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Repeatability of baseline corticosterone and acute stress responses to capture, and patterns of reproductive hormones in vitellogenic and non-vitellogenic female Fijian ground frog (*Platymantis vitiana*)

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Abstract

The survival of animal species and individuals is largely determined by their ability to express physiological stress responses to predictable and unpredictable environmental challenges. Currently, there is no empirical evidence presenting the stress endocrine responses of female frogs during breeding between different reproductive groups. In this study, non-invasive urine sampling and standard capture and handling protocol were used to quantify baseline and short-term corticosterone stress responses in vitellogenic and non-vitellogenic female Fijian ground frog (*Platymantis vitiana*) during the annual breeding period. Urinary oestrogen and progesterone metabolites were also quantified in the same frog urine samples. Repeated sampling of the female frogs (n = 20) on three occasions enabled repeatability (r) of reproductive and stress hormones to be quantified. All female frogs generated urinary corticosterone responses to the standard capture and handling stressor. Both baseline and short-term corticosterone responses were significantly higher in magnitude in the vitellogenic females in comparison to the non-vitellogenic female frogs. Vitellogenic females also showed significantly higher levels of urinary oestrogen and progesterone metabolites in comparison to the non-vitellogenic females. Baseline urinary corticosterone, short-term corticosterone responses, urinary oestrogen and progesterone metabolites were highly repeatable for both female groups. The results highlight the importance of reproductive and stress hormones during the breeding period in female ground frogs. Future studies should determine the role of potential biological stressors (such as interactions with invasive species) that could be mediating the observed differences in stress endocrine responses of the vitellogenic and non-vitellogenic female frogs.

Key words: Female reproduction; corticosterone; *Platymantis vitiana*; stress; physiological stress response; Repeatability; Reproductive hormones

1.0 Introduction

Maximising the reproductive effort is paramount to the success of any animal species. Factors that can contribute to reproductive success can be related to life-history theory, which investigates behavioural and physiological traits, and strategic decisions that over a lifetime can impact organism fitness (Ricklefs and Wikelski, 2002). Reproduction varies between animal groups and species and many cases where animals only reproduce at certain times of the year, across years, or seasonally (Klose et al., 2006). Therefore, it is important to understand how animals cope with environmental challenges during the breeding period and how the physiological stress response varies between different reproductive groups. The stress endocrine system plays a crucial role in providing this physiological stress (hormone) response to environmental challenges in animals. Understanding the patterns and magnitudes of physiological stress hormone responses of animals during times of reproduction, specifically for amphibians in this case, is important. Approximately a third of amphibian species worldwide are currently experiencing major declines, facing extinction and have potentially already gone extinct (Stuart et al., 2004). Thus, it is particularly important for amphibian conservation to focus effort towards monitoring physiological stress in free-living populations and understanding the role that stress hormones play in amphibian reproduction. Non-invasive endocrinology is a tool that is used to measure and monitor the changes in hormone metabolite concentrations in animals with minimal disturbances. This relates to assessing stress hormones as indices of physiological stress responses to physical and psychological stressors in wild and captive animals (Cockrem, 2005). Non-invasive techniques of monitoring hormone concentrations move from using traditional invasive

techniques such as plasma blood sampling and use techniques revolving around mainly excreta (urine and faeces) as well as hair and saliva (Narayan, In-press). It is known that the stress hormone corticosterone plays key roles in metabolism, foraging behaviour, immune responses, interactions with reproductive hormones and mating displays in amphibians (Bliley and Woodley, 2012). The hypothalamo-pituitary-interrenal (HPI) axis is the main centre of the physiological stress hormone response in amphibians and also found in other lower vertebrates (Schreck, 2010). Corticotropin releasing hormone (CRH) is produced from the hypothalamus, which then acts upon the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) into the blood. The ACTH release results in the discharge of corticosterone from the interrenal cortex that is responsible for behavioural and physiological responses to particular stimuli (Cockrem, 2007; Narayan et al., 2011a). It is known that when a physiological stress hormone response occurs, non-essential functions at that time may be temporarily halted such as immune responses and reproductive hormone production (Bliley and Woodley, 2012). Corticosterone is particularly important for responses to competition, for mobilizing energy stores, and regulating physiological responses to biological stressors, such as predator encounters and even to extreme climatic events, such as storms (French et al., 2007; Romero, 2002).

In amphibians, the hierarchy of the hypothalamus pituitary gonadal axis (HPG) includes the main target organs such as gonads, liver and central nervous system (CNS). Endogenous and exogenous stimuli on the CNS lead to secretion of gonadotropin releasing hormone (GnRH), which is produced by cells of the hypothalamus. In turn, GnRH acts on the gonadotrophs of the pituitary, thus stimulating the secretion of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) into blood circulation. The gonadotropins increase synthesis and release of androgens. In male frogs, the main sources of androgens are the

testes, where androgen synthesis principally occurs in the interstitial (Leydig) cells. However, it must be noted that oestradiol is also produced in males but at very low levels (Duellman and Trueb, 1994). In female anurans, sex steroids are regulated by the same endocrine components via the HPG-axis as in males. However, the female ovary produces much higher levels of oestradiol compared to males because the enzyme aromatase converting testosterone into oestradiol is much more pronounced and active (Duellman and Trueb, 1994). Sex hormone production is one of the endogenous mechanisms that are in phase with gametogenesis and involves gonadal tissue [Sertoli cells and interstitial tissue in male testes, follicle cells and corpora lutea in female ovaries]. Oestradiol or oestrogen is the hormone responsible for the stimulation of yolk production [i.e. deposition of energy as yolk into developing eggs] (Ho, 1987). Vitellogenin is a female specific protein produced by the liver of non-mammalian vertebrates including amphibians and incorporated into eggs in the ovary where it forms the basis for egg yolk (Olmstead et al., 2009). Production of vitellogenin is induced by circulating oestradiol (Olmstead et al., 2009). Progesterone levels peak at ovulation (egg maturation). Generally during the active breeding season female frogs have high circulating plasma oestradiol and progesterone levels and the elevation of these hormones occurs when the female has mature eggs (Wilczynski et al., 2005). These correlations are also seen in other yolk producing vertebrates, including fish, reptiles and birds (Duellman and Trueb, 1994), implying an evolutionary highly conserved role of the reproductive hormones. Amphibians are a relatively understudied group, and the area of hormone to reproduction relationship is inspired by testable hypotheses based on studies of other terrestrial vertebrates. The ability to correlate physiological measures with estimates of reproductive success identifies areas of amphibian research that will profit from the continued attention (Houck, 1998; Sutherland, 1998).

The relationship between reproduction and stress is a common association, considering many anuran species may also exhibit territorial or competitive behaviour to reproduce, particularly in the case of males (Mendonça et al., 1985). In the case of seasonal breeders, the question still remains as to whether the corticosterone stress response is consistent in relation to different reproductive stages during the breeding season. Earlier, Coddington and Cree (1995) found that corticosterone responses in plasma to captivity stress in vitellogenic frogs of the southern brown tree frog (*Litoria ewingi*) did not significantly correlate with reproductive hormone levels including oestradiol, testosterone or progesterone. They suggested that the particular stage of vitellogenesis could potentially modulate the stress response to captivity stressor. They then hypothesised that female frogs with moderate to high oestradiol (in relation to stages of vitellogenesis) with elevated corticosterone may not actually inhibit sex steroid secretion, as opposed to the inverse relationship between testosterone and corticosterone demonstrated in some male anurans (Narayan et al., 2012c; Orchinik et al., 1988). Therefore, the question remains whether female frogs of different reproductive stages will express different levels of baseline and short-term corticosterone stress responses during the breeding period.

