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Annual changes in corticosterone and its response to handling, tagging and short-term captivity in *Nyctibatrachus humayuni*

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Glucocorticoids (GCs) are primarily involved in mobilising energy reserves to various physiological processes including reproduction. During situations of stress, GCs also help in coping with stress by diverting energy away from processes such as growth and reproduction. Hence, studying annual and seasonal changes in GCs of wild populations can help in understanding their role in stress management and reproduction. The quantification of GCs in wild animals involves capturing, handling and restraining, which could be stressful. Moreover, different species may exhibit differential sensitivity to different stressors. Hence, determining species-specific sensitivities and responses to different stressors may help in developing effective conservation measures. In this context, we studied the annual and seasonal variations in corticosterone metabolites of the Bombay night frog, *Nyctibatrachus humayuni*. In addition, the effects of handling, marking and short-term captivity (24 h) on corticosterone metabolite levels of *N. humayuni* were determined. Our results show that urinary corticosterone metabolites (UCM) varied significantly annually and between the sexes; in males, the levels were highest during the breeding season, whilst in females, the levels were highest just before the breeding season. Interestingly, UCM levels of both the sexes were not affected by tagging with visual implant elastomer (VIE), and by short-term captivity, suggesting that these manipulations were not stressful in terms of corticosterone responses.

Keywords: *Nyctibatrachus humayuni,* glucocorticoids, sex-specificity, short-term captivity, tagging

INTRODUCTION

Glucocorticoids (GCs) play a key role in many physiological processes including energy mobilisation and mediation of stress responses (Sapolsky et al., 2000; Romero, 2002). In stable environments, GCs play an important role in mobilising energy reserves for various physiological processes including development and reproduction (Wingfield et al., 1998; Moore & Jessop, 2003). During situations of stress, the metabolic actions of GCs also help in the survival of an organism by temporarily suspending other physiological and behavioural processes such as development and reproduction (Wingfield et al., 1998; Moore & Jessop, 2003; Busch & Hayward, 2009).

The secretion of GCs is regulated by hypothalamus-pituitary-adrenal axis (HPA), whereas reproductive processes are controlled by hypothalamus-pituitary-gonadal axis (HPG). Hence, a tight association between the HPG and HPA axes is essential for successful survival and reproduction (Carr, 2011; Narayan & Hero, 2013). Theoretical predictions combined with experimental evidence have suggested the existence of an inverse correlation between HPG and HPA axes, i.e. when the levels of sex steroids are high, the levels of corticosterone are low, and vice-versa (Carr, 2011; Narayan et al., 2012). An animal's response to situations of stress is mediated by the HPA axis through the secretion of glucocorticoids. Excess of glucocorticoids is suppressive to the secretion and action of sex steroids (Carr, 2011; Leary et al., 2004, 2008; Leary & Harris, 2013; Leary et al., 2015). However, a moderate increase in plasma GCs is observed in many vertebrates during reproduction (Romero, 2002; Moore & Jessop, 2003; Narayan et al., 2013; Joshi et al., 2018). Thus, the relationship among stress, GCs and reproduction is much more complex than suspected (Moore & Jessop, 2003).

The profiling of GCs during the annual reproductive cycle helps in the assessment of reproductive health and facilitates conservation efforts (Narayan, 2013). Further, determining the annual and seasonal profiles of GCs between the sexes is essential as the accumulation and expenditure of energy often differ between the sexes during reproduction (Beck et al., 2003; Homan et al., 2003). The estimation of GCs in wild animals often involves the use of invasive techniques such as

