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# Repeatability of baseline corticosterone and short-term corticosterone stress responses, and their correlation with testosterone and body condition in a terrestrial breeding anuran (*Platymantis vitiana*)

Edward J. Narayan [a,*] John F. Cockrem [b] and 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 14 North, New Zealand.

[*] Corresponding author. Tel: + 61 (0)401697287

*E-mail address:* e. narayan@ga.griffith.edu.au; edward_nryn@yahoo.com

**Abstract**

Repeatability of physiological response variables, such as the stress hormone corticosterone, across numerous sampling occasions is an important assumption for their use as predictors of behaviour, reproduction and fitness in animals. Very few studies have actually tested this assumption in free-living animals under uncontrolled natural conditions. Non-invasive urine sampling and standard capture handling protocol have enabled the rapid quantification of baseline corticosterone and short-term corticosterone stress responses in anuran amphibians. In this study, established non-invasive methods were used to monitor physiological stress and urinary testosterone levels in male individuals of the terrestrial breeding Fijian ground frog (*Platymantis vitiana*). Adult male frogs (n = 20) were sampled at nighttimes on three repeated occasions at intervals of 14 days during their annual breeding season on Viwa Island, Fiji. All frogs expressed urinary corticosterone metabolite responses to the capture and handling stressor, with some frogs showing consistently high urinary corticosterone responses than others. Ranks of corticosterone values at 0, 4 and 8 h, and the corrected rank were highly significant ($r$ = 0.75 – 0.99) between the three repeated sampling occasions. Statistical repeatabilities were high for baseline corticosterone ($r$ = 0.973) and for corticosterone values at 2 h ($r$ = 0.862), 4 h ($r$ = 0.861), 6 h ($r$ = 0.820) and 8 h ($r$ = 0.926), and also for the total (inclusive of baseline corticosterone values) and the corrected integrated responses (index of the acute response) [$r$ = 0.867 and $r$ = 0.870]. Urinary testosterone levels also showed high statistical repeatability ($r$ = 0.78). Furthermore, variation in baseline and short-term corticosterone stress responses was greater between individuals than within individuals. Baseline urinary corticosterone was significantly negatively correlated with the corrected integrated corticosterone response ($r$ = -0.3, $p$ < 0.001) but non-significantly with body-condition ($r$ = -0.04) and baseline urinary testosterone ($r$ = -0.07). While, the corrected integrated corticosterone response was positively correlated (non-significantly) with baseline

urinary testosterone ($r$ = +0.04) and body-condition ($r$ = +0.08). Urinary testosterone levels and body-condition were significantly negatively correlated ($r$ = -0.23, $p$ < 0.001). The results suggest that male frogs with higher levels of testosterone could have depleted energy reserve during the breeding period. The acute corticosterone responses help in replenishing energy that is needed for breeding and survival. The results also provide some support to the 'cort-fitness' hypothesis as highlighted by the negative correlation between baseline corticosterone and body-condition. It is most likely that the acute corticosterone response is adaptive and linked positively with reproductive fitness and survival in male anurans.

***Keywords:*** baseline corticosterone; short-term (acute) corticosterone response; body condition; testosterone; repeatability; amphibians; *Platymantis vitiana*; fitness

## 1.0 Introduction

Stress can be defined as the experience of facing challenges that require behavioural, biochemical and physiological responses of the organism (Morgan and Tromborg, 2007). Anything that challenges a system in homeostasis could be regarded to as a stressor. Glucocorticoid hormones (such as corticosterone and cortisol) mediate stress in animals by causing changes in glucose metabolism, reproductive hormones and behaviour (Cook et al., 2012; Narayan et al., 2010b; Wack et al., 2012). Glucocorticoids are present at baseline levels in the blood and the concentrations increase when the animal perceives a stressor, which causes activation of the hypothalamo-pituitary adrenal (HPA) axis in larger vertebrates or the hypothalamo-pituitary interrenal (HPI) axis in lower vertebrates (such as amphibians and reptiles). The acute or short-term stress hormone response causes key physiological and behavioural changes that allow the animal to overcome the stressor (See (McEwen and

Wingfield, 2007) for a detailed conceptualization of physiological stress responsiveness in animals).

Stress hormones are often consistent with certain behavioural and crucial life-history processes such as development, aggression, breeding, migratory behaviour and predator evasion (Cockrem and Silverin, 2002; Long and Holberton, 2004; Narayan et al., 2010b; Surbeck et al., 2012). This suggests that stress hormones play crucial role(s) in the regulation of important eco-physiological and behavioural processes in animals and they could also be linked to fitness (such as lifetime reproductive fitness) and survival (Bonier et al., 2009b; Rivers et al., 2012). There are currently two contradicting hypothesis that explains the relationship between stress hormones (glucocorticoids) and individual fitness. Firstly, the 'cort-fitness hypothesis', which states that glucocorticoids are negatively associated with an individual's fitness thus individuals with the highest levels of baseline glucocorticoids will have the lowest fitness (Bonier et al., 2009a). As highlighted by (Rivers et al., 2012), the 'cort-fitness hypothesis' works around the assumption that increased levels of baseline glucocorticoids during period of any environmental stress could lead to a redistribution of important resources away from normal activities that could be detrimental towards the animal's survival during and after the stress event. A second hypothesis is known as the 'cort-activity hypothesis', which states that elevated levels of baseline glucocorticoids are linked directly with individual fitness through physiological and behavioural modifications undertaken to cope with the stress event. For example, increased levels of baseline glucocorticoids are linked with increased anti-predator activities and increased locomotion in some animals (Breuner and Hahn, 2003; Comendant et al., 2003; Cote et al., 2006). Physical traits, especially body-condition (BC) is also used widely by ecologists as an important determinant of an individual animal's fitness (Green, 2001). Body condition is used as a

proxy of energy reserves in animals (Schulte-Hostedde et al., 2005). Several studies have assessed the relationships between glucocorticoids and BC in animals to determine how the stress endocrine status correlates with fitness [See (Moore and Jessop, 2003) for a detailed review]. For example, baseline levels of plasma corticosterone in marine iguanas (*Amblyrhynchus cristatus*), garter snakes (*Thamnophis sp.*), and two species of agamid lizard (*Amphibolurus nuchalis, Pagona barbatus*) were negatively correlated with BC (Bradshaw, 1986; Cree et al., 2000; Moore et al., 2000; Moore and Mason, 2001; Romero and Wikelski, 2001). The short-term or acute corticosterone response has also been correlated with BC in some animals, for example in the western fence lizards (*S. occidentalis*), individuals in good BC produced lower levels of corticosterone in response to capture stress in comparison to individuals in poor condition (Dunlap and Wingfield, 1995). In amphibians, it has been shown in some species that depletion of energy reserves due to male vocalization during breeding is thought to increase baseline corticosterone and male frogs with high baseline corticosterone also tend to have poor body condition (Leary and Harris, 2013).

