Changes in serum and urinary corticosterone and testosterone during short-term capture and handling in the cane toad (Rhinella marina)

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Published
2013

Journal Title
General and Comparative Endocrinology

DOI
https://doi.org/10.1016/j.ygcen.2013.06.018

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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.
Changes in serum and urinary corticosterone and testosterone during short-term capture and handling in the cane toad (Rhinella marina)

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\begin{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 (\textit{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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\end{abstract}
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 adrenocorticotropic 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 metabolism 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 20–30 degree angle below the sternum, directed towards the toad’s head. Blood was collected in less than 1 min after handing, which was consistent for all toads. Blood (500–1000 µl) 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 (300–500 µl 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

Urine 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 antisera (CJM06, UC Davis California) that was diluted 1:45,000, horseradish peroxidase-conjugated corticosterone label that was diluted 1:120,000 and corticosterone standards (1.56–400 pg well−1). Urinary testosterone concentrations in toad urine were determined using a polyclonal anti-testosterone antisera (R156/7, UC Davis California) that was diluted 1:45,000, horseradish peroxidase-conjugated testosterone label that was diluted 1:20,000 and testosterone standards (0.78–200 pg well−1). Cross reactivity of the CJM06 anti-corticosterone antisera and R156/7 anti-testosterone antisera 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 EIA were achieved by demonstrating; (a) parallelism between serially diluted pooled toad serum or urine sample and the

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample size</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control</td>
<td>10</td>
<td>Urine collected at 0, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 h. Blood collected at 8 h</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>Urine and blood collected at time = 0 h only.</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>Urine collected at 0 and 0.5 h. Blood collected at 0.5 h.</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>Urine collected at 0, 0.5 and 1 h. Blood collected at 1 h.</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>Urine collected at 0, 0.5, 1 and 2 h. Blood collected at 2 h.</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>Urine collected at 0, 0.5, 1, 2 and 3 h. Blood collected at 3 h.</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>Urine collected at 0, 0.5, 1, 2, 3 and 4 h. Blood collected at 4 h.</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>Urine collected at 0, 0.5, 1, 2, 3, and 4 h. Blood collected at 5 h.</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>Urine collected at 0, 0.5, 1, 2, 3, 4, and 5 h. Blood collected at 6 h.</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>Urine collected at 0, 0.5, 1, 2, 3, 4, 5, 6 and 7 h. Blood collected at 7 h.</td>
</tr>
</tbody>
</table>
2.3. Statistical analysis

Urinary corticosterone and testosterone values were log-transformed before analysis. The percentage change in serum corticosterone and testosterone concentrations from 100% to 50% of the 0 h value were 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; F3,366 = 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; F3,63 = 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 urinary 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; F3,366 = 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; F3,63 = 37.01, p < 0.0001]. First

![Table 2](https://example.com/table2.png)

Table 2 Shows the quality control parameters for the serum and urinary corticosterone and testosterone enzyme-immunoassays.

<table>
<thead>
<tr>
<th>Quality control parameters</th>
<th>Serum corticosterone</th>
<th>Urinary corticosterone</th>
<th>Serum testosterone</th>
<th>Urinary testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution factor</td>
<td>1:4</td>
<td>1:2</td>
<td>1:3</td>
<td>1:4</td>
</tr>
<tr>
<td>Recovery equation</td>
<td>y = 0.6835x + 0.8455, y = 0.9258x + 0.2232, r² = 1 (n = 6)</td>
<td>y = 0.6786x + 0.8439, r² = 1 (n = 6)</td>
<td>y = 0.7745x + 0.8345, r² = 1 (n = 6)</td>
<td></td>
</tr>
<tr>
<td>Intra-assay CV (70% binding control)</td>
<td>3.2%</td>
<td>3.1%</td>
<td>2.4%</td>
<td>2.6%</td>
</tr>
<tr>
<td>Intra-assay CV (30% binding control)</td>
<td>2.1%</td>
<td>2.2%</td>
<td>2.2%</td>
<td>2.4%</td>
</tr>
<tr>
<td>Inter-assay CV (70% binding control)</td>
<td>7.1%</td>
<td>7.7%</td>
<td>6.4%</td>
<td>7.2%</td>
</tr>
<tr>
<td>Inter-assay CV (30% binding control)</td>
<td>4.2%</td>
<td>5.2%</td>
<td>3.2%</td>
<td>3.5%</td>
</tr>
<tr>
<td>Assay sensitivity (pg well⁻¹)</td>
<td>1.25 ± 0.42 (n = 7)</td>
<td>1.22 ± 0.45 (n = 32)</td>
<td>0.49 ± 0.14 (n = 7)</td>
<td>0.51 ± 0.11 (n = 22)</td>
</tr>
<tr>
<td>Inter-individual variation (Mean CV %) for baseline samples (n = 6)</td>
<td>28.78%</td>
<td>26.19%</td>
<td>21.35%</td>
<td>15.92%</td>
</tr>
</tbody>
</table>

A 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 amphibia. Physiological stress hormone responses vary depending on the type and exposure to a particular stressor (Narayan et al., 2012b, 2013).

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 ± 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 ± 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.

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).
Our study has provided new evidence that there is 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), Forzan et al. (2012).

Acknowledgments

The study was supported by a Postdoctoral Research Fellowship award to E.J.N. by the Griffith University, Australia, which was hosted by the Environmental Futures Centre, Gold Coast Campus. TS, we were caught and examined under a Queensland Government Scientific Purpose Permit (WISP07106310) and Griffith University Animal Ethics Committee Permit ENV/08/10/AEC. We would like to thank the Parkwood International Golf Course for permission to work on their course. We thank Alex Simic for assisting with field sampling and literature compilation.

References