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capturing, handling, collection of blood (Narayan et al., 2011; Narayan, 2013). With the global decline in animal populations, such invasive methods should be minimised to avoid the stress that may affect their survival and reproduction. Conservation physiology tools such as urinary quantification of stress hormones have rapidly advanced ecological knowledge of rare and endemic amphibian species globally (Narayan et al., 2019). A commonly used field method for monitoring population health and reproduction is mark-recapture. Other methods used for marking smaller vertebrates are toe-clipping, pressurised fluorescent tagging, pattern markings, passive integrated transponder (PIT) tagging, VIE tagging, etc (Halliday, 2006). However, toe-clipping, pattern markings, PIT tags are invasive and can cause injuries to animals. Consequently, they can affect the survival and behaviour of animals by eliciting a stress response through GCs secretion (Narayan et al., 2011). In recent years, non-invasive endocrinology has become an integral part of conservation programmes addressing global declines in animal populations, particularly in vertebrates (Stuart et al., 2004; Narayan, 2013; Narayan et al., 2019). It involves understanding the significance of stress hormones in overall population health and fitness (Narayan, 2013). Although the role of GCs in reproduction is well known, the effects of acute and chronic stress on physiology and behaviour are not thoroughly investigated (Carr, 2011). Moreover, species-specific differences in sensitivity and responses could lead to variation in the observed pattern. Tropical forests are home to a high diversity of amphibians, many of which are endemic to the region (Gunawardene et al., 2007; Wells, 2007; Crump, 2015). However, they are least studied with regard to annual and seasonal variations in corticosterone and stress responses (Kühn et al., 1987; Narayan & Gramapurohit, 2016, 2019).

The Bombay night frog, *Nyctibatrachus humayuni* (Family: Nyctibatrachidae) is endemic to montane and sub-montane streams of the Western Ghats of peninsular India, and exhibits unique courtship and breeding behaviour (Gramapurohit et al., 2011; Willaert et al., 2016). The species exhibits sexual size dimorphism with females being larger than males. Males have femoral glands on the ventral surface of their thighs, which become prominent during the breeding season. Males are non-chorusing but exhibit a resource defence polygynous type of mating system (Joshi et al., 2017). They choose suitable spawning sites on rocks and boulders situated in the middle and along the sides of streams, as well as muddy surfaces along the banks that are moist, conducive for embryonic development, and safe against fungal pathogens and other predators. During the breeding season, they establish territories and initiate calling from these territories. Females approach these calling males and the courtship leads to an 'abbreviated amplexus' for a brief period (~ 10 min). Subsequently, females indicate the readiness to oviposit by rigorous abdominal and thigh movements following which males dismount and remain in the vicinity to watch the act of spawning. Oviposition occurs in the physical absence of amplexus and females fall-off in water or move away after spawning. Subsequently, males return to the spawning site and sit on the eggs (we believe fertilisation to occur at this moment). Interestingly, males offer parental care by attending the eggs at night (Gramapurohit et al., 2011). Information on breeding biology including the factors affecting their reproductive health is very scarce. Hence, we investigated the annual and seasonal changes in urinary corticosterone (principal glucocorticoid in amphibians) metabolites of *Nyctibatrachus humayuni* for two consecutive years spanning three breeding seasons (June 2014–October 2016). In addition, corticosterone responses to handling, tagging, and short-term captivity were studied in males and females during the breeding and non-breeding seasons of 2015 and 2016. The rationale was to determine if handling, tagging, and captivity during field observations can interfere with GC levels (quantifiable corticosterone levels) in *N. humayuni*.

MATERIALS AND METHODS

Study site and species

The study was carried out in a forest near Matheran (18° 31'N, 73° 51'E), situated in the northern Western Ghats, India. The study site covers an area of 100 m x 50 m with a stream flowing through the rocky substratum, forming an intermediate waterfall and subsequently joining a large water body at the lower end of the cliff. Written permission was obtained from the Maharashtra State Forest Department (Permission No. D-22 (8)/WL/Research/CR-966/5129/2015-2016) to capture frogs for urine sampling. All the animals were released at the site of capture after urine collection. The study was carried out following the guidelines and approval by the departmental Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Collection of urine samples