Not many studies have actually investigated physiological stress in female amphibians during breeding. One study by Homan et al. (2003) used repeated non-lethal blood sampling on spotted salamanders (*Ambystoma maculatum*) to examine sex and seasonal differences in baseline and corticosterone stress response to 30 min handling and restraint under undisturbed natural conditions. This study showed that female salamanders had a much higher baseline and short-term corticosterone stress response than males during the spring inbound pond migration. This study highlighted that corticosterone concentrations could vary depending perhaps on the reproductive stage and that responses in general for corticosterone

can be greater in relation to breeding related behaviours, explaining the variations seen between outbound and inbound migration (Homan et al., 2003). Urinary measurements of reproductive hormones (testosterone, oestrone and progesterone) have been assessed in free-living frog populations, such as in the Fijian ground frog (*Platymantis vitiana*) to demonstrate seasonal reproductive patterns (Narayan et al., 2010a). In this species, wild vitellogenic females have an annual reproductive hormone cycle, with peaks in both oestrone and progesterone from August to December. This supports that adult Fijian ground frogs have annual cycles of oocyte growth and regression with increased breeding related activity during the annual wet season (Narayan et al., 2010a). Repeated sampling of the same individuals during the breeding period and standard capture handling protocol could be used to determine the consistency in baseline and short-term stress hormone response. This concept is termed as repeatability, which signifies the consistency of any physiological, morphological or behavioural trait in animals (Lessells and Boag, 1987). The repeatability of baseline and short-term corticosterone responses to standard stressors has to be defined in terms of time, whether it be between samples, breeding season, seasons and years. Thus, it is important to use this tool (repeatability studies) to better understand how glucocorticoids actually function in survival during the breeding period.

The aim of this study was to assess the baseline and short-term urinary corticosterone responses to a standard capture and handling stressor in vitellogenic and non-vitellogenic female Fijian ground frogs (*Platymantis vitiana*) under natural conditions. It was hypothesised that the baseline and short-term corticosterone stress responses will be similar between the two female groups despite the difference in reproductive status. The levels of urinary oestrogen and progesterone metabolites, and their repeatabilities, were tested in these two female groups over three repeated sampling occasions.

2.0 Materials and Methods

2.1 Animals and urine sampling protocol

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 east off the coast of mainland Viti Levu, Fiji. Short-term corticosterone stress responses to a standard capture and handling protocol (5 min handling and placement inside plastic bags between urine sampling at 2 h intervals over 8 h) were measured in adult female frogs (n = 20) on three consecutive occasions (intervals of 14 days). Frogs were captured from 1900 – 2100 h during their annual breeding season in December 2011. All frogs were caught on the same night during each sampling occasion, with similar wet ground conditions throughout the sampling periods. Frogs were captured by hand as soon as they were located on the ground and a baseline urine sample was collected immediately. During urine collection, frogs were held above a 100 mm diameter sterile plastic cup and gently massaged to promote urination, which usually occurred within 1 min. Frogs were held manually for a maximum of 5 min and the volume of urine excreted by each frog varied from 200 µL to 3 mL. The ground frog is sexually dimorphic and adult females are on average are much larger than adult males [snout-vent length (SVL) ratio of male: female is 1: 3 or 30: 90 mm (Morrison, 2003; Narayan, 2008). Female frogs were classified as adult vitellogenic (gravid) or non-vitellogenic (non-gravid) based on the presence or absence of underbelly vitellogenic follicles (oocytes) and SVL. This was done by shining a light source through their underbelly abdomen, and creamy, round eggs were visible through their translucent skin. The smallest female caught with the ovarian follicles measuring >2 mm in diameter measured 60.1 mm. Therefore, frogs with SVL >60 mm and with vitellogenic follicles measuring >2 mm in

diameter were assigned as adult vitellogenic females (n = 10). Frogs with SVL >60 mm and with no visible vitellogenic follicles were assigned as adult non-vitellogenic females (n = 10).

2.2 Short-term capture and handling stress protocol

For the short-term corticosterone stress response measurements, a second urine sample was collected from each frog exactly 2 h after initial capture on each sampling occasion. Earlier, it was shown that urinary corticosterone concentrations in anuran amphibians increased significantly within 2 h after initial capture (Narayan et al., 2011b; Narayan et al., 2010b; Narayan et al., 2012a). After the second urine sample was collected, each frog was held for 5 min and then placed in a resealable plastic bag and transferred back into individual plastic containers. Frogs were removed from the plastic containers 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 searched for and re-captured on the remaining sampling occasions at intervals of 14 days (the effects of toe-clipping on the HPI-axis generally subsided in less than 10 days, Narayan pers comm.) at the same times each night as the first sampling occasion. All 20 female frogs were successfully re-captured within one night during the second and third sampling occasions. All female frogs were subjected to the standard capture and handling protocol on the second and third sampling occasions as described earlier. The underbelly oocytes of the female frogs were inspected and frogs were released *in-situ* upon completion of the study.

2.3 Urinary enzyme-immunoassays

A corticosterone (CORT) enzyme-immunoassay (EIA) that was previously validated for the Fijian ground frog (Narayan et al., 2010b) was used to measure corticosterone metabolite

concentrations (referred to as urinary corticosterone) in female ground frog urine. The EIA used a polyclonal anticorticosterone antiserum (CJM06) diluted 1: 45 000, horseradish peroxidase conjugated corticosterone label diluted 1: 120 000 and corticosterone standards (1.56–400 pgwell^{-1}). The different types of corticosterone metabolites in frog urine have not yet been quantified thus based on the cross reactivities of the EIA reagents used (100% with corticosterone, 0.9% with tetrahydrocorticosterone metabolite), the likely primary hormone being measured is corticosterone but does not rule out contributions even in a very small way from other metabolites (Narayan et al., 2010b). Concentrations of urinary oestrone (EC) metabolites were determined using a polyclonal anti-oestrone antiserum (R522/2) diluted 1: 45 000, horseradish peroxidase-conjugated oestrone glucuronide label diluted 1: 45 000 and oestrone glucuronide standards (0.39–100 pg/well). Concentrations of urinary progesterone (P) metabolites were determined using a monoclonal anti-progesterone antiserum (CL425) diluted 1: 15,000, horseradish peroxidase conjugated progesterone label diluted 1: 40 000 and progesterone standards (0.39–100 pg/well). The antibody cross-reactions have been reported for oestrone (R522/2) as 60.1% with oestradiol-17b and over 100% with oestrone conjugate (Callard, 1978) and for progesterone (CL425) as > 50% with most 4-pregnene- and 5-a pregnan-metabolites (Graham et al., 2001; Szymanski et al., 2006).