Since stress hormones are important for stress mitigation in animals hence their concentrations should be elevated during ecologically important or challenging periods, such as breeding to cope with the stressor. The application of corticosterone as a biomarker of physiological stress in animals relies on the assumption that an individual with a low corticosterone titre will always be ranked lower than individuals with a high corticosterone titre if sampling was conducted on one or more occasions. This consistency among rank order is a test of repeatability [*r*] (Romero and Reed, 2008). Only a handful of studies have actually tested this assumption by performing repeated sampling of the same individual under natural conditions (Cockrem et al., 2009; Cook et al., 2012; Ouyang et al., 2011a; Rensel and Schoech, 2011; Wada et al., 2008). There are examples from the laboratory [e.g. rodents

(Guimont and Wynne-Edwards, 2006)] and in aquaculture supporting the repeatability of glucocorticoids [See detailed review (Overli et al., 2005)]. On the other hand, some studies on birds have shown that baseline and stress induced corticosterone concentrations were not repeatable between times of the day, and between seasons or years (Ouyang et al., 2011a; Romero and Reed, 2008). In simple terms, if glucocorticoid titres and the stress protocol are not repeatable within individuals it becomes an unreliable metric for inferring physiological stress and thus potentially useless despite presumably thousands of papers measuring corticosterone as a predictor of individual fitness in animals.

A non-invasive method of assessing corticosterone metabolites *via* urinary enzyme-immunoassay (EIA) in anuran amphibians has been established (Narayan et al., 2010b). This non-invasive method has been used widely for assessing physiological stress in anurans under both free-living and captive conditions (Kindermann et al., 2012; Narayan et al., 2012b; Narayan and Hero, 2011; Narayan et al., 2012c; Narayan et al., 2010b; Narayan et al., 2011a; Narayan et al., 2012e). Urinary levels of corticosterone metabolites can also increase during exposure to different thermal regimes and pathogenic diseases, such as chytridiomycosis (Kindermann et al., 2012; Narayan et al., 2012a). Recent studies in anuran amphibians have also shown using the non-traditional model anuran species, the cane toad (*Rhinella marina*), that both baseline and short-term urinary corticosterone metabolite stress responses are repeatable under captive and field conditions (Narayan et al., 2013; Narayan et al., 2012d). To our knowledge, the repeatability of corticosterone and testosterone has not been tested in any endemic frog species under uncontrolled natural conditions. The study species, Fijian ground frog (*Platymantis vitiana*), is endemic to the Fiji Islands. It is listed as Endangered (B1ab(v) ver 3.1) under the International Union for Conservation of Nature (IUCN) 2008 standards [see: (Zug et al., 2004)]. The ground frog has an annual breeding cycle (Narayan et

al., 2008b) and seasonal patterns of changes in corticosterone and reproductive hormones (testosterone, progesterone and oestradiol) were shown in earlier studies using non-invasive urinary EIAs (Narayan et al., 2010a, b). Earlier, we also measured variation in mean monthly concentrations of urinary corticosterone metabolites between individual male ground frogs that was explained in relation to the differences in energy required or expended during breeding calls in individual male frogs (Narayan et al., 2010b). The current study will provide evidence regarding the function of baseline and short-term corticosterone metabolite stress responses during the breeding period.

In this study, baseline and short-term urinary corticosterone responses were quantified in adult male individuals of the Fijian ground frog using urinary measurements and standard capture and handling protocol. Statistical repeatabilities of the baseline and stress induced urinary corticosterone metabolite concentrations were determined by sampling the same individual frogs on three repeated occasions. Furthermore, ranks of corticosterone values for the baseline sample and during the period of the short-term stress response was determined to confirm whether individuals ranked as high or low on one occasion were consistently ranked between sampling occasions. The baseline and short-term corticosterone stress responses were correlated with body-condition (testing the 'cort-fitness' hypothesis) and also with urinary testosterone levels of the male frogs

## 2.0 Materials and Methods

### *2.1 Field sampling*

The study population of the Fijian ground frog (*Platymantis vitiana*) lives within a forest adjacent to plantations on Viwa Island ($18^{o}000S$, $175^{o}000E$), a small (60 ha) island located

900 m off the coast of mainland Viti Levu, Fiji (Narayan et al., 2008a). Baseline urinary corticosterone metabolites and short-term urinary corticosterone metabolite responses to a standard capture and handling stress protocol (5 min handling and placement inside plastic bags between hourly urine sampling over 8 h) was measured in adult male frogs (n = 20) on three consecutive occasions. Frogs were captured from 1900 – 2100 h during their annual breeding season in December 2011 (Narayan et al., 2010a). All frogs were caught on the same night during each sampling occasion, with similar wet ground conditions observed throughout the sampling periods. Frogs were captured by hand as soon as they were located on the ground and a urine sample was collected manually within 30 sec – 1 min of capture (Narayan et al., 2010b). We were working with an endangered species and as such the frogs could not be dissected to confirm sex, sex was assigned using other features. For example, males are larger than females, lack underbelly oocytes and have distinct 'stress calls' (Narayan et al., 2008a). The male frogs had a mean SVL of 45.3 $\pm$ 0.5 mm (n = 20) and mean body-weight of 32.5 $\pm$ 0.2 g (n = 20). Female frogs had a mean SVL of 57.5 $\pm$ 0.5 mm. Additionally, urinary estrone conjugate (EC) and progesterone (P) levels were measured in the same frog urine samples using established methods to confirm sex (Narayan et al., 2010a). The levels of EC and P were comparable to the hormone levels found in male ground frogs in a previous study (data not shown) and these hormone levels were 95 % lower than that found normally in female ground frogs (Narayan et al., 2010a).

*2.2 Short-term capture and handling protocol*

Earlier, it was shown that urinary corticosterone metabolite concentrations in amphibians increased significantly within 2 h after initial capture (Narayan et al., 2012b; Narayan et al., 2010b; Narayan et al., 2011b). After the first (baseline) urine sample was collected, each frog was held for 5 min and then placed in a resealable plastic bag (constituting a mild

stressor) and transferred back into a plastic container. Frogs were removed from this container for urine collection followed by holding for 5 min and placement in a fresh resealable bag back in the plastic container at two hourly intervals up to 8 h after capture. Afterwards, each frog was marked using toe-clips following established methods (Hero, 1989) and released *in-situ*. Frogs were re-captured on the remaining two sampling occasions at intervals of 14 days at the same times each night as the first sampling occasion. All frogs (n = 20) were re-captured within two nights of intensive sampling between 1900 – 2100 h during the second and third repeated sampling occasions. The capture handling protocol was done immediately after collection of baseline urine samples from individually captured frogs during each sampling occasion.

