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Highlights

• Patterns of serum and urinary CORT and T are comparable in amphibians. • Serum CORT increased between 0–0.5 h, reaching maximal levels at 6–8 h. • Urinary CORT increased with a lag-time of 1 h. • Serum T decreased between 0–7 h and increased between 7–8 h. • Urinary T decreased with a lag-time of 0.5 h, with no change between 4–8 h.

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General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen Changes in serum and urinary corticosterone and testosterone during short-term capture and handling in the cane toad (*Rhinella marina*)Edward J. Narayan^{a,*}, John Cockrem^b, Jean-Marc Hero^a^a Environmental Futures Centre, School of Environment, Griffith University, Gold Coast Campus, QLD 4222, Australia^b Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North, New Zealand

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ABSTRACT

Non-invasive endocrine monitoring with minimally invasive biological samples, such as urine, is being used widely for conservation biology research on amphibians. Currently, it is unknown how closely urinary measurements correspond with the traditional serum hormone measurements. We compared urinary and serum concentrations of corticosterone (CORT) and testosterone (T) in adult male cane toads (*Rhinella marina*) using a standard capture and handling (short-term stressor) protocol. Free-living male cane toads were captured and sampled for baseline urine (0 h) with a second urine sample taken at 0.5 h and hourly between 1 and 8 h. A single blood sample was collected from each toad after the final urine sampling and capture handling. The mean serum CORT concentration increased between 0 and 0.5 h, reaching the highest level between 6 and 8 h. The mean urinary CORT concentration increased with a lag-time of 1 h and continued to increase up to 8 h. The mean level of serum T decreased between 0 and 7 h and increased between 7 and 8 h. Mean urinary T concentration decreased with a lag-time of 0.5 h. Urinary T levels did not change between 4 and 8 h. Mean serum T levels reached 50% of the original 0 h value at 1 h while mean serum CORT levels reached 200% of the original 0 h value within 0.5 h. Mean urinary T levels reached 50% of the original 0 h value within 3 h while mean urinary CORT levels reached 200% of the original 0 h value within 3 h. The inter-individual variation in baseline serum and urinary CORT and T levels were highly comparable, suggesting that baseline urine sample provides a reliable indicator of the physiological status of the animal. Overall, the results have demonstrated that urine sampling and standard capture handling protocol provide reliable measures of baseline corticosterone and testosterone, as well as short-term stress hormone responses in amphibians.

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1. Introduction

Conservation physiology is a broad field of biological science that encompasses a number of physiological disciplines such as endocrinology, environmental and ecological physiology and neurophysiology in order to address current conservation problems and the consequences of conservation actions (Narayan, in press; Wikelski and Cooke, 2006). One of the most useful tools associated with conservation physiology is the measurement and monitoring of stress hormones in animals in response to environmental stressors. Non-invasive endocrinology is a key component of amphibian conservation physiology and minimally invasive methods have been used widely for measuring hormone metabolite concentrations to assess reproductive hormones and physiological stress in wild and captive amphibians with minimal disturbance (Narayan et al., 2013). Minimally invasive sampling techniques in amphibians

diverge from using traditional methods such as blood sampling and uses techniques associated with monitoring stress particularly with feces, urine or saliva (see latest review based on amphibian conservation physiology (Narayan et al., 2013)). The traditional means of serial blood collection is considered invasive, as it could generate a physiological stress hormone response during sampling and blood sampling procedures such as toe-clipping and cardiac puncture can lead to tissue damage (Deviche et al., 2001). The most commonly used minimally invasive means of monitoring stress in amphibians are with urine. Urine is easily acquired manually using a sterile plastic cup, micro-pipette or catheter tubing depending on the size of the species (Germano et al., 2009; Kindermann et al., 2012; Narayan et al., 2010a) and is far easier to obtain than feces, especially in free-living populations. There are numerous examples of urinary corticosterone and testosterone metabolites being monitored in accordance with variations in a capture handling stressor, exposure to multiple experimental stressors, pathogenic disease, altitudinal gradients and temperature treatment (Graham et al., in press; Kindermann et al., 2012; Narayan et al., 2012a, 2013, 2011b). Amphibians are a concern for the worldwide diversity

* Corresponding author.

