, 2011c).

2.2. Urinary corticosterone enzyme-immunoassay

An enzyme-immunoassay (EIA) was adapted from Narayan et al. (2010b)) to quantify corticosterone metabolite concentrations in toad urine. The corticosterone EIA was validated (both physiologically and under laboratory conditions) for toad urine in our previous studies (Narayan et al., 2011a, 2011b). Intra- (within) and inter- (between) assay coefficients of variation (CV) were determined from high- (~70%) and low- (~30%) internal binding controls run on all assays. Intra-assay CVs were 2.5% and 3.5%, for low- and high-percentage-bound controls respectively. Inter-assay CVs were 6.7% and 8.2% for low- and high-percentage-bound controls respectively. The overall assay sensitivity was calculated as the value 2 standard deviations from the mean response of the blank (zero binding) samples and was 1.12 ± 0.42 pg/well ($n = 40$). Urinary steroid metabolite concentrations were standardized to creatinine (Cr) levels to control for water content (Narayan et al., 2010a) and are reported as pg hormone metabolite/μg Cr. Creatinine