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Urinary corticosterone metabolite responses to capture and captivity in the cane toad (*Rhinella marina*)

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ABSTRACT

Urinary corticosterone metabolite responses to capture have recently been shown for the first time in amphibians, and in the present study urinary corticosterone metabolite responses to capture and to confinement in captivity were measured in adult cane toads (*Rhinella marina*) in Queensland, Australia. An adrenocorticotropic hormone (ACTH) challenge was used to provide a biological validation for urinary corticosterone metabolite concentrations measured by radioimmunoassay (RIA). Urinary corticosterone metabolite increased 1–2 days after ACTH but not saline injection and then returned to initial values, indicating that the RIA could detect changes in corticosterone secretion in toads. Urinary corticosterone metabolite responses to short-term capture and restraint in plastic bags were first apparent 2 h after capture of wild toads. Toads held communally in captivity for 5 days had elevated urinary corticosterone metabolite concentrations. Mean corticosterone concentrations declined significantly after a further 7 days in individual housing chambers. There was no sex difference in urinary corticosterone metabolite responses of toads to ACTH challenge, short-term capture or captivity. The relative amount of variation in the mean corticosterone responses was quantified by calculating coefficients of variation (CV) for each mean corticosterone response. Mean corticosterone at 0 min was more variable for captive toads than wild toads. Furthermore, initial corticosterone concentrations (0 min) were more variable than concentrations during the ACTH challenge, short-term capture and captivity. There was little change in the amount of variation of mean corticosterone levels between male and female toads with increasing time in captivity (12–29 days). This study has shown individual corticosterone responses of amphibians for the first-time, and has provided a novel method for quantifying the relative amount of variation in amphibian corticosterone responses.

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1. Introduction

Stress can be defined as a state when the hypothalamo–pituitary–adrenal axis is activated with increased secretion of glucocorticoids in response to a stressor [7]. In amphibians, the hormonal stress response begins with the release of corticotropin releasing hormone (CRH) from the hypothalamus, which acts on the anterior pituitary gland to stimulate the release of adrenocorticotropic hormone (ACTH) into the peripheral blood circulation. ACTH then stimulates the release of glucocorticoids (primarily corticosterone from the adrenocortical cells) with corticosterone causing physiological, metabolic and behavioural changes in response to the stressor [29].

Amphibians, like other vertebrates, have plasma corticosterone responses to capture and restraint [19,20,24,30]. Whilst reports of plasma corticosterone responses of amphibians have shown mean responses of groups of individuals, individual corticosterone responses may be quite different from a mean response. However, there are no reports of individual corticosterone responses in amphibians to date. The determination of the individual variation in amphibian corticosterone concentrations is thus needed to better understand patterns of corticosterone secretion in relation to fitness and to environmental variables [2,6,12,13].

Difficulties associated with sequential blood sampling after physical restraint or anesthesia limits the use of blood for the assessment of stress responses in amphibians [27]. Recently, we established methods to collect urine samples for the non-invasive measurement of corticosterone stress responses in the endangered Fijian ground frog (*Platymantis vitiana*) [27], thereby providing opportunities for more extensive studies of corticosterone responses in amphibians. We selected cane toads (*Rhinella marina*, previously *Bufo marinus*) [14], as our current study species because of their large size, abundance, easy handling and relatively inexpensive captive husbandry requirements. The aims of this study were to use an ACTH challenge to enable a biological validation

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of a radioimmunoassay for the measurement of urinary corticosterone metabolites in toad urine, and to characterise urinary corticosterone metabolite responses of individual toads to capture and to short-term captivity. Relative amounts of variation between individual animals were also quantified by the calculation of coefficients of variation in corticosterone responses for the first time in amphibians.

2. Materials and methods

2.1. Study species and husbandry

The cane toad is a large non-native terrestrial amphibian species found in high numbers in some regions of Australia. Cane toads were introduced into Queensland, Australia in 1937 to control insect pests of sugarcane fields. However, they are now an invasive species and are considered to be a pest. Wild toads were collected at night (1800–2200 h) in April 2010 from a park in Southport, Queensland under approvals from the Animal Ethics Committee (AEC) of Griffith University, Australia (ENV/08/10/AEC), and from the Queensland Government Environmental Protection Agency, Australia (scientific purposes permit WISP07106310). Toads were sexed using the criteria of [26]. Toads were transported to the laboratory and held in temperature-controlled room. Toads that were kept for more than 8 h were housed in sterilized plastic boxes with clear sides and meshed lids ($50 \times 30 \times 20$ cm for communal chambers and $15 \times 15 \times 10$ cm for individual/home containers). The toads were maintained at constant temperature conditions of 25 °C with 85% relative humidity, and the overhead fluorescent lights in the lab was kept off at night-time and kept on during day-time hours to mimic the natural photoperiod. The toads were fed with two commercially purchased house crickets (*Acheta domesticus*) per animal three times each week, and distilled water in a sterilized Petri dish was constantly available in each container. The containers were cleaned with 30% ethanol and distilled water after each urine sampling session to prevent any disease outbreak in captivity.