E-mail addresses: e.narayan@griffith.edu.au, edward_nryn@yahoo.com (E.J. Narayan).

decline, with approximately a third of all amphibian species experiencing major declines, facing extinction or have perhaps already gone extinct (Stuart et al., 2004). Non-invasive endocrinology has been used for both model and threatened species, in simulated laboratory scenarios and *in situ* (Kindermann et al., 2012, 2013; Narayan et al., 2010a).

Currently, there is no experimental demonstration of the relationship between urinary based hormone monitoring and the traditional blood analysis. Previous studies have examined the relationship between plasma hormones and excretory hormone metabolites in other animals, such as birds. For example, (Crespi et al., 2013) measured the relationship between plasma corticosterone and fecal corticosterone metabolites in the chicken after an adrenocorticotrophic hormone (ACTH) and dexamethasone injection to stimulate and suppress adrenal activity respectively. Results showed that the concentrations of fecal metabolites (to both tests) corresponded to the changes of biologically active hormone in plasma, but with fecal peaks obtained with a delay of 4 h compared to plasma. Furthermore, (Kindermann et al., 2013) showed that fecal corticosterone reflects serum corticosterone in Florida Sandhill Cranes (*Grus canadensis*) in response to both confinement and ACTH stimulation.

In this study, we compare the urine and serum concentrations of corticosterone and testosterone in adult male cane toads (*Rhinella marina*) during exposure to a standard capture and handling protocol. Model species, such as the cane toad, are useful to validate the method of urinary hormone metabolite monitoring as they are robust, easy to sample, and are easily accessible. We demonstrate the pattern of changes in serum and urinary levels of corticosterone and testosterone in the male toads with respect to exposure to the standard capture and handling stress protocol.

2. Methods

2.1. Animals and sample collection

Adult male toads were collected from the Parkwood International Golf Course, Queensland. The toads were located at night from 1800 to 2200 h by a visual encounter with support of a head torch. Sampling was performed over a 2 week period of hot and dry nights in April 2012 when the toads were most likely to be outside their breeding period. Toads were sexed using a visual inspection criteria described earlier (Malisch et al., 2008). To obtain baseline urinary corticosterone and testosterone concentrations, we collected urine samples from adult male toads ($n = 8$) immediately upon capture in the field (this time was termed, $t = 0$ h). Urine was collected using the sampling protocol described earlier (Narayan et al., 2008). Each toad was gently handled and urine was obtained usually between 30 s and 1 min of initial handling. Each toad was then sampled using the standard capture and handling (short-term stressor) protocol. Each toad was manually restrained

for 5 min, which further stimulated the HPI-axis and then transferred inside resealable plastic bags that were kept in a large holding container. A second urine sample was collected from each toad exactly 0.5 h after initial capture (0 h), and further urine samples were collected at times 1, 2, 3, 4, 5, 6, 7 and 8 h after capture in a similar manner. Urine samples were transported to the laboratory and kept at -20°C for a month prior to being assayed. A single blood sample was also collected from each toad at the end of the urine sampling at $t = 8$ h. Blood was obtained in the field by a minimally invasive cardiac puncture technique with a sterile 25 gage hypodermic needle. Briefly, the toad was held using one hand so that its ventral side or underbelly abdomen was visible to the handler. The underbelly abdomen of the toad was wiped using a cotton wool (dipped in 10% ethanol). The heart was palpated and the needle was inserted into the skin at a $20\text{--}30$ degree angle below the sternum, directed towards the toad's head. Blood was collected in less than 1 min after handling, which was consistent for all toads. Blood ($500\text{--}1000\text{ }\mu\text{l}$) from each toad was transferred immediately into labeled Eppendorf tubes and kept on ice in the field (<12 h). The blood samples were centrifuged ($5000g$; 10 min) overnight in the laboratory to obtain serum ($300\text{--}500\text{ }\mu\text{l}$ serum per blood sample). Toads were toe-clipped ($n = 1$ front toe; Narayan et al., 2011a) to avoid repeat capture of the same individual after the nights sampling and released into their original habitat. During the following ($n = 10$) nights, we collected remaining toads to obtain urine and blood for time periods as shown in Table 1. All toads were toe-clipped and were released immediately into their original habitat after sampling.