The plates were coated with 50 µL of antibody in EIA coating buffer (50 mM bicarbonate buffer, pH 9.6) and incubated overnight (12 – 15 h) at 4^{o} C. For all assays, standards, internal controls and urine samples were diluted in EIA buffer (39 mM $NaH_2(PO_4)_2$-H_2O, 61 mM $NaHPO_4$, 15 mM NaCl and 0.1% bovine serum albumin, pH 7.0). For all assays, 50 µL of standards, internal controls and urine samples were added to each well of the coated Nunc Maxi-Sorp^{TM} plates. About 50 µL of the corresponding horseradish peroxidase label was then added to each well, and the plates incubated at room temperature for 2 h. Plates were washed

and 50 µL of a substrate solution (0.01% tetramethylbenzidine and 0.004% hydrogen peroxide in 0.1 M acetate citric acid buffer, pH 6.0) was added to each well. Stop solution (50 µL of 0.5 mol/L H_2SO_4) was added based on the visual inspection of plates so that the optical density of the zero wells would read between 0.7 and 1 (usually after at least 10 min incubation at room temperature). Nonspecific binding was accounted for by subtracting the blank absorbance from each reading. Standard curves were generated and a regression line fitted by the method of least squares and used to determine hormone concentrations in the frog urine samples.

2.4 Enzyme immunoassay validations

Serial dilutions of pooled frog urine were parallel to standard curves for the three steroids (EC, P and CORT). The quantitative recovery of each steroid was measured by adding different amounts of steroid standards to pooled frog urine. Recoveries of EC, P and CORT were $y = 0.875x + 0.479$, $r^2 = 0.966$, $n = 6$; $y = 0.868x + 0.234$, $r^2 = 0.997$, $n = 6$; and $y = 1.019x - 0.833$, $r^2 = 0.991$, $n = 6$, respectively. Assay sensitivities for EC, P and CORT were 0.6 ± 0.1 (n = 21), 1.85 ± 0.27 (n = 15) and 1.11 ± 0.26 pg/well (n = 10) respectively. Urinary concentrations of CORT, EC and P were standardised to creatinine (Cr) levels to control for water content (Narayan et al., 2010a) and were reported as pg/µg Cr.

2.5 Statistics

Statistical analyses were performed using SYSTAT version 13. All of the data were tested for normality using the D'Agostino & Pearson omnibus normality test. Body condition (BC) was calculated using Fulton‘s index [$K=M/L^3$, where M = body-weight and M = snout-vent length] (Peig and Green, 2010). Data are presented as individual points or as mean $\pm$ S.E. Probability values of $p < 0.05$ were considered to be significant.

2.5.1 Comparison of baseline urinary corticosterone between reproductive status, repeats and body-condition

Baseline urinary corticosterone concentrations were compared between the reproductive status (vitellogenic and non-vitellogenic females) using a General Linear Model (GLM) with repeats (1, 2 and 3), body-condition and reproductive status (vitellogenic or non-vitellogenic) as the explanatory variables. Post-hoc comparisons between the reproductive status with baseline corticosterone and body-condition were done using Kruskal-Wallis Test. Pearson correlation was done between baseline urinary corticosterone and body-condition.

2.5.2 Comparison of short-term urinary corticosterone responses between reproductive status, time and repeats

Short-term urinary corticosterone responses were analysed using 3-way Analysis of Variance (ANOVA) with reproductive status, time (0, 2, 4, 6 and 8 h) and repeat (1, 2 and 3) as the factors. Interaction terms in the 3-way ANOVA included Reproductive status*Repeat, Reproductive status*Time, Repeat*Time and Reproductive status*Repeat*Time. Post-hoc comparison between reproductive status and short-term corticosterone responses was done using Kruskal-Wallis Test.

2.5.3 Comparison of baseline urinary oestrogen and progesterone between reproductive status, repeats and body-condition

Baseline urinary oestrogen and progesterone concentrations were compared between the reproductive status (vitellogenic and non-vitellogenic females) using two separate General Linear Models (GLM) with repeat, body-condition and reproductive status as the factors. Post-hoc comparisons between the reproductive status with baseline oestrone conjugate and progesterone were done using Kruskal-Wallis Test. Pearson correlations were done between

body-condition with oestrone conjugate and progesterone. Pearson correlations were also done between baseline urinary corticosterone with oestrone conjugate and progesterone, and also between oestrone conjugate and progesterone.

2.5.4 Repeatability

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 reproductive and stress hormones variables were calculated using an established method (Lessells and Boag, 1987) and also used in recent repeatability studies (Cockrem et al., 2009; Narayan et al., 2012b; Narayan et al., 2013a).

3.0 Results

3.1 Reproductive status, body-condition and reproductive hormones in the female frogs

The non-vitellogenic females carried follicles measuring on average <2 mm in diameter throughout the three sampling periods. The average number of follicles observed in the vitellogenic females was 21.53 $\pm$ 0.91 (repeat 1), 24.60 $\pm$ 0.89 (repeat 2) and 26.2 $\pm$ 0.86 (repeat 3). The diameter of follicles for the vitellogenic females ranged from 3 – 4 mm (repeat 1), 4 – 5 mm (repeat 2) and 5 – 6 mm (repeat 3). There was no significant change in the body condition (BC) of the vitellogenic female frogs during the sampling period ($F_{2, 29}$ =

1.187, $p > 0.05$). There was a small increase in body condition of the non-vitellogenic female frogs during the sampling period ($F_{2, 29} = 5.022$, $p < 0.05$). Post-hoc comparison of mean body-condition of non-vitellogenic females between the second and third sampling period was significant (BC repeat 2 = 0.93 c.f. BC repeat 3 = 0.95; $p < 0.05$). Pairwise comparison of mean body-condition with the reproductive status showed that body-condition was significantly different between the vitellogenic and non-vitellogenic females ($p < 0.001$). Pearson correlations were significant between body-condition with baseline urinary oestrone conjugate ($r = 0.544$) and progesterone metabolites ($r = 0.498$).

The GLM analysis showed a significant effect of reproductive status on baseline urinary oestrone conjugate ($F_{4, 55} = 91.80$, $p < 0.0001$). However, the repeats and body condition did not significantly affect baseline urinary oestrone conjugate ($p > 0.05$ for both comparisons). Vitellogenic females had much higher (almost double) concentrations of urinary oestrone in comparison to the non-vitellogenic females throughout the three repeated sampling periods (Fig. 1A - Mean urinary EC repeat 1: 653.4 $\pm$ 29.96 pg/µg Cr c.f. 302.6 $\pm$ 15.06 pg/µg Cr; repeat 2: 671.8 $\pm$ 31.63 pg/µg Cr c.f. 297.9 $\pm$ 13.86 pg/µg Cr; repeat 3: 703.2 $\pm$ 33.80 pg/µg Cr c.f. 336.2 $\pm$ 14.76 pg/µg Cr). There was a significant difference in mean urinary oestrone concentrations of the vitellogenic female frogs throughout the sampling period (Fig. 1A; $F_{2, 29} = 18.42$, $p < 0.001$). The post - hoc comparison showed significant difference in mean urinary oestrone concentrations for vitellogenic females between repeats 1 and 3 and between repeats 2 and 3 ($p < 0.05$; Fig. 1A). Likewise, for non-vitellogenic females there was a significant difference in mean urinary oestrone concentrations throughout the sampling period (Fig. 1A; $F_{2, 29} = 15.31$, $p < 0.001$). The post - hoc comparison showed significant difference in mean urinary oestrone concentrations of non-vitellogenic females between repeats 1 and 3 and between repeats 2 and 3 ($p < 0.05$; Fig. 1A).