Regular field visits were carried out at 10–day intervals during the breeding season, and once a month during the non-breeding season. Each visit consisted of 1–4 days of field observations, depending on the level of breeding activity. Observations were conducted between 1900–2300 h using LED lamps. The field site was thoroughly searched for adults and individuals were sexed based on the presence (males) or absence (females) of femoral glands on the ventral surface of their thighs. Snout-vent length (SVL) and body mass were recorded for each frog followed by tagging with visual implant elastomer (VIE; Northwest marine technology, Washington; see below). Snout-vent length was recorded to the nearest 0.1 mm using a digital calliper whereas body mass was recorded to the nearest 1.0 mg using a digital balance. Urine was sampled using a capillary tube (diameter = 0.78 mm, GC 100TF-15; Clark Electromedical Instruments, UK). A urine sample (50–200 µL) was collected from each frog in under 3 min and the frogs were released if the urine could not be collected (as prolonged handling could elicit stress responses). The samples were kept on ice packs, transported to the laboratory and stored at -20 °C until used for the assay.

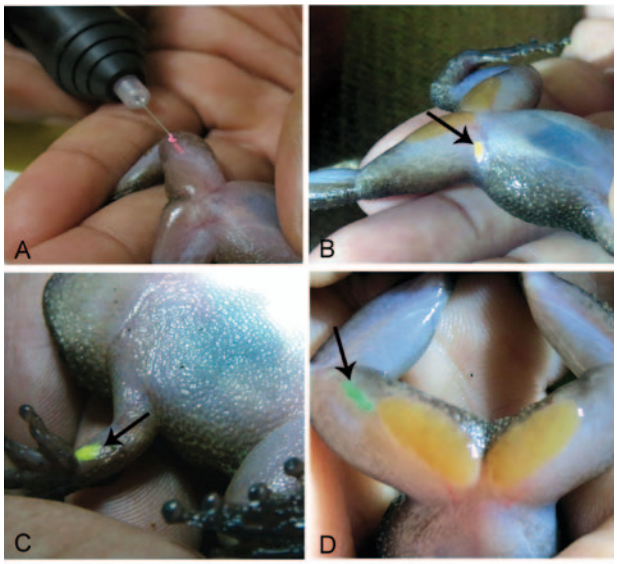

Figure 1. Visual implant elastomers (VIE) used for tagging individual frog, **(A)** insertion of VIE using a syringe, **(B–D)** individuals tagged with different VIE colours. (In B–D, arrow shows VIE tagged area)

Handling, tagging, and short-term captivity stress

To assess the effect of handling and tagging, individuals were captured and tagged with VIE (Fig. 1). The elastomer is of inert material and consists of a colour component and a curing agent mixed in a ratio of 1:10, injected subcutaneously using a syringe (Fig. 1A) and visualised using a UV torch. It does not enter into the body fluid thereby eliminating toxicity-related issues. It is comparatively easy to use and lasts for a long time, thus acting as an efficient and effective method of tagging (Halliday, 2006). Four colour tags (yellow, pink, orange and green) were used either singly or in combination at one or more parts of the body (Figs. 1B, C, D). The tags were generally inserted on the ventral surface of the body for better visibility and the most common parts were forelimbs, hindlimbs, digits and thigh joints. The process of tagging was generally completed in ~ 3 min. Urine was sampled from untagged frogs immediately after their capture and from tagged ones during subsequent visits. Handling of frogs involved capture, measuring body size, urine collection, and tagging. The entire process of handling and tagging was completed in ~ 5 min. To study the effect of short-term captivity, males and females were captured during the breeding (n = 15) and non-breeding (n = 10) seasons. The urine was sampled immediately (0 h) to obtain baseline corticosterone levels. Subsequently, frogs were placed in well-aerated zip-lock polythene bags with a little quantity of stream water and transported to a room near the field site for short-term captivity. Urine was sampled in captivity for 24 h at an interval of 6 h. All the frogs were released at the field site after 24 hours.