2.2. Urinary and serum corticosterone and testosterone enzyme-immunoassays

Urinary corticosterone and testosterone enzyme-immunoassays (EIA) were based to our earlier works (Narayan et al., 2010a, b). Urinary corticosterone concentrations in toad urine were determined using a polyclonal anti-corticosterone antiserum (CJM06, UC Davis California) that was diluted 1:45,000, horseradish peroxidase-conjugated corticosterone label that was diluted 1:120,000 and corticosterone standards ($1.56\text{--}400\text{ pg well}^{-1}$). Urinary testosterone concentrations in toad urine were determined using a polyclonal anti-testosterone antiserum (R156/7, UC Davis California) that was diluted 1:25,000, horseradish peroxidase-conjugated testosterone label that was diluted 1:40,000 and testosterone standards ($0.78\text{--}200\text{ pg well}^{-1}$). Cross reactivity of the CJM06 anti-corticosterone antiserum and R156/7 anti-testosterone antiserum were reported in earlier works (Narayan et al., 2010a,b).

Toad serum samples were also assayed for corticosterone and testosterone concentrations using the same EIA protocols as described above. Laboratory validations of the urinary and serum EIAs were achieved by demonstrating; (a) parallelism between serially diluted pooled toad serum or urine sample and the

Table 1

Shows the experimental design, including groups and sample sizes of adult male toads used in this study.

Group	Sample size	Treatment
1 Control	10	Urine collected at 0, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 h. Blood collected at 8 h
2	8	Urine and blood collected at time = 0 h only.
3	8	Urine collected at 0 and 0.5 h. Blood collected at 0.5 h.
4	8	Urine collected at 0, 0.5 and 1 h. Blood collected at 1 h.
5	8	Urine collected at 0, 0.5, 1 and 2 h. Blood collected at 2 h.
6	8	Urine collected at 0, 0.5, 1, 2 and 3 h. Blood collected at 3 h.
7	8	Urine collected at 0, 0.5, 1, 2, 3 and 4 h. Blood collected at 4 h.
8	8	Urine collected at 0, 0.5, 1, 2, 3, 4 and 5 h. Blood collected at 5 h.
9.	8	Urine collected at 0, 0.5, 1, 2, 3, 4, 5 and 6 h. Blood collected at 6 h.
10	8	Urine collected at 0, 0.5, 1, 2, 3, 4, 5, 6 and 7 h. Blood collected at 7 h.

respective standard curves and, (b) by significant recovery of exogenous steroid standards added to toad urine and serum samples. Dilution rates for toad urine and serum were based on the concentration of pooled samples that resulted in 50% binding on the parallelism curve. Recovery was expressed as a linear regression formula ($y = mx + b$, where y = amount of hormone observed, x = amount of hormone expected, b = y intercept and m = slope of the line) and the multiple correlation coefficient was squared to produce the coefficient of determination (r^2). Assay sensitivity was calculated as the value two standard deviations from the mean response of the blank (zero binding) samples. Laboratory EIA validation results are provided in Table 2. Urine steroid conjugates were standardized to creatinine (Cr) levels to control for water content based on the methods described earlier (Narayan et al., 2008). Urinary testosterone and corticosterone concentrations were reported as pg/ μ g Cr while serum testosterone and corticosterone concentrations were reported as ng/ml.

2.3. Statistical analysis

Statistical analyses were performed using Prism (Graphpad Software Inc.) and Systat (Ver 13.0). Urinary and serum corticosterone and testosterone concentrations were transformed to logarithms before analysis to check the homogeneity of variances using Levene's tests.

Serum hormone concentrations were transformed using the formula $Y = \sqrt{\text{sqrt}(Y)}$ to check for homogeneity of variances and then one-way analysis of variance (One-way ANOVA) was used to compare the mean serum corticosterone concentration between times. Post-hoc comparisons between time periods were done using Bonferroni's multiple comparison tests. Serum testosterone concentrations were transformed and analyzed in a similar way.