The GLM analysis showed a significant effect of reproductive status on baseline urinary progesterone ($F_{4, 55}$ = 130.74, p < 0.0001). However, the repeats and body condition did not significantly affect baseline urinary progesterone (p > 0.05 for both comparisons). Vitellogenic females had much higher (almost triple) concentrations of urinary progesterone in comparison to the non-vitellogenic females throughout the three repeated sampling periods (Fig. 1B - Mean urinary P repeat 1: 172.9 $\pm$ 6.43 pg/µg Cr c.f. 68.90 $\pm$ 3.91 pg/µg Cr; repeat 2: 197.6 $\pm$ 7.14 pg/µg Cr c.f. 44.20 $\pm$ 3.25 pg/µg Cr; repeat 3: 219.2 $\pm$ 7.65 pg/µg Cr c.f. 33.80 $\pm$ 4.30 pg/µg Cr). There was a significant difference in mean urinary progesterone concentrations of the vitellogenic female frogs throughout the sampling period (Fig. 1B; $F_{2, 29}$ = 43.20, p < 0.001). Post-hoc comparison showed significant difference for mean urinary progesterone concentrations of the vitellogenic females between all three repeats (p < 0.05; Fig. 1B). Likewise, for non-vitellogenic females there was a significant difference in mean urinary progesterone concentrations throughout the sampling period (Fig. 1B; $F_{2, 29}$ = 15.31, p < 0.001). The post - hoc comparison showed significant difference for mean urinary progesterone concentrations of non-vitellogenic females between repeats 1 and 2 and between repeats 1 and 3 (p < 0.05; Fig. 1B).

3.2 Comparison of baseline urinary corticosterone between reproductive status, repeats and body-condition

The GLM analysis showed a significant effect of reproductive status on baseline urinary corticosterone ($F_{4, 55}$ = 28.74, p < 0.0001). However, the repeats and body condition did not significantly affect baseline urinary corticosterone (p > 0.05 for both comparisons). Vitellogenic females had much higher (almost double) concentrations of mean baseline urinary corticosterone in comparison to the non-vitellogenic females throughout the three repeated sampling periods (Mean baseline urinary CORT repeat 1: 31.0 $\pm$ 2.35 pg/µg Cr c.f.

14.5 ± 2.26 pg/µg Cr; repeat 2: 29.3 ± 2.18 pg/µg Cr c.f. 13.00 ± 1.66 pg/µg Cr; repeat 3: 30.8 ± 2.30 pg/µg Cr c.f. 13.20 ± 1.66 pg/µg Cr). Pearson correlation was significant for comparison between baseline corticosterone and body condition (r = 0.537). Furthermore, Pearson correlation was also significant for comparison between baseline corticosterone with oestrone conjugate (r = 0.756) and progesterone (r = 0.749).

3.3 Comparison of urinary corticosterone responses between reproductive status, repeats and body-condition

The 3-way ANOVA results showed a significant effect of reproductive status ($F_{1, 270}$ = 215.43, p < 0.001) and time ($F_{1, 270}$ = 125.39, p < 0.001) on mean urinary corticosterone stress responses, as well as significant interaction between reproductive status and time ($F_{4, 270}$ = 3.065, p = 0.017). However, the interactions between repeat*time, repeat*reproductive status and reproductive status*repeat*time were not significant (p > 0.05 for all comparisons). Urinary corticosterone stress responses of the individual vitellogenic female frogs were consistent between the three sampling occasions (Fig. 2). Some frogs showed consistently high responses (for example, frog #1 showed using dotted lines in Fig. 2), and others showed consistently low urinary corticosterone responses (for example, frog #4 showed using dashed lines in Fig. 2). Likewise, the individual non-vitellogenic female frogs showed consistent short-term urinary corticosterone stress responses between the three repeated sampling occasions (Fig. 3). Some frogs showed consistently high responses (for example, frog #7 showed using dotted lines in Fig. 3), and others showed consistently low urinary corticosterone responses (for example, frog #8 showed using dashed lines in Fig. 3).

3.4 Repeatability in baseline urinary corticosterone, oestrogen, progesterone and short-term corticosterone responses of the female frogs

Baseline (0 h) urinary oestrogen were highly statistically repeatable for both vitellogenic (r = 0.863, $p < 0.0001$) and non-vitellogenic female frogs (r = 0.763, $p < 0.0001$). Baseline (0 h) urinary progesterone were highly statistically repeatable for both vitellogenic (r = 0.855, $p < 0.0001$) and non-vitellogenic female frogs (r = 0.753, $p < 0.0001$). Baseline urinary corticosterone concentrations of the vitellogenic female frogs were highly statistically repeatable (r = 0.963, $p < 0.0001$). Short-term urinary corticosterone stress responses had high statistical repeatabilities at 2 h (r = 0.865, $p < 0.0001$), 4 h (r = 0.866, $p < 0.0001$), 6 h (r = 0.820, $p < 0.0001$) and 8 h (r = 0.915, $p < 0.001$), and for the total and corrected integrated corticosterone responses (r = 0.869, $p < 0.0001$; r = 0.890, $p < 0.0001$ respectively).

Likewise, baseline (0 h) urinary corticosterone concentrations of the non-vitellogenic female frogs were highly statistically repeatable (r = 0.853, $p < 0.0001$). Short-term urinary corticosterone stress responses had high statistical repeatabilities at 2 h (r = 0.785, $p < 0.0001$), 4 h (r = 0.855, $p < 0.0001$), 6 h (r = 0.810, $p < 0.0001$) and 8 h (r = 0.825, $p < 0.001$), and for the total and corrected integrated corticosterone responses (r = 0.815, $p < 0.0001$; r = 0.805, $p < 0.0001$ respectively).

4.0 Discussion

This study has shown for the first time in anuran amphibians that the magnitude of baseline and short-term corticosterone stress responses are different between the reproductive stages of female frogs during breeding. Urinary oestrogen and progesterone levels were also used as an additional method to confirm the reproductive stages of the female frogs. Progesterone and oestrogen play important roles in reproduction in anuran amphibians. They vary in response

to different reproductive stages during the breeding season. Lynch et al. (2006) studied the changes in gonadal hormone levels in female Tungara frogs (*Physalaemus pustulosus*). It was found that the species has cyclic fluctuations in gonadal hormone levels, and the plasma levels of oestrogen and progesterone change significantly in accordance to reproductive stage during one reproductive cycle. Elevations of the plasma oestrogen and progesterone occur at the same reproductive stage that is reported that females display the maximum frequency of reproductive behaviours, the amplexed stage. This is similar to the paradigm that there are temporal relationships between the appearance of reproductive hormones and reproductive behaviours (Mendonça et al., 1985).