Validation of enzyme immunoassay (EIA)

A series of internal controls were used to ensure precise detection as well as cross-reactivity. To ensure the detection of corticosterone in the frog urine, we pooled the urine samples and ran the dilutions, which showed parallel displacements with curves of the corticosterone standards. Urine samples were assayed neat as percent binding of the urine pool was 40 %. The assay sensitivity was 3.84 ± 0.64 pg/well (n = 4). Further, we performed accuracy-recovery checks to confirm the detectability of corticosterone metabolites. Recovery was checked using a spike recovery test, in which different concentrations of corticosterone standards were added to the pooled urine. A recovery curve was plotted using observed values against expected values of standards and expressed using a regression equation, Y = mX + C, where Y is the amount of corticosterone observed, X is the amount of corticosterone expected and m is the slope of the curve. Ideal values for the slope should be in the range of 0.85 to 1.15. Recovery for corticosterone standards in the frog urine was 98.87 % while the slope value was 0.91. Inter and intra-assay coefficients of variation were 7.95 % and 3.66 %, respectively.

Biological validation

Biological validation for urinary corticosterone metabolite (UCM) EIA was carried out using a corticotropin (ACTH; A0298 - Sigma Aldrich) challenge in males and females during the non-breeding season (December and April respectively). Males (n = 4) and females (n = 4) were captured from the wild at 2000 h and the urine samples were collected immediately (0 h). Frogs were quickly transported to a room near the field site for ACTH challenge. Each frog was injected intraperitoneally with ~ 150 µL ACTH (at a concentration of 0.45 µg/g of body mass) and kept undisturbed in a well-aerated zip-lock polythene bag. Subsequently, urine samples were collected up to 30 h at 6 h interval.

Enzyme immunoassays (EIA)

Urinary corticosterone metabolites (UCM) were quantified following the protocol established by Narayan et al. (2010) with some modifications. The polyclonal antibody and horseradish peroxidase (HRP) conjugate for corticosterone (CJM06) were procured from the Clinical Endocrinology Laboratory, University of California, Davis. Cross-reactivity of the corticosterone antibody was 100 % with corticosterone, but only 14.25 % and 0.9 % with deoxycorticosterone and tetrahydrocorticosterone, respectively. Antibody and HRP dilutions were 1: 35,000 and 1: 120,000 respectively. The assays were carried out using direct ELISA with 96-well micro-plates. The plates were coated with 50 µL of antibody diluted in coating buffer (carbonate bicarbonate buffer, pH 9.6) and incubated at 4 °C for 12–16 h. Subsequently, plates were thoroughly rinsed with a washing solution (167 mM NaCl and 0.05 % Tween 20), then 50 µL of samples, standards and positive controls (C1 and C2 to compute inter and intra-assay coefficients of variation) were added to the wells followed by the addition of HRP conjugate to each well. Positive controls consisting of C1 (ten times diluted urine pool; 1:10) and C2 (neat urine pool) were prepared using a urine pool (mixture of all urine samples). The samples, standards, C1 and HRP conjugate were diluted using assay buffer (39 mM NaH_2PO_4 $2H_2O$,

61 mM Na_2HPO_4, 15 mM NaCl and 0.1 % bovine serum albumin, pH 7.0). The plates were then incubated at room temperature for 2 h. Subsequently, plates were rinsed thoroughly with a washing solution followed by the addition of 50 µL substrate solution (0.01 % 3, 3', 5, 5' tetramethylbenzidene, 0.006 % H_2O_2 and 0.05 M phosphate-citrate buffer, pH 5.0) and incubated for 20 min at room temperature. The reaction was stopped using 0.2 M H_2SO_4 and the optical density (OD) was read at 450 nm using an ELISA plate reader (Thermo Scientific Multiskan SK, Ascent software-version 2.6). The titres of corticosterone metabolites were expressed as pg/µg of creatinine.

Creatinine assay

The creatinine index was used as a measure of the rate of hormone metabolism. Creatinine was estimated using Jaffe's reaction, which employs alkaline picrate reagent to detect the creatinine concentration in the urine sample (Taussky & Kurzmann, 1954).