Urinary corticosterone and testosterone values were log ($Y = \log[Y]$) transformed to check for normality using D'Agostino & Pearson omnibus normality test. Urinary corticosterone and testosterone values were analyzed separately using General Linear Model (GLM) with time as the factor, and urinary corticosterone or urinary testosterone as the dependant variable. Post-hoc comparisons between time periods for each hormone were done using Kruskal–Wallis Test.

Percentage change in serum corticosterone and testosterone could only be calculated from the mean values at each time period (0, 0.5, 1, 2, 3, 4, 5, 6, 7 or 8 h) since each toad was sampled only once for blood. The percentage increases in serum corticosterone concentration from 100% to 200% and 500% of 0 h value were obtained from the mean baseline concentration. The percentage decrease in the concentration of serum testosterone from 100% to 50% of the 0 h value was obtained from the mean baseline concentration. Percentage changes in urinary corticosterone and testosterone were calculated using means as were the serum percentages so that the urinary and serum percentages could be compared. The percentage increases in urinary corticosterone from 100% to 200% and 500% of

0 h value were obtained from the mean baseline concentration. The percentage decrease in concentration of urinary testosterone from 100% to 50% of the 0 h value was obtained from the mean baseline concentration.

We also calculated the coefficient of variation (CV%) for baseline serum and urinary corticosterone and testosterone to show that even at baseline, the variation in urine reflected the variation in serum (see the result Table 2).

3. Results

3.1. Urinary and serum corticosterone

All toads generated short-term urinary corticosterone stress responses to the standard capture and handling protocol and most toads showed increasing levels of urinary corticosterone after 1 h while some toads showed increasing levels of urinary corticosterone between 0 and 0.5 h (Fig. 1A). There was a significant effect of time on urinary corticosterone concentrations [ANOVA; $F_{3,366} = 880.1$, $p < 0.0001$]. The mean urinary corticosterone concentration increased significantly beyond the 0 h (baseline sample) with a lag-time of 1 h and continued to increase up to 8 h (Kruskal–Wallis Test; $p < 0.05$ for all comparisons of time periods with 0 h, except for comparison between 0 h with 0.5 and 1 h; Fig. 1B).

There was a significant effect of time on serum corticosterone concentrations [One-way ANOVA; $F_{9,63} = 107.0$, $p < 0.0001$]. First significant increase in mean serum corticosterone concentration c.f. 0 h was observed at 0.5 h and mean serum corticosterone concentrations were significantly different from baseline (0 h) sample for all time periods, reaching highest level between 6 and 8 h (Bonferroni's Multiple Comparison Test; $p < 0.05$ for all comparisons; Fig. 1B). Comparison between each time period also showed that the mean serum corticosterone concentration was not significantly different between 4 and 8 h (Bonferroni's Multiple Comparison Test; $p > 0.05$ for all time comparisons; Fig. 1B).

3.2. Urinary and serum testosterone

The individual concentrations of urinary testosterone decreased after 0.5 h for most toads while some toads showed decreasing levels of urinary testosterone after 1 h during exposure to the capture and handling protocol (Fig. 2A). There was a significant effect of time on urinary testosterone concentrations [ANOVA; $F_{3,366} = 221.11$, $p < 0.0001$]. The mean urinary testosterone concentration decreased significantly c.f. 0 h with a lag-time of 0.5 h (Kruskal–Wallis Test; $p < 0.05$ for all comparisons of time periods with 0 h, except for comparison between 0 and 0.5 h; Fig. 2B). Mean urinary testosterone levels did not change much between 4 and 8 h (Fig. 2A).

There was a significant effect of time on serum testosterone concentrations [One-way ANOVA; $F_{9,63} = 37.01$, $p < 0.0001$]. First

Table 2

Shows the quality control parameters for the serum and urinary corticosterone and testosterone enzyme-immunoassays.