2.2. Study design

2.2.1. Biological validation

Biological validation of the urinary corticosterone metabolite radioimmunoassay (RIA) was achieved using an ACTH challenge in toads caught in the wild and housed for 14 days in individual containers. A saline challenge was used as a control for the ACTH challenge to verify that an increase urinary corticosterone metabolite concentration after the ACTH challenge was primarily due to activation of the HPA axis by the ACTH. A total of six toads of each sex were used for the ACTH challenge and six toads of each sex for the saline control treatments. ACTH (0.446 mg ACTH g^{-1} bodyweight (Sigma Chemical Co., A-0298) in 100 μL saline vehicle (0.9% NaCl) or saline solution (100 μL 0.9% NaCl) was administered at 1900 h to each toad by injection into the coelomic cavity (at the junction of the underbelly and thigh, away from the vital organs) using a 25-g needle. The injections were administered within 1 min from the time toads were removed from their home containers. Toads were returned immediately after injection to their home containers and remained undisturbed for 3 h. A urine sample was collected 3 h after the challenge, and further samples were collected at 1900 h on each of the next 5 days.

2.2.2. Corticosterone response to short-term capture and restraint

Wild adult toads ($n = 5$ male and 12 female) were captured on one night between 1800 and 2200 h. The environmental conditions were hot and dry and toads were caught outside their active breeding period. Toads were captured by hand as soon as they were located on the ground and a urine sample was collected immediately (0 h sample). For urine collection, a sterile 200 μL pipette tip was gently inserted (2 mm length) into the toad's cloaca to collect urine via capillary action. The urine sampling required handling of the animal for a maximum of 2 min. This minimally-invasive urine collection technique was adapted from our previous studies of urinary hormones in Fijian ground frogs [27,28]. The volume of urine collected from each toad varied from 0.01 to 3 mL. Each urine sample was then transferred into a labelled 1.5 mL polypropylene Eppendorf tube. After the initial 0 h urine sample was collected, each toad was placed inside a clean zip-lock bag and transported back to the laboratory. The lights in the laboratory were kept off during the night and were turned on at the time of dawn to mimic the natural photoperiod. A second urine sample was collected from each toad in the laboratory 2 h after initial capture, with light for the collection of the sample provided by a head torch. Afterwards, each toad was returned to its original zip-lock bag, and placed inside individual plastic containers. Subsequent urine samples were collected from each toad at hourly intervals up to 6 h in a similar fashion. Each toad was sexed and toe-clipped, following the methods of [25] and [29], for identification, and later released at the original field site after the laboratory sampling was completed. Urine samples were kept frozen in an ice box with dry ice (<0 °C) in the field and then kept in a −80 °C freezer until assayed.

2.2.3. Corticosterone response to short-term captivity

Adult male and adult female toads ($n = 11$ of each sex, different toads from those used in the short-term capture stress response study) were caught between 1900 and 2200 h three nights after the short-term capture and restraint study in hot and dry conditions. A urine sample was collected upon initial capture (0 h sample), in the same way as the first sample in the short-term capture stress response study. The toads were transported to a laboratory room and immediately transferred into large communal housing chambers, with one chamber for males and one for females. The lights in the laboratory room were turned on and off to mimic the natural photoperiod. A urine sample was collected from each toad after 5 days in the communal containers. Afterwards, each toad was transferred into an individual container that was used as a home chamber for another 24 days. Further urine samples were collected from each toad at 12, 19, 26, and 29 days after initial capture. All urine samples were collected between 1900 and 2200 h.

2.3. Corticosterone assay and creatinine measurements

Corticosterone concentrations in neat urine samples were measured by radioimmunoassay using the method of [8]. This assay has previously been used to measure corticosterone in rat urine [3,36] and hormonal values were presented as urinary corticosterone metabolites. Same terminology has been used in this study. 10 μL of urine sample was incubated for 2 h at room temperature (22–25 °C) with iodinated corticosterone and antiserum from an MP Biomedicals, USA, corticosterone radioimmunoassay kit. Precipitant solution (MP Biomedicals, USA) was added and each sample vortexed for 5 min, then 50 μL egg white (10 g/L dried egg white (Sigma) in PBSG) was added to increase adhesion of the pellet to the tube after centrifugation. The samples were incubated for 15 min at room temperature to separate bound and free corticosterone, then centrifuged for 15 min, the supernatant aspirated and the pellets were counted on a LKB Wallac 1261 Multigamma gamma counter. The sensitivity of the corticosterone assay was determined by the hormone concentration at the mean – 2 standard deviations from the zero hormone point on the standard curves. The assay sensitivity, expressed as pg corticosterone/ml urine,

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