### *2.3 Urinary corticosterone and testosterone enzyme-immunoassays*

A urinary corticosterone metabolite enzyme-immunoassay (EIA) that was previously validated for the Fijian ground frog (Narayan et al., 2010b) was used to measure corticosterone metabolite concentrations in male ground frog urine. The urinary testosterone metabolite EIA was based on our previous methods (Narayan et al., 2010a). Intra-assay CVs were 2.4 % and 3.4 % for low- and high-percentage-bound controls respectively. Inter-assay CVs were 6.5 % and 8.1 for low- and high-percentage-bound controls respectively. The overall assay sensitivity was 1.05 $\pm$ 0.22 pg/well (n = 70). Urinary hormones were standardised to creatinine (Cr) levels to control for water content (Narayan et al., 2010a) and were reported as pg/µg Cr. Due to the cross-reactivity of the corticosterone and testosterone EIA we refer to the hormones measured as urinary corticosterone or testosterone metabolites throughout.

### *2.4 Statistical analysis*

Statistical analyses were performed using Prism (Graphpad Software Inc.). All of the data were tested for normality using the D'Agostino & Pearson omnibus normality test, and the urinary corticosterone and testosterone metabolite data were $\log_{10}$ transformed prior to analysis. Data are presented as individual points or as mean $\pm$ S. E. Probability values of $p < 0.05$ were considered to be significant. Body condition (BC) was calculated using Fulton‘s index [BC=Body-weight/$SVL^3$] (Peig and Green, 2010). Spearman (*r*) correlation was used to correlate baseline urinary corticosterone with urinary testosterone, body-condition and the integrated (total and corrected) corticosterone response. Frog body-condition was also correlated with the integrated corticosterone response and with urinary testosterone.

#### *2.4.1 Comparisons of baseline urinary corticosterone and testosterone, and the integrated corticosterone responses within and between sampling occasions*

$\text{Log}_{10}$ transformed baseline urinary corticosterone data were analysed using two way repeated measures analysis of variance (ANOVA) with time (0 – 8 h) and treatment (repeated sampling occasions) as the grouping factors. Comparisons between times within each treatment and between treatments for each time were examined with post-hoc Tukey's Multiple Comparison Test. $\text{Log}_{10}$ transformed baseline urinary testosterone data were analysed using One way repeated measures ANOVAs. The areas under the urinary corticosterone response curves were determined in Prism using the trapezoid rule and termed as the total integrated corticosterone metabolite response (Cockrem and Silverin, 2002). The total area under the graph minus the area attributed to baseline corticosterone metabolite concentrations at 0 h was expressed as the corrected integrated corticosterone metabolite response (Cockrem and Silverin, 2002). The integrated corticosterone metabolite responses

were expressed as pg/µg Cr.h. One way repeated measures ANOVAs were used to compare integrated corticosterone metabolite responses between the three sampling occasions.

### *2.4.2 Coefficients of variation*

Coefficient of variations (CV %, where CV = Standard Deviation/mean x 100) were used comparing variation in urinary corticosterone, and CVs in baseline and short-term corticosterone stress responses were calculated for each sampling occasion. Variation within and between frogs in parameters of their baseline corticosterone, short-term corticosterone stress responses and integrated corticosterone responses were compared.

### *2.4.3 Repeatability of baseline urinary corticosterone and testosterone, and short-term corticosterone stress responses*

Statistical repeatability is a measure that describes the proportion of variance in a variable that occurs among rather than within individuals. Repeatability for a variable can be calculated from a one way analysis of variance in which repeatability, *r*, is given by the formula: $r = s^2_A/( s^2 + s^2_A)$, where $s^2_A$ is the among ($_A$) group variance component and $s^2$ is the within ($_w$) group variance component. These variance components are calculated from the mean squares (MS) in the analysis of variance as $s^2 = MS_W$ and $s^2_A = (MS_A - MS_W)/n_0$ where $n_0$ is a coefficient related to the sample size per group in the analysis of variance. Statistical repeatabilities of corticosterone metabolite variables were calculated by the method of (Lessells and Boag, 1987) and was used in recent studies (Cockrem et al., 2009; Narayan et al., 2013; Narayan et al., 2012d).

The frogs were ranked on each of the three occasions according to their 0, 4 and 8 h urinary corticosterone concentrations and to their corrected integrated corticosterone responses over

the duration of the capture handling stressor. Corrected integrated corticosterone responses represent the amount of corticosterone secreted over the 24 h response period in addition to the corticosterone that would have been secreted if the initial corticosterone concentrations had been maintained for 24 h. Spearman correlations were done between the first with second and third sampling occasions for urinary corticosterone concentrations at 0, 4 and 8 h and for corrected integrated corticosterone responses.

## 3.0 Results

### *3.1 Comparisons of baseline urinary corticosterone and testosterone, and the integrated corticosterone responses within and between sampling occasions*

Urinary corticosterone metabolite stress responses of the individual male Fijian ground frogs were consistent between the three sampling occasions (Fig. 1). Some frogs showed consistently high urinary corticosterone responses (for example, frog #8 in Fig. 2), and others showed consistently low urinary corticosterone metabolite responses (for example, frog #5 in Fig. 2).

Mean baseline (0 h) urinary corticosterone metabolite concentrations were not significantly different between the three repeated sampling occasions, which indicated that corticosterone metabolite concentrations had returned to baseline between the first, second and third sampling occasions. This clarifies that the period between repeat sampling was sufficient to mitigate any confounding effects of the toe-clipping marking technique (Narayan et al., 2011a). Mean urinary corticosterone metabolite concentrations at 2, 4, 6, and 8 h period of the short-term capture and handling protocol did not differ significantly between the first and second sampling occasions or between the second and third occasions but differed

significantly at 4 h and 6 h between the first and third sampling occasion ($p < 0.05$ for all comparisons).

Comparisons of sampling times (0, 2, 4, 6 or 8 h) between the three repeated sampling occasions showed significant effects of sampling occasions ($F_{2, 190} = 22.8$, $p < 0.001$) and time ($F_{4, 95} = 84.6$, $p < 0.001$), and a significant interaction between sampling occasions and time ($F_{8, 95} = 2.5$, $p < 0.05$). Comparisons between sampling times (0, 2, 4, 6 or 8 h) within each repeated sampling occasion showed significant effects of time ($F_{4, 228} = 312.10$, $p < 0.001$), no significant effect of sampling occasion ($F_{4, 95} = 1.40$, $p > 0.05$) and no significant interaction between sampling times and sampling occasion ($F_{8, 95} = 0.40$, $p > 0.05$). For each of the three sampling occasions, mean urinary corticosterone metabolite concentrations were significantly different between all time periods ($p < 0.05$ for all three sampling occasions) except for 4 h versus 8 h (for the first sampling occasion only) and 6 h versus 8 h (for the first, second and third sampling occasions).

Mean urinary testosterone metabolite levels were significantly different between the three repeated sampling occasions (One way repeated measures ANOVA $F_3 = 40.0$, $p < 0.0001$). Mean urinary testosterone concentration for each repeated sampling occasion was as follows: Repeat 1 = 105.9 $\pm$ 5.72 pg/µg Cr (n = 20); Repeat 2 = 108.3 $\pm$ 5.77 pg/µg Cr (n = 20); Repeat 3 = 123.0 $\pm$ 6.48 pg/µg Cr (n = 20).