Quality control parameters	Serum corticosterone	Urinary corticosterone	Serum testosterone	Urinary testosterone
Dilution factor	1:4	1:2	1:3	1:4
Recovery equation	$y = 0.6835x + 0.8455$, $r^2 = 1$ ($n = 6$)	$y = 0.9258x + 0.2232$, $r^2 = 0.9975$ ($n = 6$)	$y = 0.6786x + 0.8439$, $r^2 = 1$ ($n = 6$)	$y = 0.7745x + 0.8345$, $r^2 = 1$ ($n = 6$)
Intra-assay CV (70% binding control)	3.2%	3.1%	2.4%	2.6%
Intra-assay CV (30% binding control)	2.1%	2.2%	2.2%	2.4%
Inter-assay CV (70% binding control)	7.1%	7.7%	6.4%	7.2%
Inter-assay CV (30% binding control)	4.2%	5.2%	3.2%	3.5%
Assay sensitivity (pg well ⁻¹)	1.25 ± 0.42 ($n = 7$)	1.22 ± 0.45 ($n = 32$)	0.49 ± 0.14 ($n = 7$)	0.51 ± 0.11 ($n = 22$)
Inter-individual variation (Mean CV %) for baseline samples ($n = 6$)	28.78%	26.19%	21.35%	15.92%

Fig. 1. Individual urinary corticosterone responses (A) of $n = 10$ male toads (group 1) during exposure to the standard capture and handling protocol. (B) shows the mean $\pm$ S.E.M. serum and urinary concentrations of corticosterone. Sample size of $n = 10$ –82 toads at each time point for urine samples. Sample size of $n = 8$ toads at each time point for the serum samples.

Fig. 2. Individual urinary testosterone responses (A) of $n = 10$ male toads (group 1) during exposure to the standard capture and handling protocol. (B) shows the mean $\pm$ S.E.M. serum and urinary concentrations of testosterone. Sample size of $n = 10$ –82 toads at each time point for urine samples. Sample size of $n = 8$ toads at each time point for the serum samples.

significant decrease in mean serum testosterone concentration c.f. 0 h was observed at 0.5 h and serum concentrations were significantly different from baseline sample for all time periods (Bonferroni's Multiple Comparison Test; $p < 0.05$ for all comparisons; Fig. 2B). Mean serum testosterone level increased between 7 and 8 h.

3.3. Percentage change in serum and urinary corticosterone and testosterone

The time required for percentage increase in mean serum corticosterone to 200% and 500% of the mean baseline (0 h) value were within 0.5 h and 1 h respectively (Fig. 3A). The time required for percentage increase in mean urinary corticosterone to 200% and 500% of the 0 h value were within 3–7 h respectively (Fig. 3A). The time required for the percentage decrease of mean serum testosterone to 50% of 0 h value was 1 h (Fig. 3B) and within 3 h for urinary testosterone (Fig. 3B).

4. Discussion

The results show that the urinary corticosterone and testosterone enzyme-immunoassays reliably detected the changes in serum corticosterone and testosterone in the male cane toads. Urinary testosterone concentrations decreased (50% in 3 h) at a similar rate compared to the increase (200% in 3 h) in urinary corticosterone. As shown in Table 1, the mean CV% is comparable between the serum and urinary corticosterone and testosterone, which further supports the utility of urine sampling for non-invasive reproductive and stress endocrine monitoring in amphibians.

Physiological stress hormone responses vary depending on the type and exposure to a particular stressor (Narayan et al., 2012b,

Fig. 3. Serum (A) and urinary (B) corticosterone and testosterone mean values shown as percentage change from 0 h (100%) for the adult male cane toads. The gridlines intersect the mean% value (y-axis) with time (x-axis).