Statistical analysis

All the data were checked for normality using Shapiro-Wilk's W test before subjecting to statistical analyses. Parametric tests were employed to analyse normal data whereas non-parametric tests were used to analyse non-normal data. Annual changes in the UCM levels of males and females were analysed using a Kruskal-Wallis test followed by a Mann-Whitney U test for multiple pairwise comparisons. Changes in UCM levels over time during captivity were analysed using one-way analysis of variance (ANOVA) followed by Scheffe's test for multiple pair-wise comparisons. The UCM levels between tagged and untagged individuals were compared using Mann-Whitney U test. All tests were two-tailed and the significance level was set to 0.05. Statistical tests were carried out using SPSS 19 or PAST 3.

RESULTS

Biological validation

Administration of corticotropin in males resulted in an increase in UCM levels from 117.60 ± 47.12 pg to 2326.50 ± 591.70 pg (1878.30 % increase) after 6 h, which then declined after 12 h. Similarly, basal levels of UCM in females were 141.05 ± 21.49 pg (0 h), which peaked at 6 h following corticotropin administration to 3670.79 ± 461.02 pg (2502.40 % increase) and declined subsequently after 12 h.

Annual and seasonal variation in UCM levels of *N. humayuni*

In males, UCM varied significantly during different months of the year (H = 87.93, p < 0.05; Fig. 2A). The levels were highest in June in comparison to those in the other months (p < 0.05). The levels were moderately high but comparable from July to September, and declined from October to May. Further, UCM levels also differed significantly among the three seasons (H = 58.85, p < 0.05; Fig. 2B). Urinary corticosterone metabolite levels were highest during the breeding season (June–October)

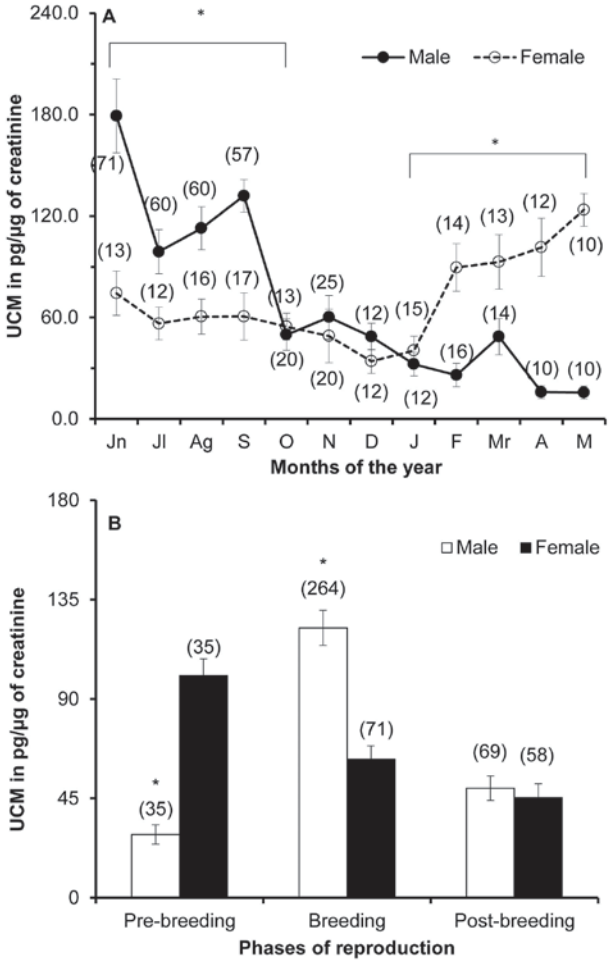

Figure 2. Variation in **(A)** annual and **(B)** seasonal cycles of urinary corticosterone metabolites in *N. humayuni*. Data are presented as Mean ± SE. Numbers in parenthesis indicate sample sizes.
UCM - urinary corticosterone metabolites. (asterisks over the line/bars indicate significant difference at 0.05 level)

followed by the post-breeding season (November–February) while the levels were lowest during the pre-breeding season (March–May). In females, UCM levels differed significantly during different months of the year (H = 44.80, p < 0.05; Fig. 2A). The levels were highest in May (just before the breeding season), while moderate from June to October, and lowest in December and January (Fig. 2A). The levels increased gradually from March to May. Seasonal variation was also observed in the UCM levels of females (H = 23.84, p < 0.05; Fig. 2B). The levels were highest during the pre-breeding season and declined during the breeding and post-breeding seasons.

