



Urinary corticosterone responses to capture and toe-clipping in the cane toad (*Rhinella marina*) indicate that toe-clipping is a stressor for amphibians

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ABSTRACT

Toe-clipping, the removal of one or more toes, is a common method used to individually mark free-living animals. Whilst this method is widely used in studies of amphibians, the appropriateness of the method, and its potential detrimental effects have been the subject of debate. Here, we provide for the first time, evidence that toe-clipping is a stressor in a wild amphibian. We measured urinary corticosterone responses of male cane toads (*Rhinella marina*) to capture and handling only, and to toe-clipping under field conditions. Urinary testosterone concentrations and white blood cell proportions were also measured. Urinary corticosterone metabolite concentrations increased 6 h after capture and handling only and remained high for 24 h; corticosterone returned to baseline levels after 48 h and remained low at 72 h post capture and handling. Corticosterone concentrations in toads subjected to toe-clipping increased at 6 h to significantly higher concentrations than after capture and handling only, then decreased more slowly than after capture and handling, and were still elevated (approximately double basal level) 72 h after toe-clipping. Testosterone did not change significantly after capture and handling only, whereas after toe-clipping testosterone decreased at 6 h and remained low at 72 h. There were weak short-term effects of toe-clipping compared with capture and handling only on white blood cell proportions. We have clearly shown that toe-clipping is a distinctly stronger stressor than capture and handling alone. This indicates that there is an ethical cost of toe-clipping, and this should be considered when planning studies of amphibians.

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1. Introduction

Stress is a state in which the hypothalamo–pituitary–adrenal (HPA) axis is activated in response to a stressor resulting in increased secretion of glucocorticoids [5]. An animal's successful physiological response to stressful environmental factors or events, either social or physical, is important for maintaining its homeostatic balance. However, activation of the HPA axis may be associated with inhibition of the hypothalamo–pituitary–gonadal (HPG) axis and of growth [18]. Corticosterone is one of the major glucocorticoids produced by the inter-renal tissue of amphibians [25]. Earlier, we biologically validated a urinary corticosterone enzyme-immunoassay (EIAs) using adrenocorticotrophic hormone (ACTH) challenge in cane toads (*Rhinena marina*), and demonstrated “raise and return to baseline” profiles in urinary corticosterone levels [30]. One of the major drawbacks associated with

plasma glucocorticoid assessment is that levels of plasma corticosterone rise quickly following capture of wild animals [37], thus making it difficult to obtain baseline measurements in field situations. Corticosterone stress responses can now be readily measured in amphibians using non-invasive urine samples [27,30]. The measurement of leukocyte (white blood cell) counts in blood smears is also considered by some authors to be useful for the assessment of stress [8]. In vertebrates, there are five leukocyte types (lymphocytes, neutrophils (heterophils in birds and reptiles) eosinophils, basophils and monocytes), and the neutrophil to lymphocyte ratio (N–L) may reflect experimentally elevated plasma corticosterone levels in amphibians [8]. Recently, urinary corticosterone and leukocyte populations were compared in the endangered Fijian ground frog (*Platymantis vitiana*) after capture from the wild and transportation into an outdoor enclosure and both of these stress evaluation methods clearly demonstrated the physiological stress responses of frogs during the study [28].

Mark-recapture techniques are commonly used in animal ecology studies, and over the past decade the use of these techniques have become a hotly debated topic [11,12,21,33,34] as most marking techniques are considered to be invasive [32]. There are several

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techniques available for amphibian mark-recapture studies such as PIT-tagging, radio tracking (external and internal), pressurized fluorescent marking, toe-clipping, spooling, pattern mapping and visible implant tags [35]. Toe-clipping is one of the most commonly used amphibian marking techniques because it is cost effective and requires a minimal handling time in recaptures following marking. Toe-clipping involves removing one or more toes at the joint from the anterior and posterior limbs of anuran amphibians in a unique combination [15] that allows individuals to be recognised for repeated capture. This technique is often criticized as it is believed to affect foraging [9] and locomotion [31] and to reduce body-weight [9]. However, [2] found no effect of toe-clipping on the survival of metamorphs of western Palearctic water frogs. [32] showed that removal of multiple toes leads to reduction in return rates (recapture of marked individuals) by 4–11% for each toe removed after the first. The criticisms of toe-clipping, however, have been weighed against the knowledge gained about species ecology and conservation status from repeated and long term mark-recapture studies [11]. The relationships between urinary corticosterone and relative leukocyte numbers in amphibians with respect to toe-clipping have not been assessed. Furthermore, there have also been no studies of pain in amphibians in relation to toe-clipping.

The aim of the current study was to determine whether toe-clipping is a stressor in a wild amphibian species. We examined stress responses by determining changes through time in urinary corticosterone concentrations of adult male cane toads (*R. marina*) subjected to a standard short-term capture and handling stress protocol. Urinary corticosterone responses of toads that were captured and handled only were compared with responses of toads that were captured, handled and toe-clipped. We also investigated changes in urinary testosterone and WBC proportions in relation to corticosterone metabolite measures.