2011b). Many studies have examined how animals respond to different experimental stressors using blood samples and more recently with minimally invasive endocrine sampling techniques (see the latest reviews Creel et al. (2013), Crespi et al. (2013), Narayan et al., (in press)). The current study has shown that in response to the standard capture and handling stressor, there was an increase in both the serum and urinary corticosterone and a concomitant decrease in both serum and urinary testosterone. Capture and handling have been used widely in studies with amphibians (Narayan and Hero, 2011b, 2012c) to mimic the animal's physiological stress hormone response to naturalistic stressors such as the sight of a predator. Therefore, this method certainly holds merit for future investigations based on the physiological stress responses of amphibians to acute and chronic environmental stressors, such as thermal variability, deforestation, pathogenic fungus, predation and so forth. Most importantly, the use of minimally invasive urine sampling and capture handling protocol will enable the assessment of both intra- and inter-individual variation (Narayan et al., 2011a, 2012b) in the physiological status and physiological reaction of species to environmental change. Thus these methods will be useful for assessing the physiological status and making predictions regarding vulnerability of free-living populations hence beneficial for the broad research fields of conservation biology and wildlife management.

Previous studies have also investigated the effects of experimental stressors on plasma and urinary corticosterone in other animals. For example, (Brennan et al., 2000) tested the effects of persistent inescapable stress (tail shocks) on the plasma and urinary corticosterone concentrations in Sprague Dawley rats. Exposure to inescapable stress resulted in elevations in plasma corticosterone concentrations as well as urinary corticosterone levels. Urinary concentrations of corticosterone remained elevated for 3 days after terminating the stress protocol. The elevations of corticosterone in rat plasma were only apparent in the morning while the levels in the night were not different between the stressed and non-stressed rats. Thus serial urine sampling provides a minimally invasive way for studying the adrenocortical activity of the study animal over the entire day; however blood samples at certain times of the day could miss important effects of acute stressors. While mean serum levels of corticosterone appeared to be reaching an asymptote within 8 h, the mean urinary corticosterone concentration seemed to continue to increase. This corresponds to the rate of clearance of the hormone metabolites (Abelson et al., 2009). Blood plasma represents the total corticosterone/testosterone, which includes both free corticosterone/testosterone and binding globulin (Ludders et al., 2001). There is competition between corticosterone and testosterone for the same binding globulins (Deviche et al., 2001; Ward et al., 2007). Thus, assuming no change in binding globulin concentration, a decrease in total plasma testosterone is expected to result in some testosterone dissociating from the binding globulin, in turn freeing the binding sites to which corticosterone can bind. If the total plasma corticosterone concentration does not change, the process should result in more corticosterone bound to the binding globulins, i.e., less free ("bio-available") corticosterone that is available for diffusion into cells.

Our study has provided new evidence that there is a lag-time associated with detectable changes in both corticosterone and testosterone in urine in comparison with serum in cane toads. In their study, (Abelson et al., 2009) used Sprague Dawley rats to show that radioactivity in serum samples was highest at 90 min and a rapid decline thereafter. Free corticosterone is removed from the plasma and metabolized via the kidneys (in urine for 6 h) and finally in feces after at least 12 h via the bile and liver. Thus there is most likely an endpoint point for plasma corticosterone while urinary levels continue to increase. The endpoint point for plasma corticosterone could be related to perhaps increasing metabolism or

clearance of the biologically active (free) corticosterone in order to avoid high concentrations of corticosterone in the blood, which can have serious implications. In earlier studies, the lag-times of urinary corticosterone and testosterone have been demonstrated for amphibians using ACTH and human-chorionic gonadotropin (hCG) hormone challenge respectively (Narayan et al., 2010a,b). A lag-time for the detection of both corticosterone and testosterone for urinary metabolites in comparison with serum is very important because (a) it provides baseline information or snapshot into the physiological status of the study species, and (b) it provides a crucial time-frame for field capture and transfer into the laboratory prior to experimental manipulations.

In conclusion, our study has demonstrated the reliability and usefulness of minimally invasive urine sampling and standard capture handling protocol for assessing the physiological status, short-term stress hormone response and changes in reproductive hormones in amphibians. Apart from being minimally invasive, urinary methods provide a more integrated measure of the status of the animal's adrenocortical and reproductive hormone axis. Overall, these methods are recommended over traditional blood sampling for assessing the reproductive and stress endocrinology of amphibians.

Uncited references

Dehnhard et al. (2003), Forzán et al. (2012).

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