4.1 Biological significance of baseline corticosterone

Baseline corticosterone can vary depending on a number of different factors including individual variation, life-history experience as well as general extrinsic factors such as resource availability or links with reproductive effort (Romero, 2002). Studies on birds, such as Ouyang et al. (2011a) found that baseline corticosterone concentrations in blood plasma are repeatable within season for the great tit (*Parus major*) and tree swallows (*Tachycineta bicolour*). However, baseline corticosterone was not repeatable between seasons or years. Consistency in baseline corticosterone is dependent on the reproductive status of the individuals, which also reflects differences in the reproductive hormone concentrations. Lutterschmidt et al. (2009) showed that reproductively active timber rattlesnakes (*Crotalus horridus*) had significantly elevated baseline corticosterone in comparison to the non-reproductive individuals and the lowest overall baseline concentrations were observed through summer sampling (July) in comparison to spring and fall. They suggested that pregnancy is most likely to be positively associated with higher energy demands for survival during this period (Lutterschmidt et al., 2009). In another study, Ouyang et al. (2011b)

showed that individuals of both sexes of free-living house sparrows (*Passer domesticus*) had low baseline corticosterone before the breeding period and high baseline corticosterone during the breeding period. They hypothesized that the plasticity in baseline corticosterone led to an increase in the reproductive success (increased off-spring production) of the house sparrows. They also suggested that the energy input into reproduction from both parents was reflected in the overall high baseline corticosterone levels. Earlier, Mendonça et al. (1985) demonstrated a similar result for male bull frogs (*Rana catesbeiana*) that during chorus have higher corticosterone than males not in the chorus. High baseline corticosterone concentrations can also be used as a proxy to identify populations that are under the impacts of prolonged or chronic stress (CORT-Fitness hypothesis), but the long-term patterns of baseline corticosterone concentrations and fitness parameters (such as body-condition and reproductive output) in many free-living species is lacking to appropriately test this concept (Bonier et al., 2009; Narayan et al., 2013b). Homan et al. (2003) previously investigated seasonal and related sex differences in the baseline and short-term corticosterone responses using repeated blood sampling in the spotted salamanders (*Ambystoma maculatum*). Sex related differences in baseline corticosterone concentrations were observed during migration to the breeding pond where females had higher baseline corticosterone concentrations than males. Their results suggested that female salamanders were preparing for this ecologically challenging reproductive period that accounted for higher baseline and short-term corticosterone stress responses during inbound pond migration. Overall, it appears that a high baseline corticosterone concentration is associated with reproductive effort and may potentially be linked to reproductive fitness (correlated positively with body condition) and greater reproductive success.

4.2 Biological significance of short-term corticosterone stress responses

The significance of higher magnitude of short-term corticosterone stress responses in vitellogenic females suggests that a greater corticosterone response during breeding may in fact be beneficial. As previously mentioned, reproductive Timber rattlesnakes (*Crotalus horridus*) expressed significantly higher acute corticosterone stress responses to capture stress in comparison to non-reproductive or post parturient females (Lutterschmidt et al., 2009). This appears to indicate that reproductive stage has an impact on the magnitude of the stress hormone response, which is accompanied by changes in baseline corticosterone and reproductive hormones. Lutterschmidt et al. (2009) showed that there were seasonal and reproductive stage differences in baseline and short term corticosterone stress responses in timber rattlesnakes. It was also found however that the baseline corticosterone was positively correlated with oestradiol in females. A similar result was also found in the current study. Woodley and Moore (2002) investigated the plasma corticosterone response to acute stress according to reproductive condition in female tree lizards. It was found in vitellogenic females that the baseline corticosterone was associated with changes in ovarian weight, which suggests that during vitellogenesis corticosterone may have some role in ovarian development (Woodley and Moore, 2002). Increased corticosterone in the vitellogenic females in correlation with increasing ovary weight could potentially be linked with the changes in oestrogen levels. Furthermore, oestradiol increases interrenal weight and plasma corticosterone in the desert iguana (Callard, 1978; Woodley and Moore, 2002). Woodley and Moore (2002) suggested that oestrogen could potentially modulate the corticosterone binding globulins hence impacting upon the amount of total and free corticosterone circulating in the blood. In mammals, females tend to have higher glucocorticoid levels than males perhaps because oestrogen stimulates the synthesis of the corticosterone binding globulin (Woodley and Moore, 2002). Thus, in vitellogenic females the high levels of oestrogen could be

potentially linked with high baseline corticosterone and high short-term corticosterone stress responses. Therefore, it leads us to the indication that the regulation of the interrenal axis in female anurans during breeding is complex and linked with the levels of reproductive hormones, reproductive status and the environment.

4.3 Repeatability of corticosterone during breeding

The baseline and short-term corticosterone stress responses, like the reproductive hormones, were repeatable in the vitellogenic and non-vitellogenic ground female frogs during breeding season. The ecological significance of having a highly repeatable baseline and more importantly, high magnitude short-term stress hormone responses for vitellogenic females during the breeding season could be related to many factors. In terms of repeatability in both the baseline and short term stress response, it can potentially be variable between seasons and amongst years, especially for different species (Romero, 2002; Romero and Reed, 2008). Recently, Ouyang et al. (2011a) tested the repeatability of corticosterone (both baseline and short-term stress hormone responses) within seasons and amongst years in the blood plasma of the great tit (*Parus major*) and the tree swallow (*Tachycineta bicolour*). Repeatability of the baseline corticosterone was found within the breeding season for both sexes of the Great tit as well as the female tree swallows. No repeatability of baseline corticosterone was found among seasons or years, which indicates that there is repeatability over a short-time scale for baseline corticosterone. Differences in the baseline levels of stress hormones could be due to variations in the amount of energy and time put into reproduction to maximise fitness, otherwise it may be a reflection of environmental conditions (Ouyang et al., 2011b). Others point out that the calculation of repeatability may be inconsistent, as a study by Rensel and Schoech (2011) found repeatability in the corrected integrated corticosterone stress responses (which subtracts the variation from the baseline data) in Florida scrub jays (*Aphelocoma*

coerulescens) but no repeatability in the actual baseline corticosterone. Furthermore, both (Ouyang et al., 2011a; Rensel and Schoech, 2011) studies had low repeatability across the years. In another study, Romero and Reed (2008) measured baseline corticosterone titers five times under six experimental conditions for house sparrows (*Passer domesticus).* Repeatability for individuals was only found during the night during all three conditions (long and short day photoperiod and prebasic moulting) as well as during the day for short days. This provides further evidence that repeatability of baseline corticosterone concentrations is dependent on a number of extrinsic and intrinsic factors (Romero and Reed, 2008).

Overall, repeatability analysis is very useful for understanding the mechanisms and significance of variation of phenotypes and can be used to assess the flexibility or plasticity of stress hormone responses. For this experiment, repeatability was confirmed within a three week sampling period over the breeding season. The repeatability of short-term corticosterone stress responses in Fijian ground frogs between different seasons and among years however remains unknown. As the main focus of repeatability in this sense is the consistency in baseline and short-term stress response, the interaction this holds with the reproductive state (and thus the production of reproductive hormones) during times of breeding seems to be quite complex. Narayan et al. (2010a) discussed earlier that adult Fijian ground frogs have annual cycles of gonadal growth and regression during spring and summer. Using the data from the current experiment it is conclusive that both baseline and short-term corticosterone responses are repeatable in both groups of female ground frogs, the question remains whether corticosterone titres are repeatable between seasons and years and how they correlate with reproductive hormone cycle and reproductive output.