Mean total integrated corticosterone metabolite responses were significantly different from the corresponding mean corrected integrated corticosterone metabolite responses between the three repeated sampling occasions (One way repeated measures ANOVA $F_3 = 57.2$, $p < 0.0001$ and $F_3 = 27.6$, $p < 0.0001$; Fig. 3). Post-hoc comparisons showed that the mean

integrated corticosterone metabolite responses were not significantly different between the first, second and third sampling occasions.

### *3.2 Coefficients of variation*

Measures of variation between and within frogs were obtained by calculating coefficients of variation (CVs) for the individual frog means, and determining mean values of the CVs for individual frogs (Table 1). Variation between frogs, measured by the CVs of the mean of the individual frog means, was greater for the corrected integrated corticosterone concentrations than the variation in the total integrated corticosterone response. Variation within frogs, measured from the CVs of individual frog, was also higher for the corrected integrated corticosterone concentrations than the variation in the total integrated corticosterone response. Variation between frogs in baseline urinary corticosterone concentrations was greater than variation within frogs (Table 1).

### *3.3 Repeatability of baseline urinary corticosterone and testosterone, and short-term corticosterone stress responses*

Baseline (0 h) urinary corticosterone metabolite concentrations of the male frogs were highly statistically repeatable ($r = 0.973$, $p < 0.0001$). Short-term urinary corticosterone metabolite stress responses had high statistical repeatabilities at 2 h ($r = 0.862$, $p < 0.0001$), 4 h ($r = 0.861$, $p < 0.0001$), 6 h ($r = 0.820$, $p < 0.0001$) and 8 h ($r = 0.926$, $p < 0.001$), and for the total and corrected integrated corticosterone metabolite responses ($r = 0.867$, $p < 0.0001$; $r = 0.870$, $p < 0.0001$ respectively). Baseline (0 h) urinary testosterone metabolite concentrations of the male frogs were also highly statistically repeatable ($r = 0.78$, $p < 0.0001$). Spearman correlations indicated that corrected corticosterone responses of the toads were more consistent between sampling occasions than were baseline urinary corticosterone

concentrations (Table 2). Furthermore, the 4 h and 8 h ranks were also more consistent than baseline corticosterone (see Table 2).

*3.4 Correlations between baseline urinary corticosterone, urinary testosterone, acute corticosterone response and body condition*

Baseline urinary corticosterone levels were negatively correlated with urinary testosterone (Spearman $r$ = - 0.07, $p > 0.05$; Fig. 4A) and body condition (Spearman $r$ = -0.04, $p > 0.05$; Fig. 4B). Urinary testosterone levels were significantly negatively correlated with body condition (Spearman $r$ = -0.23, $p < 0.001$; Fig. 4C). The corrected integrated corticosterone responses were significantly negatively correlated with baseline urine corticosterone (Spearman $r$ = -0.32, $p < 0.001$; Fig. 5A) but positively correlated with body condition (Spearman $r$ = +0.08, $p < 0.05$; Fig. 5B) and urinary testosterone (Spearman $r$ = +0.04, $p > 0.05$; Fig. 5C).

**4.0 Discussion**

The results have demonstrated that baseline corticosterone and short-term corticosterone metabolite stress responses (assessed via urinary EIA) are repeatable during breeding in adult male individuals of the Fijian ground frog (*Platymantis vitiana*) under uncontrolled natural conditions. Furthermore, the significance of the baseline and short-term corticosterone stress responses were tested by correlating each physiological response variable with urinary testosterone and body condition. Baseline urinary corticosterone showed non-significant negative correlation with body condition and urinary testosterone. The baseline urinary corticosterone was also significantly negatively correlated with the corrected integrated corticosterone response (index of the acute corticosterone response). Urinary testosterone was also significantly negatively correlated with body condition. While the corrected

integrated corticosterone response was slightly positively correlated with body condition and urinary testosterone.

The significant negative correlation between urinary testosterone and body condition suggests that high levels of testosterone during breeding could lead to energy depletion in frogs thus contributing to a decrease in body condition. This negative correlation between testosterone and body condition has also been shown in some species of birds (Geslin et al., 2004). According to (Pérez-Rodríguez et al., 2006), the relationship between testosterone and body condition could also be mediated by the stress endocrine system, especially the circulating levels of corticosterone (Wingfield and Ramenofsky, 1999). (Pérez-Rodríguez et al., 2006) also stated that the negative correlation between corticosterone and testosterone could be the physiological link between body condition and specific behaviours (such as mating). The negative correlation between urinary testosterone and body-condition could also reflect the energetically costly nature of the breeding process in male frogs. This relationship has been demonstrated in the male tungara frogs [*Physalaernus pustulusu*] (Marler and Ryan, 1996). For example, during the breeding season, male tungara frogs spend little time foraging while calling at the breeding sites (Ryan, 1985) Calling is energetically costly and male frogs need to replace their lost energy in order to survive (Marler and Ryan, 1996). Male individuals of the Fijian ground frogs also call during the breeding season from elevated perches in trees, low shrubs and rocks. The advertisement call is a short, sharp whistle (*Narayan, pers comm*). It is possible that the acute corticosterone responses help the male frogs in re-gaining energy during breeding that enables them to complete the mating process. Thus, even though transient negative correlations were found between baseline urinary corticosterone and body condition, the corrected integrated corticosterone responses were positively correlated with urinary testosterone and body condition. The acute corticosterone stress responses may

represent the physiological trait of male frogs that enables them to replenish their energy reserves between calling and foraging. For example, it has been shown in the male tungara frogs that individuals in a chorus, where food is limited, have to leave the chorus to begin foraging to fulfil the minimal energy threshold of lipid and glycogen stores (Marler and Ryan, 1996). It is known that the acute corticosterone response promotes locomotion in amphibians and reptiles (Homan et al., 2003; Tyrell and Cree, 1998). The non-significant negative correlation between baseline urinary corticosterone and body condition provides some support to the 'cort-fitness' hypothesis, whereby an individual or population in worse condition or under reduced relative fitness will have higher levels of baseline corticosterone, as compared to individuals or populations with lower levels of baseline corticosterone (Bonier et al., 2009a). It is most likely that the acute corticosterone response is adaptive and positively related to fitness and reproduction in amphibians as shown in many other animals [See detailed review by (Breuner et al., 2008)]. Whether acute corticosterone responses are required for energy supply only during reproduction or in combination for survival after breeding in male frogs warrants further investigation.