Effect of handling, tagging and short-term captivity on UCM levels

The levels of UCM were comparable in tagged and untagged individuals of both the sexes (U = 1.43, p > 0.05; Fig. 3). Subsequent to tagging, frogs engaged in courtship and calling activities soon after the release. During the breeding season, UCM levels were comparable in males ($F_{1, 45}$ = 1.32, p > 0.05; Fig. 4A) and females ($F_{1, 43}$ = 0.74,

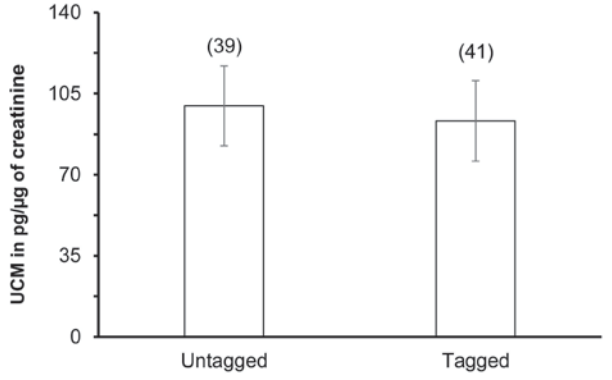

Figure 3. The effect of tagging on the urinary corticosterone metabolite levels of *N. humayuni*. Data are presented as Mean ± SE. Numbers in parenthesis indicate sample sizes. UCM - urinary corticosterone metabolites.

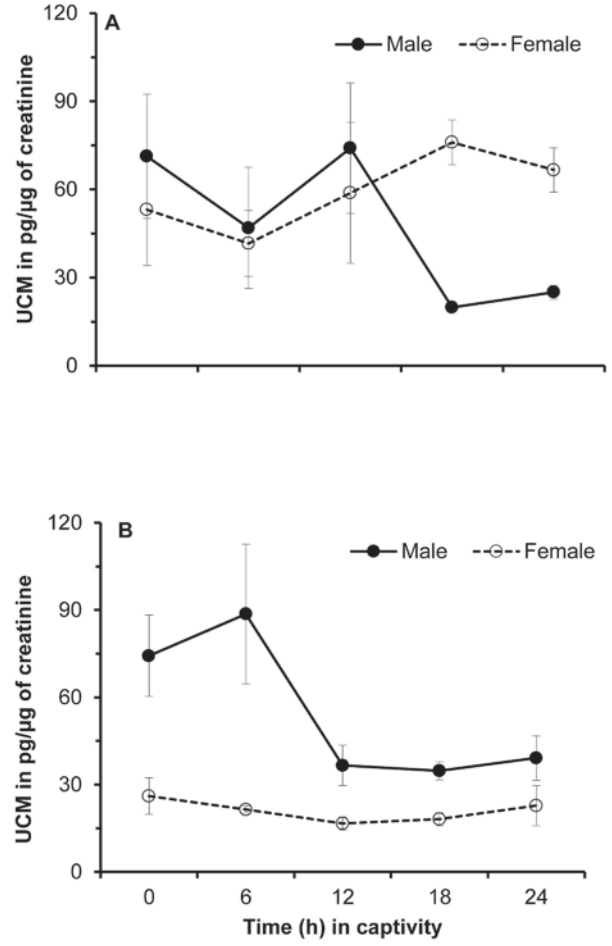

Figure 4. Effect of short-term captivity on the levels of urinary corticosterone metabolites in *N. humayuni*; during **(A)** breeding and **(B)** non-breeding seasons. Data are presented as Mean ± SE. Numbers in parenthesis indicate sample sizes. UCM - urinary corticosterone metabolites.

$p > 0.05$; Fig. 4A) before and after a short-term captivity. Similarly, the UCM levels were comparable in males ($F_{1, 43} = 2.11$, $p > 0.05$; Fig. 4B) and females ($F_{1, 42} = 0.27$, $p > 0.05$; Fig. 4B) in response to a short-term captivity during the non-breeding season.

DISCUSSION

Here we show that corticosterone metabolites exhibited annual and seasonal variation in males and females of *N. humayuni*, with peak levels associated with reproduction or associated activities. Our study found that UCM of males and females of *N. humayuni* increased at different times of the year. In males, the highest levels were observed during the breeding season (June–September), whereas in females they peaked just before the breeding season (April–May). Furthermore, the levels did not increase in response to handling and tagging or short-term captivity in both the sexes. Levels of corticosterone were high in both sexes of *Rana catesbeiana*, *Rana esculenta*, *Bufo japonicus*, *Platymantis vitiana* during the breeding season, whereas the levels were moderate in *Bufo bufo* and *Dicroglossus occipitalis* (Licht et al., 1983; Jolivet-Jaudet et al., 1984; Mendonca et al., 1985; Kühn et al., 1987; Paloucci et al., 1990; Zerani et al., 1991; Giacoma et al., 1994; Romero, 2002; Narayan et al., 2010). However, in our study, peak levels of corticosterone in male and female *N. humayuni* were observed during different phases of the reproductive cycle. In males, levels were high during the breeding season, while in females the levels were high just before the breeding season. Sex-specific differences in the baseline levels of corticosterone could be due to the differential expenditure of energy associated with reproduction. Males need to establish territories during the breeding season just before mating, a process that is energetically demanding (Taigen & Wells, 1985; Wells, 2007; Joshi et al., 2017). Female reproduction is also energetically demanding, in particular, ovarian growth and vitellogenesis, which require mobilisation of energy reserves, a process that occurs before the breeding season (Saidapur, 1989; Wells, 2007; Narayan et al., 2013; Joshi et al., 2018) Higher corticosterone levels have also been observed in vitellogenic than in non-vitellogenic females of *P. vitiana* (Narayan et al., 2010). Further, the levels of corticosterone were higher in female *Ambystoma maculatum* than those in males during migration to the breeding ponds (Homan et al., 2003). Interestingly, corticosterone levels were moderate in females but high in males of *Triturus carnifex* and *Scaphiopus couchii* during reproduction (Zerani & Gobbetti, 1993; Harvey et al., 1997; Romero, 2002). Similarly, males of *Duttaphrynus melanostictus* had higher baseline levels of corticosterone than their female counterparts during the breeding season (Narayan & Gramapurohit, 2016).

Understanding species biology requires field observations, handling, tagging and sometimes short-term captivity. Although these activities could be stressful for animals, species-specific differences exist in their responses to stress (Moore et al., 1991; Narayan et al., 2011). For instance, as with *N. humayuni*, capture and handling did not cause increased corticosterone in *Rhinella marina* and *D. melanostictus* (Narayan et al., 2011; Narayan & Gramapurohit, 2016). In contrast, capture and handling caused elevated corticosterone in *Desmognathus ochrophaeus* (Ricciardella et al., 2010). Similarly, corticosterone levels increased in response to

acute handling of *Urosaurus ornatus* (Moore et al., 1991). These differences in response to handling and capture could be ascribed both to differences in biology and to the methodology. Alternatively, less rigorous handling coupled with capturing for a short period of time could have contributed to a lack of increase in corticosterone of *N. humayuni*.