4.4 Conclusion

This experiment has provided a snap-shot of reproductive and stress hormone activity in female Fijian ground frogs in relation to breeding. The capture handling and non-invasive urine sampling protocol quantified the pattern and magnitude of baseline and short-term urinary corticosterone responses in the vitellogenic and non-vitellogenic female groups. The higher levels of baseline and short-term corticosterone stress responses in the vitellogenic female ground frogs signifies the diverse roles that this glucocorticoid plays for physiological alertness, behaviour, immune system responses and metabolism during the crucial breeding season. This signifies that the physiological stress hormone response enables the female ground frogs to overcome potential threats such as interactions with the invasive cane toads [*Rhinella marina*] (Narayan et al., 2012a) as well as providing necessary eco-physiological and behavioural adjustments for successful reproduction.

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References

Bliley JM, Woodley SK. 2012. The effects of repeated handling and corticosterone treatment on behavior in an amphibian (ocoee salamander: *Desmognathus ocoee*). Physiology and Behavior 105:1132-1139.

Bonier F, Moore IT, Martin PR, Robertson RJ. 2009. The relationship between fitness and baseline glucocorticoids in a passerine bird. Gen Comp Endocrinol 163:208-213.

Callard IP, and G. V. Callard. 1978. General, comparative and clinical endocrinology of the adrenal gland. The adrenal gland in reptilia, part 2 Physiology In I Chester Jones and I W Henderson (eds) Vol. 2.

Cockrem J. 2007. Stress, corticosterone responses and avian personalities. J Ornithol 148:169-178.

Cockrem JF. 2005. Conservation and behavioral neuroendocrinology. Horm Behav 48:492-501.

Cockrem JF, Barrett DP, Candy EJ, Potter MA. 2009. Corticosterone responses in birds: Individual variation and repeatability in adelie penguins (*pygoscelis adeliae*) and other species, and the use of power analysis to determine sample sizes. Gen Comp Endocrinol 163:158-168.

Coddington EJ, Cree A. 1995. Effect of acute captivity stress on plasma concentrations of corticosterone and sex steroids in female whistling frogs, *litoria ewingi*. Gen Comp Endocrinol 100:33-38.

Duellman WE, Trueb L. 1994. Biology of amphibians. : John Hopkins University Press, Baltimore, MD.

French SS, McLemore R, Vernon B, Johnston GI, Moore MC. 2007. Corticosterone modulation of reproductive and immune systems trade-offs in female tree lizards: Long-term corticosterone manipulations via injectable gelling material. J Exp Biol 210:2859-2865.

Graham L, Schwarzenberger F, Möstl E, Galama W, Savage A. 2001. A versatile enzyme immunoassay for the determination of progestogens in feces and serum. Zoo Biology 20:227-236.

Hero J-M. 1989. A simple code for toe clipping anurans. Herpetological Review 20:66-67.

Ho SM, editor. 1987. Endocrinology of vitellogenesis. New York: Plenum Press. 145-169 p.

Homan RN, Reed JM, Romero LM. 2003. Corticosterone concentrations in free-living spotted salamanders (*Ambystoma maculatum*). Gen Comp Endocrinol 130:165-171.

Houck LD. 1998. Integrative studies of amphibians: From molecules to mating. Am Zool 38:108-117.

Klose S, Smith C, Denzel A, Kalko EV. 2006. Reproduction elevates the corticosterone stress response in common fruit bats. Journal of Comparative Physiology A 192:341-350.

Lessells CM, Boag PT. 1987. Unrepeatable repeatabilities: A common mistake. Auk 104:116-121.

Lutterschmidt WI, Lutterschmidt DI, Mason RT, Reinert HK. 2009. Seasonal variation in hormonal responses of timber rattlesnakes (*Crotalus horridus*) to reproductive and environmental stressors. J Comp Physiol B 179:747-757.

Lynch KS, Crews D, Ryan MJ, Wilczynski W. 2006. Hormonal state influences aspects of female mate choice in the tungara frog (*Physalaemus pustulosus*). Horm Behav 49:450-457.

Mendonça MT, Licht P, Ryan MJ, Barnes R. 1985. Changes in hormone levels in relation to breeding behavior in male bullfrogs (*Rana catesbeiana*) at the individual and population levels. Gen Comp Endocrinol 58:270-279.

Morrison C. 2003. A field guide to herpetofauna of fiji: Institute of Applied Sciences, University of the South Pacific.

Narayan CK, Morley Craig. 2008. Ecology and reproduction of the endangered fijian ground frog *platymantis vitianus* – Fiji Islands. . The South Pacific Journal of Natural Science 26.

Narayan E. In-press. Non-invasive reproductive and stress endocrinology in amphibian conservation physiology. Conservation Physiology 1.

Narayan E, Cockrem J, Hero J-M. 2012a. Individual variation in urinary corticosterone metabolite responses in two closely related species of fijian frogs. Gen Comp Endocrinol 177:55-61.

Narayan E, Cockrem JF, Hero J-M. 2013a. Are baseline and short-term corticosterone stress responses in free-living amphibians repeatable? Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 164:21-28.

Narayan E, Cockrem JF, Hero JM. 2011a. Urinary corticosterone metabolite responses to capture and captivity in the cane toad (*Rhinella marina*). Gen Comp Endocrinol 173:371-377.

Narayan E, Molinia F, Christi K, Morley C, Cockrem J. 2010a. Annual cycles of urinary reproductive steroid concentrations in wild and captive endangered fijian ground frogs (*Platymantis vitiana*). General & Comparative Endocrinology 166:172-179.

Narayan E, Molinia F, Christi K, Morley C, Cockrem J. 2010b. Urinary corticosterone metabolite responses to capture, and annual patterns of urinary corticosterone in wild and captive endangered fijian ground frogs (*Platymantis vitiana*). Aust J Zool 58:189-197.

Narayan E, Molinia F, Cockrem J, Hero J-M. 2012b. Individual variation and repeatability in urinary corticosterone metabolite responses to capture in the cane toad (*rhinella marina*). General & Comparative Endocrinology 175:284-289.

Narayan EJ, Cockrem JF, Hero J-M. 2011b. Urinary corticosterone metabolite responses to capture and captivity in the cane toad (*Rhinella marina*). General & Comparative Endocrinology 173:371-377.

Narayan EJ, Cockrem JF, Hero J-M. 2013b. 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*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 165:304-312.

Narayan EJ, Hero JM, Cockrem JF. 2012c. Inverse urinary corticosterone and testosterone metabolite responses to different durations of restraint in the cane toad (rhinella marina). Gen Comp Endocrinol 1:00386-00383.

Olmstead AW, Korte JJ, Woodis KK, Bennett BA, Ostazeski S, Degitz SJ. 2009. Reproductive maturation of the tropical clawed frog: *Xenopus tropicalis*. General and Comparative Endocrinology 160:117-123.

Orchinik M, Licht P, Crews D. 1988. Plasma steroid concentrations change in response to sexual behavior in *Bufo marinus*. Horm Behav 22:338-350.

Ouyang JQ, Hau M, Bonier F. 2011a. Within seasons and among years: When are corticosterone levels repeatable? Horm Behav 60:559-564.

Ouyang JQ, Sharp PJ, Dawson A, Quetting M, Hau M. 2011b. Hormone levels predict individual differences in reproductive success in a passerine bird. Proceedings of the Royal Society B: Biological Sciences.