The repeatability of baseline urinary corticosterone ($r$ = 0.970) and short-term corticosterone stress responses ($r$ = 0.870) is comparable to the repeatability data from our previous studies. Earlier, we reported repeatability of baseline urinary corticosterone and short-term corticosterone stress responses in the cane toad (*Rhinella marina*) as follows: captive male toads [baseline corticosterone $r$ = 0.630; corrected integrated corticosterone response $r$ = 0.728] (Narayan et al., 2012d). Wild male toads [baseline corticosterone $r$ = 0.877; corrected integrated corticosterone response $r$ = 0.743] (Narayan et al., 2013). Repeatability of corticosterone titres in amphibians seems to be much higher than those reported for other animals during breeding. This is especially impressive given that higher stress responses are

generally more variable, especially during reproduction (Adams et al., 2005). Significantly repeatability of baseline corticosterone was reported in both sexes of the great tit (*Parus major*) [$r$ = 0.26, $p$ = 0.025] during breeding and the stress-induced corticosterone concentrations were not repeatable during amongst seasons or between years (Ouyang et al., 2011a). In female individuals of the tree swallows (*Tachycineta bicolor*), significant repeatability was only seen in baseline corticosterone ($r$ = 0.44, $p$ = 0.004) within the breeding season. Based on their results, (Ouyang et al., 2011a) suggested that baseline and stress induced glucocorticoid titres are highly plastic physiological traits that are linked closely to animal behaviour and fitness. For example, baseline corticosterone levels increase over the breeding season and correlate with high fitness in both house sparrows (*Passer domesticus*) and tree swallows (Bonier et al., 2009b; Ouyang et al., 2011b). Furthermore, (Ouyang et al., 2011a) provided a detailed account of the potential reasons for differences in repeatability calculations between studies, including differences in sample sizes (small sample sizes between repeated sampling of wild animals), differences in acute stress response sampling methods and sampling times, species differences in maximal response to capture and handling, and also due to environmental factors and internal variables including how individuals perceive and process stressors. In another study, (Cook et al., 2012) found significant repeatability in stress induced plasma cortisol concentrations in the bluegill sunfish (*Lepomis macrochirus*) [$r$ = 0.432]. There was also considerable intra-individual variation in acute plasma cortisol responses, which were explained mainly by the body-condition of individual fishes (Cook et al., 2012). In our study, CVs for the integrated corticosterone responses for both within and between frogs were highest for the corrected integrated corticosterone response in comparison to the total integrated corticosterone response. The total integrated corticosterone response accounts for the variation in both baseline (0 h) corticosterone and during time periods (2 - 8 h) over the duration of the short-

term capture and handling stressor. The corrected integrated corticosterone response represents the increase in urinary corticosterone beyond the initial baseline concentrations. A greater variation in the corrected integrated corticosterone response highlights that urinary corticosterone concentrations during the period of the capture and handling stressor was more variable than the baseline urinary corticosterone. In a study by (Rensel and Schoech, 2011), it was shown that relative corrected integrated plasma corticosterone responses but not baseline were statistically repeatable ($r$ = 0.5) across all ages of the Florida scrub-jay (*Aphelocoma coerulescens*).

The results from our study highlight that baseline corticosterone and short-term corticosterone stress responses are highly repeatable during the breeding season in amphibians. This may reflect the seasonal changes in baseline and stress induced corticosterone, as well as reproductive hormone levels as the breeding season approaches for the ground frogs. It is possible that glucocorticoids and reproductive hormones in amphibians are also highly plastic (highly variable with low repeatability) over seasons and amongst years, which reflects that they are necessary for day-day living and survival. We hypothesise that as a result of past experience and the environment, the reproductive and stress hormone levels become fine-tuned and adaptive (highly repeatable) during the breeding period. Thus repeatability of reproductive and stress hormone titres should be considered as an important prerequisite for linking their expression to reproductive fitness and survival during crucial life-history phases, such as breeding in amphibians. It has been shown that amphibians and reptiles have shown that corticosterone levels generally increase during the breeding season (Licht et al., 1983; Wingfield and Grimm, 1977), which enhances metabolism and energy supply during breeding (Greenberg and Wingfield, 1987).

Overall, the repeatability in baseline and short-term corticosterone metabolite stress responses could be associated with the energy budget requirements for maintaining a good condition for successful breeding and survival in the male ground frogs. Future, studies of repeatability of corticosterone in free-living amphibian populations should measure these physiological response variables in combination with fitness parameters, such as lifetime reproductive fitness and reproductive output in order to better understand the ecological importance of corticosterone during breeding. Some interesting questions that non-invasive endocrinology methods could help to solve in future would be to test whether repeatability of baseline corticosterone and acute corticosterone responses are intrinsic features of a stable environment, or do individual responses reflect different behaviours, genotypes and how might this be linked to fitness and population resilience. We also suggest future research directions towards manipulative experiments (such as phenotypic engineering) to better understand the functional role of corticosterone in promoting trait performance and ideally for causing changes in fitness. Furthermore, we recommend for studies using plasma and/or non-invasive glucocorticoid titres to consider measuring corticosterone-binding globulin (CBG) levels to understand the repeatability of biologically active (free hormones). Future studies should also incorporate other physiological/physical metrics such as immune function, oxidative stress and body mass changes (Breuner et al., 2013), that could be used to assess how repeatable individual variation in corticosterone (total and free levels) and CBG could be ecologically important to male anurans.

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**Figure legends**

Figure 1. Individual urinary corticosterone metabolite responses of free-living adult male Fijian ground frogs (n = 20) sampled on three occasions at intervals of 14 days. Data are represented as baseline (0 h) and individual frog responses over 8 h periods at intervals of 2 h for each sampling occasion. Data are shown for 6 male frogs to show some of the different patterns of short-term corticosterone responses of the male ground frogs.

Figure 2. Individual urinary corticosterone metabolite responses of free-living adult male Fijian ground frogs (n = 20) sampled on three occasions at intervals of 14 days. Frogs with one of the highest (frog #8) and lowest (frog #5) individual corticosterone metabolite stress responses over the three sampling occasions are shown with dash and dotted lines respectively.

Figure 3. Total and corrected integrated corticosterone metabolite responses of 20 male Fijian ground frogs sampled on three occasions at intervals of 14 days. Data are represented as means ± S.E, h = hour.

Figure 4. Spearman rank correlations (*r*) of baseline urinary corticosterone with urinary testosterone (Fig. 4A) and body-condition (Fig. 4B), and correlation between urinary testosterone and body condition (Fig. 4C). Data for the three repeated sampling occasions have been pooled together for each correlation.

Figure 5. Spearman rank correlations (*r*) of the corrected integrated corticosterone response with baseline urinary corticosterone (Fig. 5A) and body-condition (Fig. 5B), and correlation between the corrected integrated corticosterone response and urinary testosterone (Fig. 5C).

Data for the three repeated sampling occasions have been pooled together for each correlation.

**Table Legends**

Table 1 Variation in urinary corticosterone metabolite responses within and between free-living male Fijian ground frogs sampled on three occasions at intervals of 14 days.

Table 2. Spearman correlations between the first with second and third sampling occasions for urinary corticosterone concentrations at 0, 4 and 8 h, and for corrected integrated corticosterone responses. Male ground frogs (n = 20) were ranked for each variable on each occasion.