2. Materials and methods

2.1. Animals and sample collection

Adult male toads were collected from the Parkwood International Golf Course, Queensland. This open grassland habitat was generally a good site for toads and their approximate density during the period of this study was 20 adult toads per 100 m². The toads were located at night from 1800 to 2200 h by visual encounter with support of a head torch. Sampling was performed during a period of hot and dry nights in April 2010 when the toads were most likely outside their breeding period.

Toads were sexed based on the visual inspection criteria of [26], whereby adult male toads had an evenly distributed pattern of small round warts on the dorsal body surface and adult female toads had larger and unevenly distributed round warts on the dorsal surface. To obtain baseline corticosterone metabolite concentrations, we collected urine samples from individual male toads ($n = 90$) immediately upon capture in the field (captured and sampled at 0 h). Urine was collected using the protocols established earlier by [27,30]. Urine sampling was relatively simple since the frogs responded to gentle handling by a micturition reflex, which usually occurred within 30 s of initial handling. The volume of urine excreted by each frog varied from 50 μ L to 3 mL. Labelled urine samples were transferred into a container with dry ice in the field and kept frozen until transported to the laboratory where they were kept at -80°C (for 1 month) until laboratory analysis. Overall, three toad groups were organised from the toads ($n = 90$) that were sampled for baseline urine; (a) Control group ($n = 10$ toads) – captured, sampled and released with no subsequent sampling, (b) Handled only treatment group ($n = 40$ toads) – captured,

sampled, released then recaptured either 6, 24, 48 or 72 h later, (c) Toe-clipped treatment group ($n = 40$ toads) – captured, sampled, toe clipped, released then recaptured either 6, 24, 48 or 72 h later. We aimed to recapture 10 toads at each time point for each of the two treatment groups. However, not all of the toads could be found and recaptured, with the recapture percentage (%) being 60–80% at each time point. Hence, the sample size for post treatment urine and blood samples for 24, 48 and 72 h ranged from 6–8 toads for each group. Three toes (the most widely used n value for toe-clipping in amphibians) for toads in the toe-clipped group were clipped using unique codes [17]. Each toad in the control and handled only group was marked with a 'blob' on the dorsal surface with a non-absorptive correction pen for short-term identification. All toads were released into their original habitats immediately after sampling. The total time from initial capture to release was 3 to 5 min for both handled only and toe-clipped toad groups. Toads were usually recaptured within a distance of 50 m from their original capture site. Upon recapture, a second urine sample was immediately taken from each toad. Labelled urine samples were kept on ice packs in the field and later in the laboratory at -80°C until assayed.

For the haematological field sampling, single blood samples were collected from each control toad on the first night and from the handled only and toe-clipped treatment toad groups at the time point of re-capture at 6, 24, 48 or 72 h after treatment. Blood was collected immediately after urine sample collection. Blood was obtained in the field by cardiac puncture with a heparinized hypodermic needle. Bleeding procedures were completed in less than 1 min after hand capture, which was consistent across experimental groups. Two to three drops (20 μ L) of blood were immediately placed onto a clean microscopic slide and a blood smear was made using a second slide in the field. The smeared slides were cover-slipped and kept in a storage box at room (ambient) temperature until laboratory analysis.

2.2. Enzyme immunoassays

Urinary corticosterone enzyme-immunoassay (EIA) was adapted from [27] and urinary testosterone EIA was adapted from [29] to quantify corticosterone and testosterone hormonal metabolite concentrations in control, handled only and toe-clipped treatment toad groups. Urinary corticosterone metabolite concentrations in toad urine were determined using a polyclonal anti-corticosterone antiserum (CJM06, UC Davis California) diluted 1:45,000 horseradish peroxidase-conjugated corticosterone label diluted 1:120,000 and corticosterone standards ($1.56\text{--}400\text{ pgwell}^{-1}$). Urinary testosterone metabolite concentrations in toad urine were determined using a polyclonal anti-testosterone antiserum (R156/7, UC Davis California) diluted 1:25,000 horseradish peroxidase-conjugated testosterone label diluted 1:40,000 and testosterone standards ($0.78\text{--}200\text{ pgwell}^{-1}$). Cross reactivity of the CJM06 anti-corticosterone antiserum and R156/7 anti-testosterone antiserum were reported earlier in [27,29], respectively.

Laboratory validations of assays were achieved by demonstrating parallelism between serially diluted pooled toad urine samples and the respective standard curves, and by significant recovery of exogenous steroid standards added to toad urine samples. Recovery was expressed as a linear regression formula ($y = mx + b$, where y = amount of hormone observed, x = amount of hormone expected, and m = slope of the line) and the multiple correlation coefficient was squared to produce the coefficient of determination (r^2). Slopes greater or less than one represented an over- or under-estimation of hormone mass, respectively. Dilution rates for toad urine were based on the concentration of pooled samples that resulted in 50% binding on the parallelism curve, which was 1:2 for corticosterone and 1:4 for testosterone. Any sample that bound

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