Peig J, Green A. 2010. The paradigm of body condition: A critical reappraisal of current methods based on mass and length. Funct Ecol 24:1323-1332.

Rensel MA, Schoech SJ. 2011. Repeatability of baseline and stress-induced corticosterone levels across early life stages in the florida scrub-jay (*Aphelocoma coerulescens*). Horm Behav 59:497-502.

Ricklefs RE, Wikelski M. 2002. The physiology/life-history nexus. Trends in ecology & evolution (Personal edition) 17:462-468.

Romero LM. 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. Gen Comp Endocrinol 128:1-24.

Romero LM, Reed JM. 2008. Repeatability of baseline corticosterone concentrations. General and Comparative Endocrinology 156:27-33.

Schreck CB. 2010. Stress and fish reproduction: The roles of allostasis and hormesis. Gen Comp Endocrinol 165:549-556.

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783-1786.

Sutherland WJ. 1998. The importance of behavioural studies in conservation biology. Anim Behav 56:801-809.

Szymanski DC, Gist DH, Roth TL. 2006. Anuran gender identification by fecal steroid analysis. Zoo Biol 25:35-46.

Wilczynski W, Lynch KS, O'bryant EL. 2005. Current research in amphibians: Studies integrating endocrinology, behavior, and neurobiology. Horm Behav 48:440-450.

Woodley SK, Moore MC. 2002a. Plasma corticosterone response to an acute stressor varies according to reproductive condition in female tree lizards (*Urosaurus ornatus*). Gen Comp Endocrinol 128:143-148.

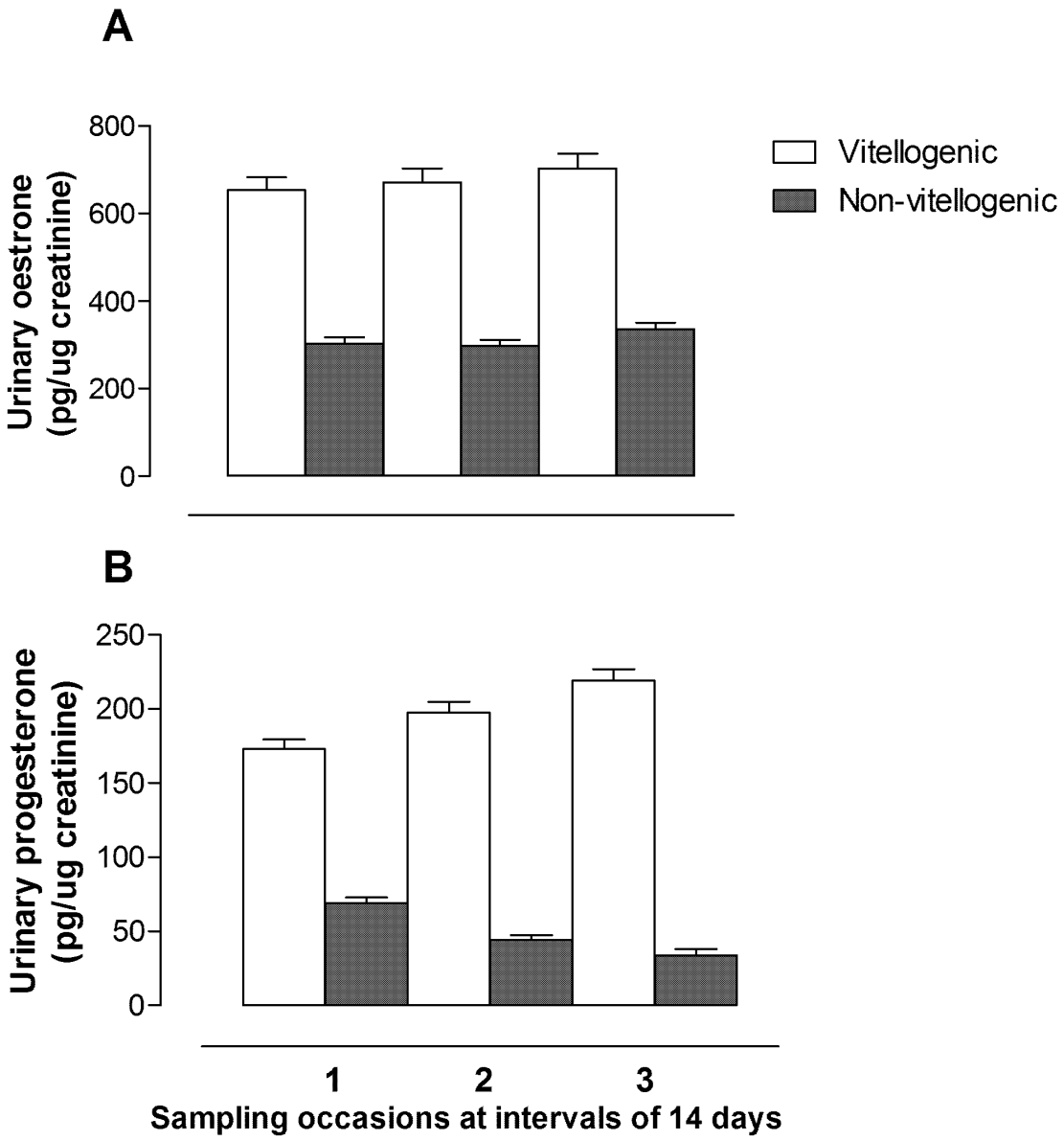
Figure Legends

Figure 1.0 Mean (± S. E.) urinary oestrone (Fig.1A) and progesterone concentrations (Fig. 1B) in vitellogenic (clear bars) and non-vitellogenic (shaded bars) female Fijian ground frogs for sampling done on three repeated occasions at intervals of 14 days during a breeding period in December 2011. Sample size for each bar is n = 10.

Figure 2.0 Individual urinary corticosterone concentrations in vitellogenic female Fijian ground frogs for sampling done on three repeated occasions (first, second and third) at intervals of 14 days during a breeding period in December 2011. Sample size of n = 10 for each repeated sampling. Frog with consistently highest corticosterone response is shown using dotted lines (Frog #1). Frog with consistently lowest corticosterone response is shown using dashed lines (Frog #4).

Figure 3.0 Individual urinary corticosterone concentrations in non-vitellogenic female Fijian ground frogs for sampling done on three repeated occasions (first, second and third) at intervals of 14 days during a breeding period in December 2011. Sample size of n = 10 for each repeated sampling. Frog with consistently highest corticosterone response is shown using dotted lines (Frog #7). Frog with consistently lowest corticosterone response is shown using dashed lines (Frog #8).