**FIGURE 1**

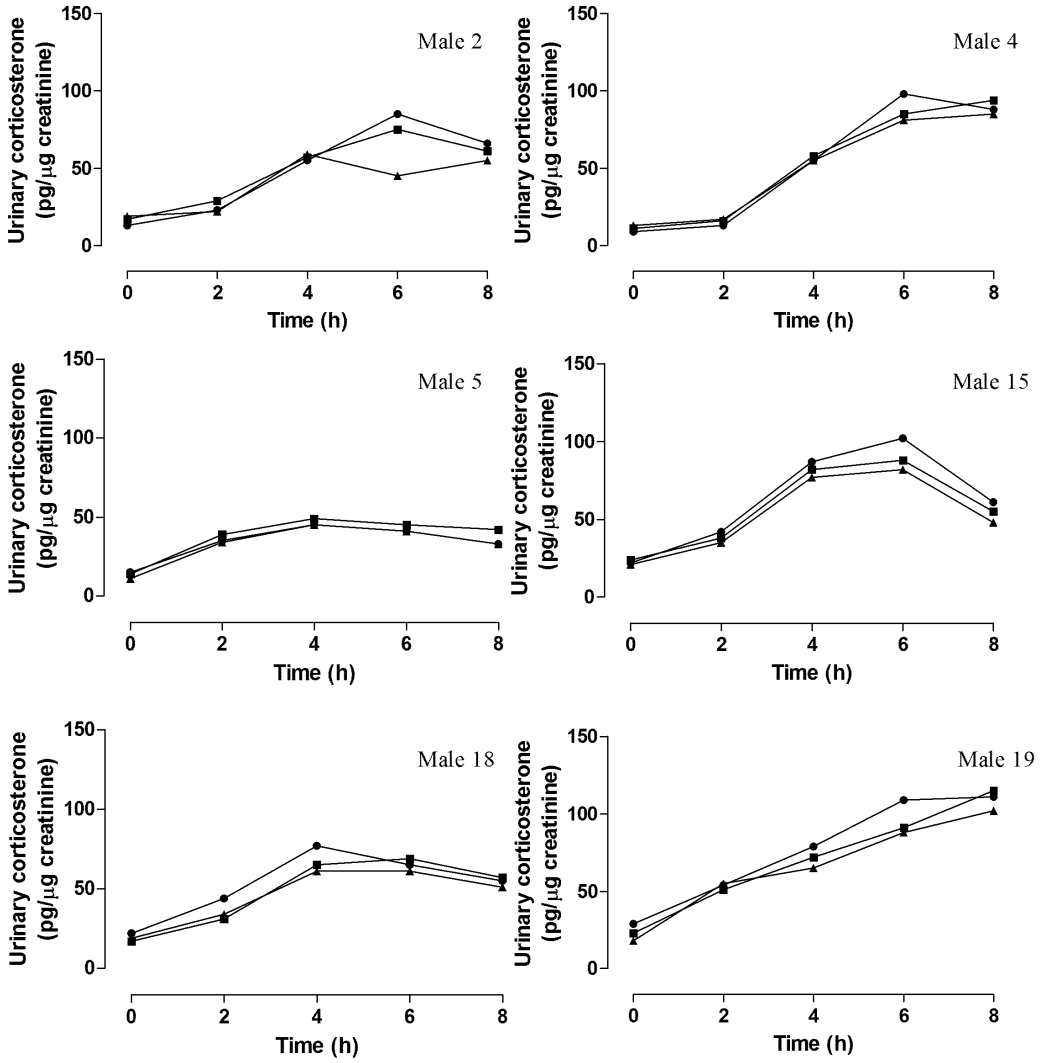
Male 2
Male 4
Male 5
Male 15
Male 18
Male 19
Urinary corticosterone (pg/μg creatinine)
Time (h)
0
50
100
150
0
2
4
6
8

**FIGURE 2**

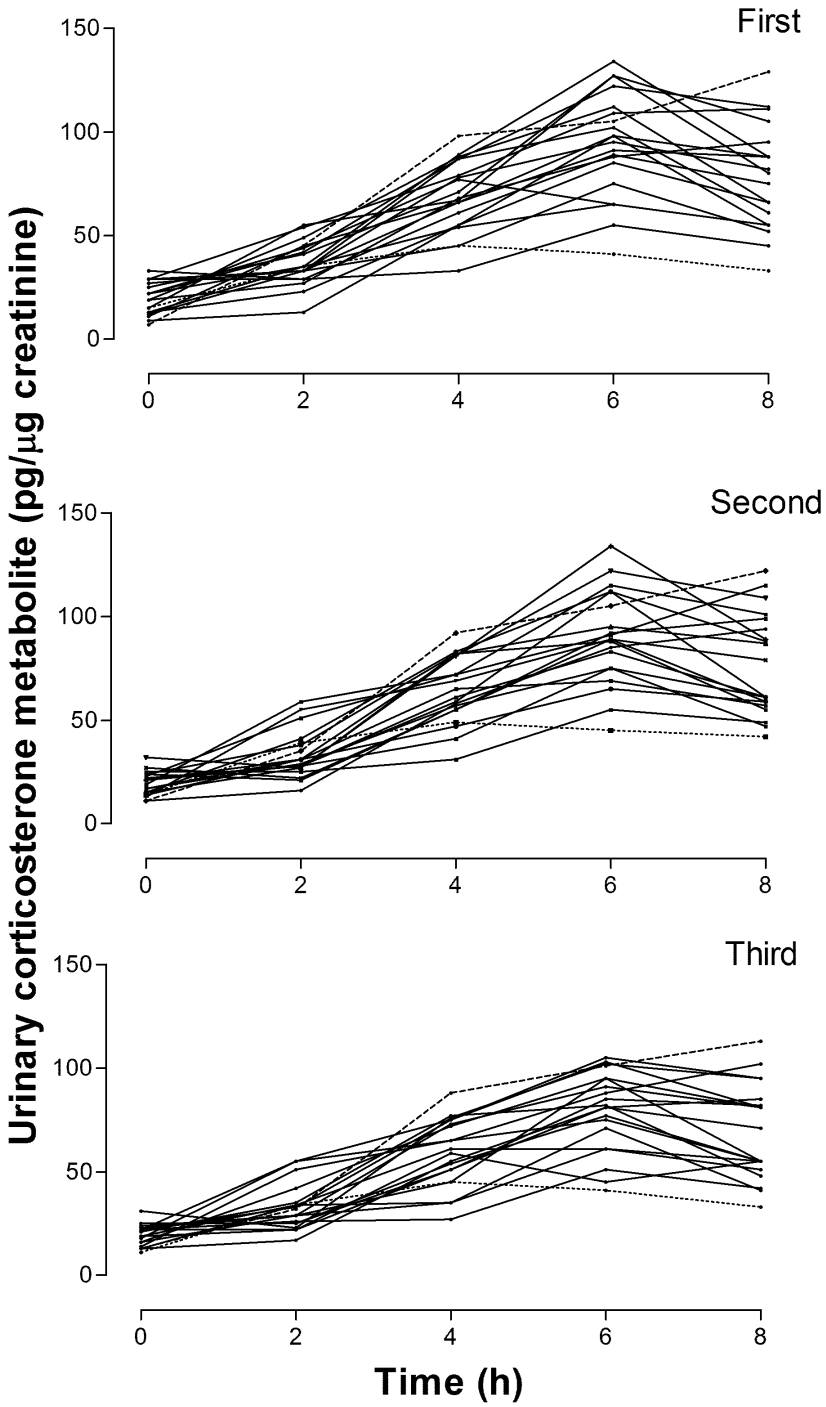
First
Second
Third
150
100
50
0
0
2
4
6
8
Urinary corticosterone metabolite (pg/μg creatinine)
Time (h)