The most common method of marking anurans is toe-clipping (Phillott et al., 2007; Schmidt & Schwarzkopf, 2010; Narayan et al., 2011), which is invasive and hence stressful. For instance, toe-clipping of *R. marina* resulted in increased corticosterone levels (Narayan et al., 2011; Fisher et al., 2013). Moreover, toe-clipping can interfere with certain activities of frogs that can, in turn, affect their survival. For instance, toe-clipping but not VIE tagging affected the locomotor and jumping performance of *Litoria nasuta* and *Carlia pectoralis* (Schmidt & Schwarzkopf, 2010). In contrast, tagging with VIE did not cause an increase in the GCs levels of *Agalychnis callidryas* (Antwis et al., 2014). Similarly, in *D. melanostictus* VIE tagging did not show significant changes in urinary corticosterone in comparison to capture handling or sham operated (Narayan & Gramapurohit, 2019). In *N. humayuni*, corticosterone levels did not increase following VIE-tagging as evidenced by similar levels in tagged and untagged individuals during subsequent captures. Moreover, the method did not interfere with any activity or behaviour as evidenced by the initiation of calling, courtship. Hence, the use of VIE for marking the frogs is effective. An added advantage of the method is its use in combination on multiple regions of the body without affecting any activities including reproduction. Hence, tagging with VIE is an effective method for marking anurans and recommended (Sapsford et al., 2015). When frogs are injected with a combination of tags on multiple body parts, their corticosterone response may vary in comparison to those frogs injected with a single tag. However, in this experiment most of the frogs were injected with two tags and hence we do not account for any such variation in their corticosterone response.

In our study, short-term captivity (24 h) of *N. humayuni* did not cause increased secretion of corticosterone during the breeding as well as the non-breeding seasons. Moreover, males of *N. humayuni* continued to call and females even spawned under captive conditions, suggesting that they did not experience any captive stress. However, the stress response could be species-specific and context dependent (Dayger et al., 2013). For instance, corticosterone levels increased during short-term captivity (24 h) of *Litoria ewingii*, *R. marina*, *P. vitiana* (Coddington & Cree, 1995; Narayan et al., 2010; 2011). In contrast, corticosterone levels did not increase during short-term captivity of *U. ornatus* (Moore et al., 1991). Similarly, corticosterone levels did not increase in *D. melanostictus* subjected to short-term captivity (Narayan & Gramapurohit, 2016). Some species may be highly sensitive to even mild stressors and respond by increasing their corticosterone whereas others may be less sensitive and hence may not respond. Consequently, species may have differential sensitivities and responses to different stressors including captivity (Moore et al., 1991). Interestingly, the sensitivity and responsiveness of the HPI axis of female garter snakes (*Thamnophis sirtalis parietalis*) varied depending upon the body condition, reproductive phase and seasonal life-history transitions (Dayger et al., 2013; Dayger & Lutterschmidt, 2017). For instance, short-term (4 h) captivity caused an increase in corticosterone levels of females with poor but not good body condition (Dayger et al., 2013). Further, corticosterone levels increased more rapidly in den-collected females than those in migrating females during the breeding season, while its levels increased only in den-collected females but not in migrating females during the non-breeding season (Dayger & Lutterschmidt, 2017). Thus, the effect of stress may also differ with the reproductive stage and the season. In the present study, *N. humayuni* were maintained in a room situated near the study site. As a result, the frogs might not have experienced significant changes in the environmental conditions, which could contribute to minimising the stress response during captivity as suggested by Levine (2000). Taken together, stress sensitivity and responsiveness is species-specific and could vary with life-history stage, sex of the animal, breeding season and geographical range. In addition, methodology and conditions used in assessing the stress response of different species may vary making the comparisons difficult. Hence, using a uniform methodology and captive conditions are essential to obtain accurate and unbiased results (Moore et al., 1991).

In conclusion, males and females of *N. humayuni* exhibit sex-specific differences in the annual and seasonal corticosterone cycles. The levels of corticosterone increased in males during the breeding phase, whereas the levels peaked in females just prior to the initiation of the breeding phase. Handling, tagging, and short-term captivity did not elicit a stress response in *N. humayuni*, suggesting that stress modulation may differ according to the species biology and geographical distribution.

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