158 **FIGURE 1**



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166 **FIGURE 2**

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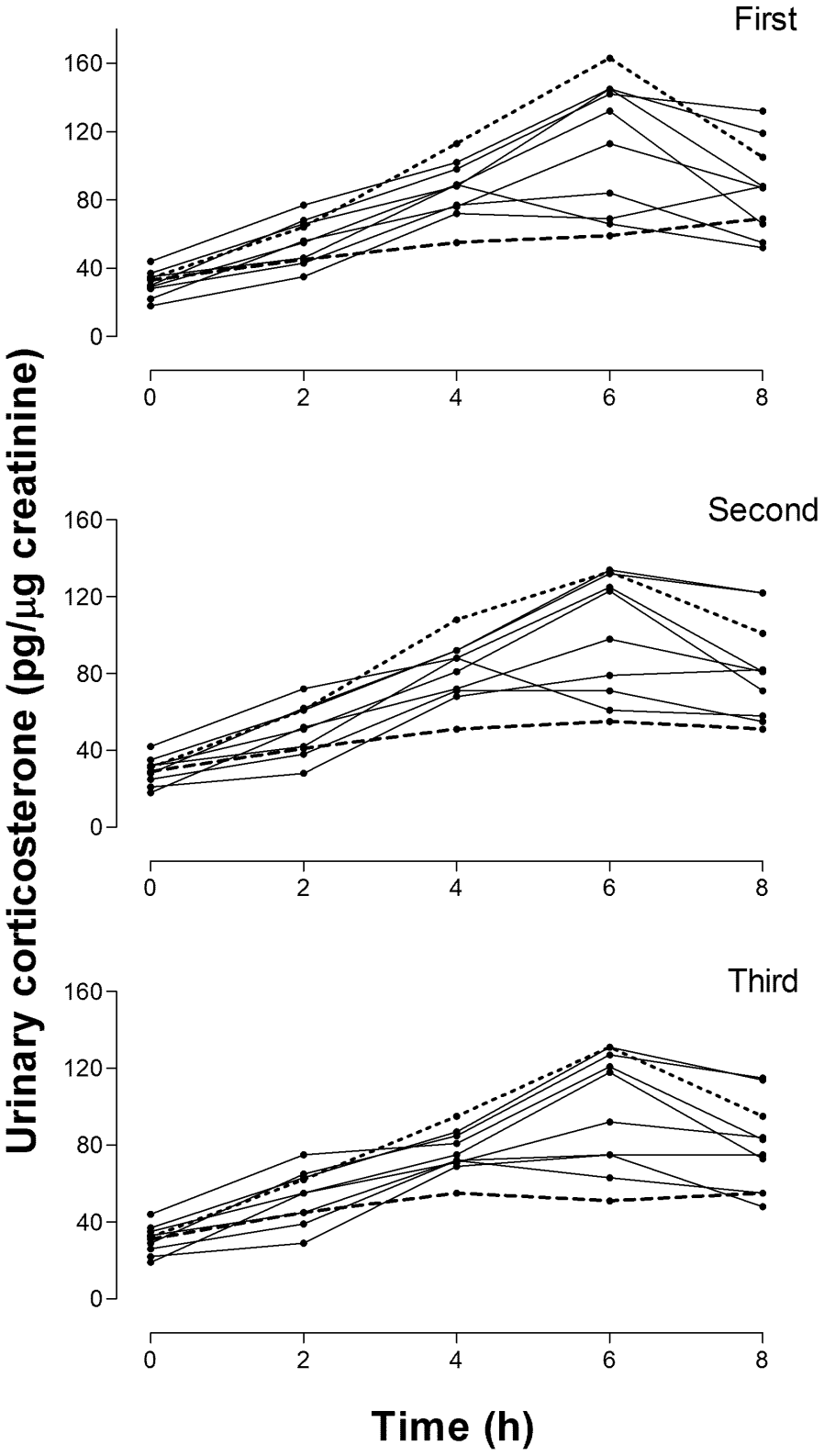

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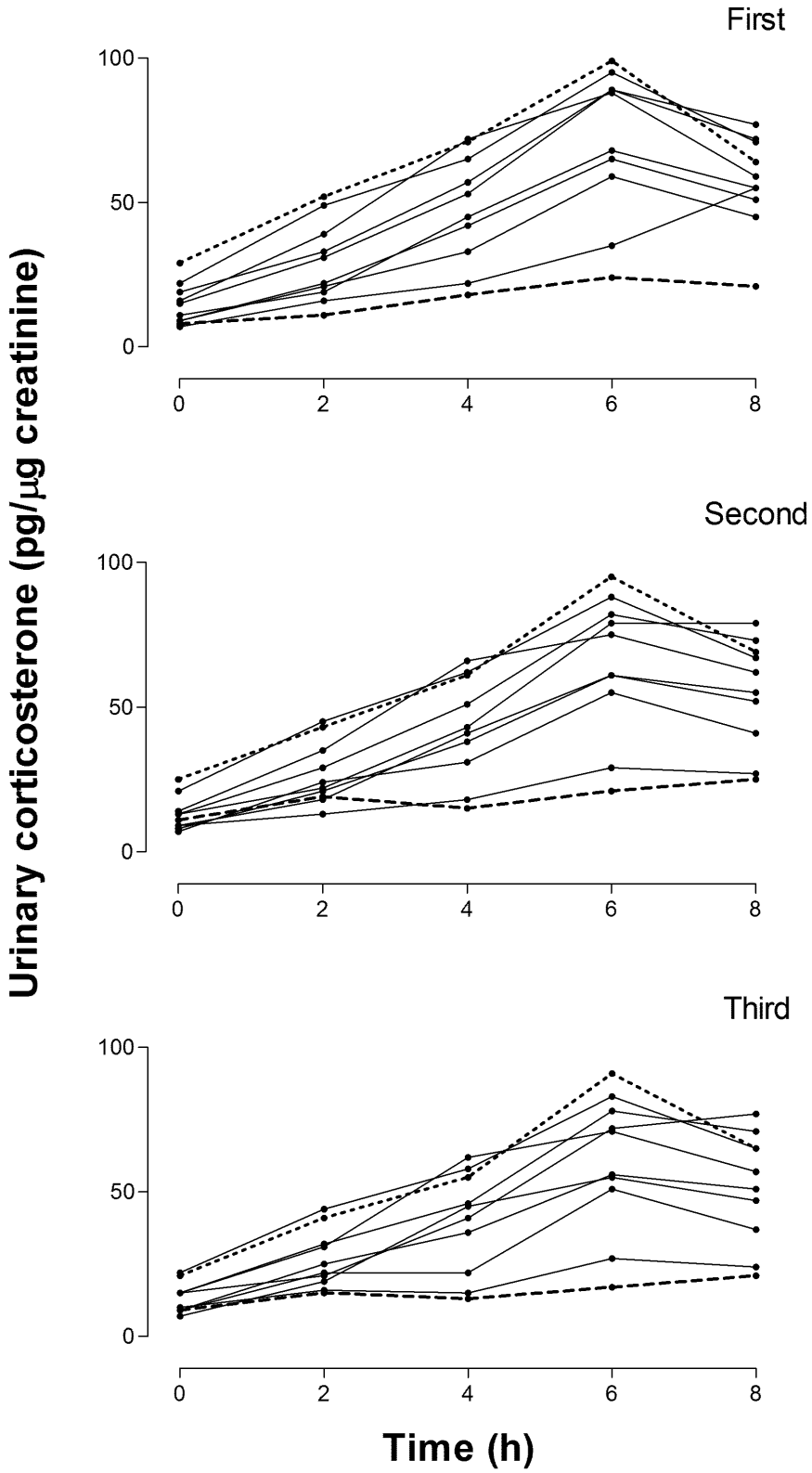
169 **FIGURE 3**



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