**FIGURE 3**

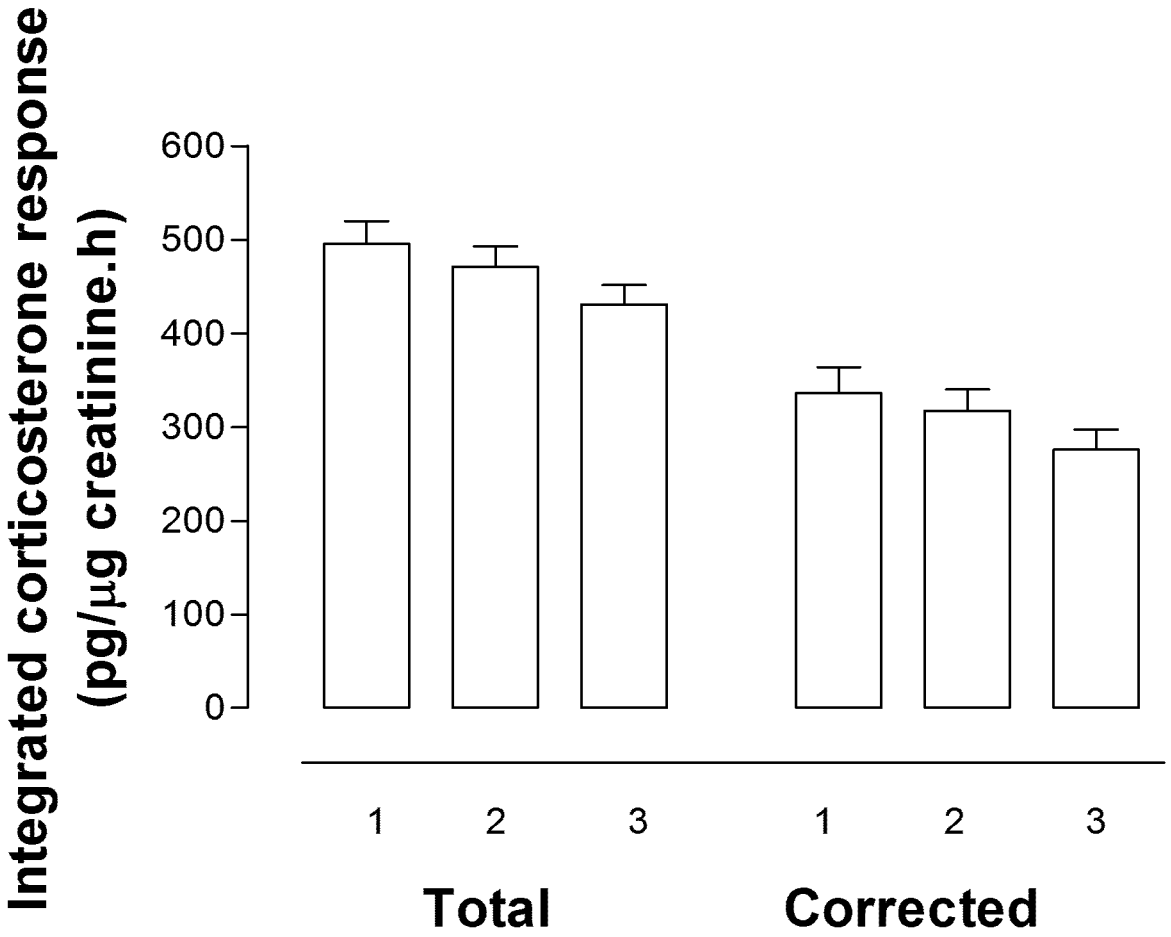
Integrated corticosterone response
(pg/µg creatinine.h)
600
500
400
300
200
100
0
1
2
3
1
2
3
Total
Corrected

**FIGURE 4**

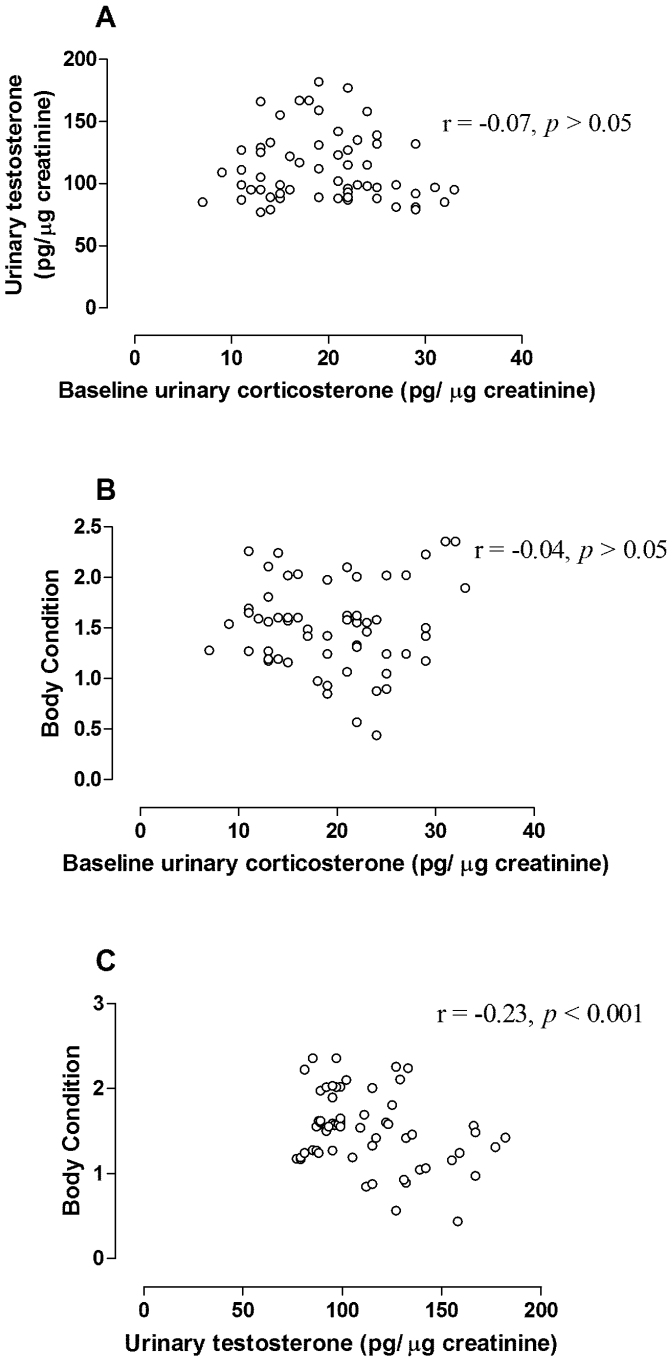
A
Urinary testosterone (pg/μg creatinine)
200
150
100
50
0
r = -0.07, p > 0.05
0
10
20
30
40
Baseline urinary corticosterone (pg/ μg creatinine)
B
Body Condition
2.5
2.0
1.5
1.0
0.5
0.0
r = -0.04, p > 0.05
0
10
20
30
40
Baseline urinary corticosterone (pg/ μg creatinine)
C
Body Condition
3
2
1
0
r = -0.23, p < 0.001
0
50
100
150
200
Urinary testosterone (pg/ μg creatinine)

**FIGURE 5**

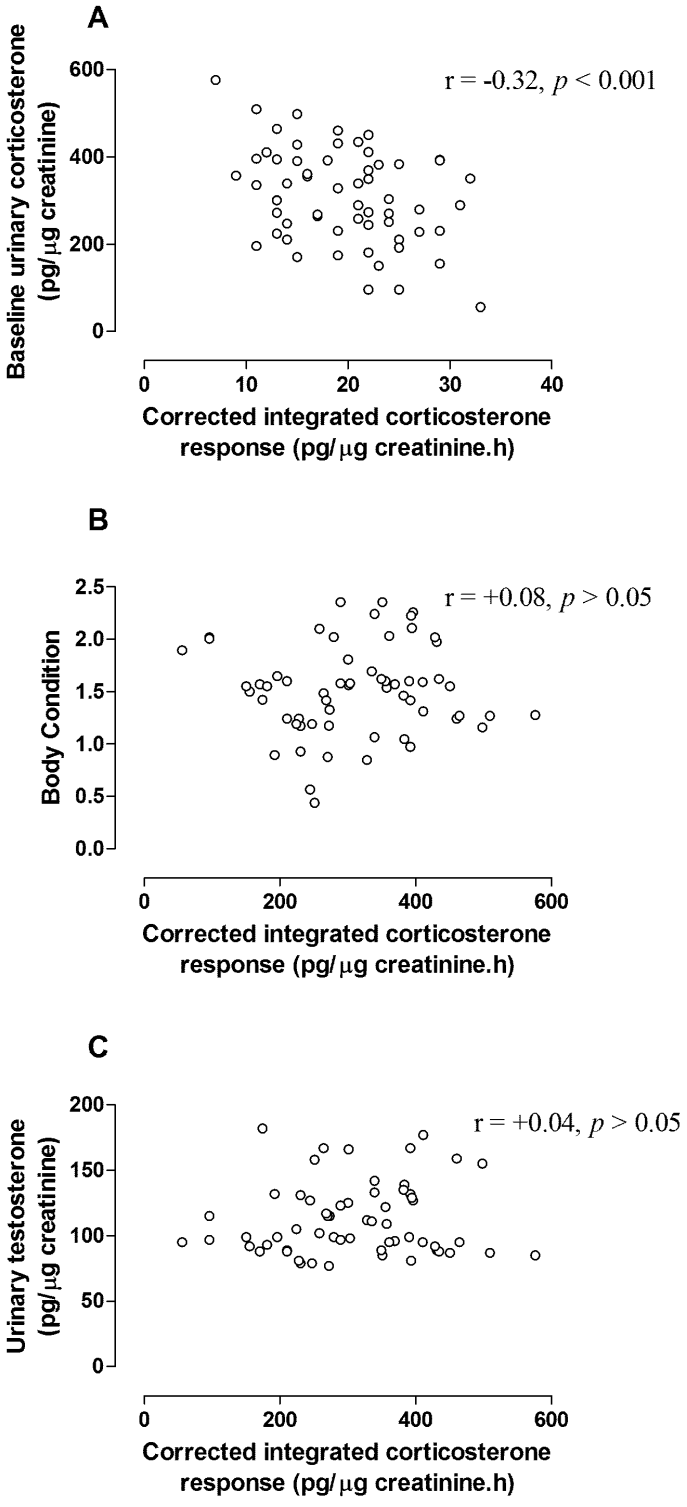
A
Baseline urinary corticosterone (pg/μg creatinine)
600
400
200
0
r = -0.32, p < 0.001
0
10
20
30
40
Corrected integrated corticosterone response (pg/μg creatinine.h)
B
Body Condition
2.5
2.0
1.5
1.0
0.5
0.0
r = +0.08, p > 0.05
0
200
400
600
Corrected integrated corticosterone response (pg/μg creatinine.h)
C
Urinary testosterone (pg/μg creatinine)
200
150
100
50
0
r = +0.04, p > 0.05
0
200
400
600
Corrected integrated corticosterone response (pg/μg creatinine.h)

**Table 1.0**

| | Corticosterone at 0 h | Corticosterone at 4 h | Corticosterone at 8 h | Total integrated response (pg/µg creatinine.h) | Corrected integrated response (pg/µg creatinine.h) |
|---|---|---|---|---|---|
| *20 male frogs sampled on each three occasion (n = 60)* | | | | | |
| Mean | 19.58 | 64.08 | 74.18 | 465.83 | 309.70 |
| S.E. | 0.80 | 2.96 | 3.11 | 13.10 | 14.09 |
| CV (%) | 31.79 | 35.72 | 30.86 | 21.79 | 35.24 |
| *Variation between male frogs (means of the individual frog means; n = 20)* | | | | | |
| Mean | 19.58 | 64.08 | 74.18 | 465.83 | 309.07 |
| S.E. | 1.31 | 3.62 | 5.33 | 21.99 | 23.67 |
| CV (%) | 29.95 | 25.29 | 32.16 | 21.11 | 34.18 |
| *Variation within male frogs (means of the individual frog CVs; n = 20)* | | | | | |
| Mean | 13.63 | 9.28 | 8.22 | 7.43 | 12.58 |
| S.E. | 1.53 | 1.21 | 1.14 | 0.65 | 1.49 |

**TABLE 2.0**

| Correlations/Rank | 0 h rank | 4 h rank | 8 h rank | Corr rank |
|---|---|---|---|---|
| 1st and 2nd repeats | | | | |
| r | 0.89 | 0.91 | 0.96 | 0.99 |
| *p* | < 0.001 | < 0.0001 | < 0.0001 | < 0.0001 |
| 1st and 3rd repeats | | | | |
| r | 0.75 | 0.83 | 0.93 | 0.92 |
| *p* | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |
| 2nd and 3rd repeats | | | | |
| r | 0.80 | 0.94 | 0.99 | 0.95 |
| *p* | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |