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# Urinary corticosterone metabolite responses to capture and visual elastomer tagging in the Asian toad (*Duttaphrynus melanostictus*)

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Herpetological research involving amphibians is increasingly using mark and recapture methods, employing various techniques such as toe-clipping and visible implant alphanumeric tags. Visual Implant Elastomer (VIE) is a new method available for herpetological surveys, involving a coloured dye inserted into the epidermal skin surface of frogs. Previously, there has been only one study which demonstrated that the VIE method does not generate a significant physiological stress response (using a faecal glucocorticoid method) in a captive amphibian species. Physiological stress can also be quantified non-invasively using urinary corticosterone metabolite (UCM) enzyme-immunoassay in amphibians. In this study, we tested the physiological stress response of a common amphibian species, wild caught Asian toads (*Duttaphrynus melanostictus*), by comparing UCM responses to capture handling, sham control or VIE marking method. Adult males (n = 38) were captured and sampled for baseline UCM (t = 0 h) then marked either using the VIE or sham (saline control), or only handled during capture. Subsequently, urine samples were collected at t = 2, 12 and 24 h for toads within each group. UCM levels were quantified using an enzyme-immunoassay (EIA) to determine differences among treatment groups and over time following capture. Toads generated acute stress responses to all three groups, showing a change in UCMs between baseline samples, 12 h, and 24 h samples. The mean UCM levels were not significantly different between the VIE method and the control groups (capture handling or sham operated). These results indicate that VIE method of tagging is no more stressful than routine handling of amphibians, hence in this context, the method does not have any additional welfare implications. Future research should explore the limitations of VIE tagging for long-term mark recapture studies, however, our current findings support its application as a minimally-invasive method for marking amphibians.

*Keywords:* amphibians, mark-recapture, welfare, stress, Visual Implant Elastomer (VIE)

## INTRODUCTION

The physiological stress response involves activation of the hypothalamo-pituitary-adrenal-axis (HPA; Cockrem, 2007) (HPI-axis in amphibians; Narayan et al., 2013) and glucocorticoid secretion, which enables animals to cope with stressful situations through metabolic, physiological, and behavioural responses (Warne et al., 2011). Over-production of glucocorticoids can cause negative consequences on the ecological fitness of animals (Coddington & Cree, 1995). Recently, non-invasive hormone monitoring techniques, such as faecal and urinary based methods, are readily available and allow an alternative method of determining physiological stress in amphibians (Narayan et al., 2011). Urinary corticosterone metabolite (UCM) assessment allows non-invasive monitoring of the stress response from a physiological perspective (Narayan et al., 2011).

Mark-recapture is routinely used in herpetological research for assessing population numbers, trends, and dynamics (Moore et al., 2010). Traditionally, toe-clipping (the removal of one or more toes; Schmidt & Schwarzkopf, 2010) has been used for amphibian mark-recapture studies (Halliday & Verrell, 1988). However, toe-clipping physically alters an animal's body, and elicits an unwarranted physiological stress response lasting several hours (Narayan et al., 2011). Whilst the toe-clipping method has historically been widely used in amphibian studies, the suitability and potentially detrimental effects of the method are a subject of debate. For example, toe-clipping is believed to affect foraging and locomotion and could also reduce body-weight (Schmidt & Schwarzkopf, 2010). Various alternative techniques are now available that are cheaper and easily accessible, such as visual implant elastomer (VIE) tags (Woods & James, 2003), passive integrated transponder (PIT) tags (Jørgensen et al., 2017), and visible implant alphanumeric (VIA) tags (Chan et al., 2006).

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VIE tags are small, flexible and bio-compatible (Northwest Marine Technology, Inc., 2018). The VIE tag is 'injected' as a liquid that cures into a pliable solid. The VIE tags are injected sub-cutaneous and remain externally visible. Unlike the method of toe-clipping to identify focal animals in the field, this technology is likely to be more humane. VIE tagging has been successfully used for individual marking in many vertebrate species such as salamanders, reptiles and fish as well as crustaceans (Davis & Ovaska, 2001; Marold, 2001; Woods & James, 2003; Curtis, 2006; Grant, 2008; Waudby & Petit, 2011). Furthermore, VIE is being used more commonly in the field for the marking of amphibians (Anholt et al., 1998; Nauwelaerts et al., 2000). Recently, Antwis et al. (2014) investigated the potential effects of VIE marking on physiological stress responses in the red-eyed tree frog (*Agalychnis callidryas*). The authors measured adrenal responses using faecal glucocorticoid metabolite concentrations. The results showed no effects of VIE method on adrenal response in the frog.

The aim of the current study was to determine whether VIE is a physical stressor that can elicit a stress response in a tropical amphibian. We examined the physiological stress by determining the changes in UCM levels of adult male *Duttaphrynus melanostictus* subjected to a standard short-term handling protocol, sham control or VIE tagging. We hypothesised that the VIE tagging would elicit a significant stress response in toads compared with the other groups. Thus, the results would verify the suitability of VIE tagging method for marking of common Asian toads.

## METHODS

**Ethics statement**

Sampling was conducted on the S.P. Pune University Campus Maharashtra, India (18°55' N and 73°82' E). Verbal permission was obtained through the security office within the office of the Registrar, which provided unrestricted access to the study site on the campus. As NPG is a faculty of the S. P. Pune University, specific written permission and access to the study site was not required. The study did not involve any endangered or protected species. Moreover, no animals were sacrificed in this study and underwent minimal handling. All the toads were released at the site of capture after urine sampling. The study was carried out following the guidelines of the departmental committee for animal ethics.

**Field methods**

We sampled a natural sub-population of *D. melanostictus* on the S. P. Pune University campus during the breeding season (late July and early August, 2013). The weather conditions were moist with some light rain on the sampling nights. Toads were generally found in open grounds, gardens and crevices near buildings and construction sites on the campus. Sampling was conducted between 1900–2100 h.

The toads were located randomly and captured (n = 38 male toads, identified by the presence of nuptial pads and reddish throats) using torches to identify eye shine. Females were not used because of limited sample size. Urine was collected immediately (within 2 min) from each toad. Each urine sample was collected via normal capillary action by gently inserting a sterile 200 µL pipette tip (2 mm length) into the toads' cloaca. Generally, the toads urinated immediately upon capture, although occasionally gentle massaging of the underbelly abdomen was required to stimulate urination. Only uncontaminated toad urine samples were used, with possible contamination risks including faeces and rain water. Urine samples (sample volume ranging from 0.5 mL to 3 mL) were aliquoted into labelled, sterile 1.5 mL polypropylene Eppendorf tubes and kept on cold ice prior to transfer into the laboratory freezer. We refer to this first urine sample (time = 2 min) as the baseline sample.

Subsequently, toads were allocated into three experimental groups; (1) capture handling only; (2) sham control or (3) VIE tag. The toads in group (1) were placed in a clean, labelled Zip-lock© bag with tiny holes made for ventilation. Toads in group (2) were given a single intra-peritoneal injection of 100 µL saline solution using a 1 mL syringe and 29-gauge needle and immediately returned to their labelled bags. Toads in group (3) were tagged by injection of VIE (Northwest Marine Technologies, Inc.) under the skin with an insulin syringe. The method was previously described by Brannelly et al. (2013). Briefly, tags were applied using 2–3 mm injections halfway between the knee and the pelvis on one hind limb. Combinations of three fluorescent silicon bead colours (pink, yellow and green) were used to uniquely tag individual toads. The locations of the tags, as identified using a handheld UV light, were recorded upon injection and again 24 h post-injection.

All toads were transported to the laboratory within 20 min of field procedures. In the laboratory, all toads were re-sampled (as described in the 'Field methods' section) for urine at 2, 12 and 24 h after field capture. All the toads were released at the site of capture after the final urine sampling.

**Urinary corticosterone metabolite (UCM) enzyme–immunoassays**

The enzyme-immunoassay (EIA) used was originally validated in our earlier research work (Narayan & Gramapurohit, 2016). Briefly, the antibodies used in this study were polyclonal, and the corticosterone antiserum (CJM006) and the conjugated horseradish peroxidase (HRP) label were standardised for a standard direct competitive EIA system (Munro & Stabenfeldt, 1984; Munro, 1985). Recovery of corticosterone standard was 89 %. The coefficients of variation for intra-run and inter-run assays were 3.2 % and 7.2 % respectively. Assay sensitivity was 0.55 pg/well.

Concentrations of UCM were determined using a polyclonal anticorticosterone antiserum (CJM006) diluted 1: 45 000, horseradish peroxidase conjugated CORT label diluted 1: 120 000 and CORT standards (1.56–400 pg well–1). Cross reactivity of the antiserum was 100 % with CORT, 14.25 % with desoxycorticosterone and 0.9 % with tetrahydrocorticosterone (Munro, 1985). Samples were assayed on NuncMaxiSorp™ 96 well plates. For each assay, the plates were coated with 50 µL of the antibody

diluted to an appropriate concentration in a coating buffer (50 mmol L-1 bicarbonate buffer, pH 9.6) and incubated at 4 °C for 12 h. Plates were then washed thoroughly using phosphate-buffered saline containing 0.5 ml L–1 Tween 20 to rinse away any unbound antibody. Stocks of standards, high- and low-binding internal controls, urine samples, and horseradish peroxidase labels were diluted to an appropriate concentration in assay buffer (39mM $NaH_2PO_4.H_2O$, 61mM $NaHPO_4$, 15mM NaCl and 0.1 % bovine serum albumin, pH 7.0). For each EIA, 50 µL of standard, internal control, and urine sample were added to each well, followed by the addition of 50 µL horseradish peroxidase label. The plates were incubated at ambient temperature for 2 h. Plates were then washed and 50 µL of a substrate buffer (0.01 % tetramethylbenzidine and 0.004 % $H_2O_2$ in 0.1 M acetate citrate acid buffer, pH 6.0) was added to each well. 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 UCM concentrations were normalised to creatinine levels to control for water content using the methods described previously (Narayan et al., 2010). UCM levels were expressed as pg/µg Creatinine (pg/µg Cr).

**Statistical analysis**

All statistical analyses were carried out using Prism GraphPad (version 5.0). All the data was tested for normality using D'Agostino and Pearson omnibus normality tests before subjecting to statistical analyses. All the tests were two tailed and the significance level was set at 0.05 Differences in the mean UCM levels among groups and time-periods (0, 2, 12 h and 24 hrs) were analysed using repeated measures ANOVA, with time as a fixed factor, followed by Dunn's multiple pair-wise comparison test.

## RESULTS

The mean baseline UCM levels of the toads tagged with VIE was 32.07 ± 4.64 pg/µg Cr (range 28.42 – 41.50 pg/µg Cr). The sham operated group displayed a UCM range of 36.78 - 65.72 UCM pg/ug Cr and a mean baseline UCM level of 36.78 + 12.63 pg/µg Cr. The capture and handling control groups displayed a UCM range of 42.17 - 64.83 UCM pg/ug Cr, and a mean baseline UCM of 42.97 + 5.09 pg/ug Cr (Fig. 1).

Individual male toads showed variability in their UCM responses to the treatments (Fig. 1). Coefficient of variation (CV) in UCM levels for each of the treatment groups ranged as follows; VIE group (28-41 %), sham operated group (82-103 %) and capture-handling control group (45-90 %).

Overall, UCM concentrations varied significantly by time-period ($F_{3, 96}$ = 5.69, P = 0.0012) and by individual within each group ($F_{32, 96}$ = 5.924, P <0.0001). However, they did not differ among treatment groups ($F_{2, 96}$ = 1.22, P = 0.3000).

Within the VIE tagged group, UCMs were significantly elevated at 12 h post capture (P = 0.0065) for comparisons between mean UCMs at time-periods 0 c.f. 12 hrs; 12 c.f. 24 h), and had returned to baseline concentrations by 24 h post-capture (p > 0.05 for comparisons between mean UCMs at time periods 0 c.f. 2 h; 0 c.f. 24 h; 2 c.f. 12 h; 2 c.f. 24 h; Fig. 2).

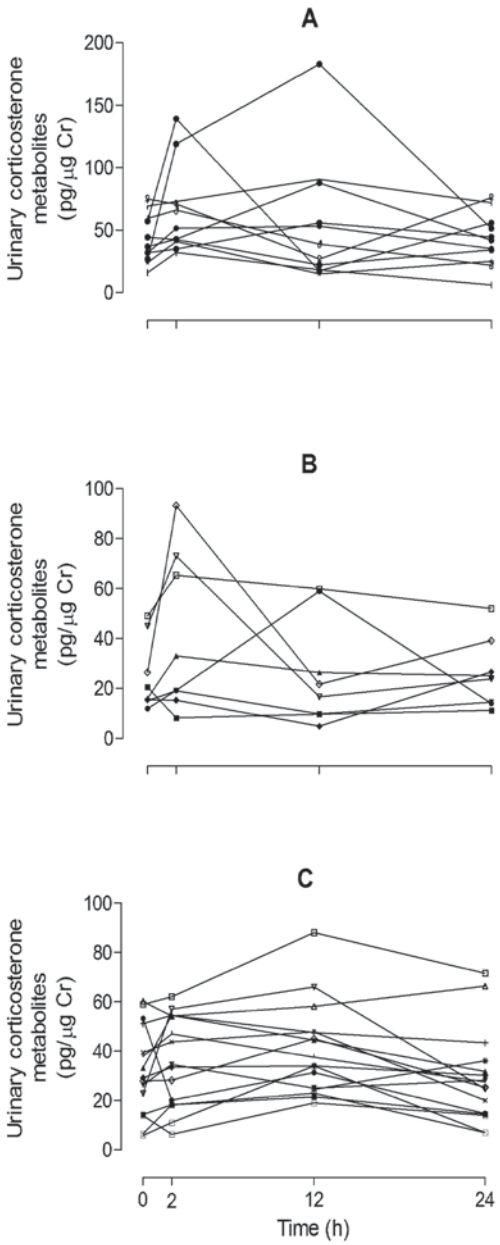


**Figure 1.** Individual urinary corticosterone metabolites in male Asian toads subjected to **(A)** capture handling control (n= 11), **(B)** sham (saline) control (n= 9), or **(C)** VIE method (n=16) at various time periods (0 h, 2 h, 12 h, 24 h) post-capture

For the sham operated and capture handling only groups, the mean UCMs levels were comparable (neither increased or decreased) between any of the time periods (0, 2, 12 or 24 h; p > 0.05; Fig. 2).

## DISCUSSION

Capture handling of wildlife in herpetological research is unavoidable as it enables the collection of crucial field data relating to the life-history and ecology of study species. As such, it is important that we understand that

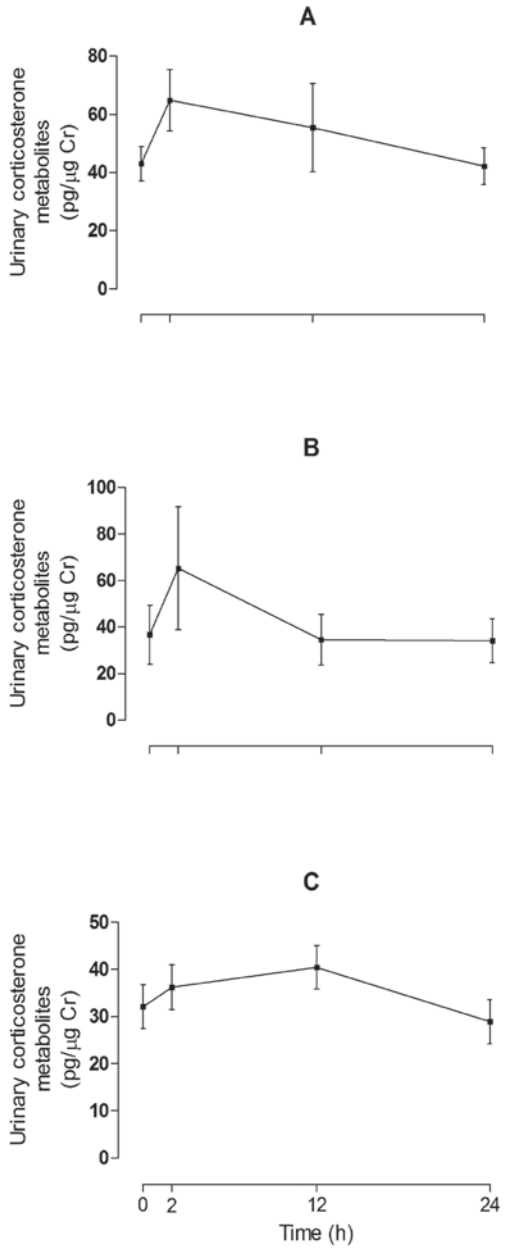


**Figure 2.** Mean (**±** S.E) urinary corticosterone metabolites in male Asian toads subjected to **(A)** capture handling control (n= 11), **(B)** sham (saline) control (n= 9), or (C) VIE tagging (n=16) at various time periods (0 h, 2 h, 12 h, 24 h) post-capture

even though ecological mark-recapture studies may not generate chronic stress in wildlife such as amphibians, wildlife can still perceive physical handling/experimental manipulation as a potential threat, and thus potentially elicit physiological stress. In this study, we tested whether the visual implant elastomer (VIE) technique generated physiological stress in male *D. melanostictus*. Our results show that although VIE tagging increased UCM levels of male toads, the mean levels of stress response were not different among groups that were subjected to either handling only or a sham (saline) injection. This result corroborates with previous work conducted on the impacts of VIE tagging on red-eyed tree frogs (Antwis et al., 2014).

Previously, adrenocorticotrophic hormone (ACTH) stimulation tests have been used on other amphibian species to determine the lag-time for the activation of HPI-axis and metabolism of UCMs, and their subsequent appearance in urine samples. It has been demonstrated that amphibians start to generate significant changes in UCMs in response to mild capture handling protocols at 2 h, and serial sampling of 2 h intervals post-treatment have been shown to result in significant changes in UCMs (Narayan et al., 2010). In our study, the sampling regime of 0 h (pre-treatment) and 2, 12 and 24 h (post-treatment) should have detected changes in UCMs reflecting the metabolism-specific lag-time of UCMs (Narayan et al., 2010). A more robust sampling design (i.e. sequential urine sampling at every 1 or 2 h interval post-treatment for over 24 h) would have in fact been more detrimental, potentially influencing and masking any notable variation in UCMs.

Short-term or acute stress response in amphibians involves the activation of the HPI-axis and release of corticosterone from the inter-renal tissue. Corticosterone release helps in glucose mobilisation so that muscle cells can expend energy. This helps amphibians in various processes, such as vocalisation, foraging behaviour, and escaping predators. However, prolonged activation of the HPI-axis can be maladaptive for amphibians and result in chronic stress, leading to severe effects on the immune response, reproductive endocrine response, and negatively affecting behaviour and survival. Our results suggest that the VIE tagging method does not lead to chronic stress in male Asian toads as their UCM levels had returned to baseline by 24 h post treatment, despite being elevated at 12 h post treatment. Additionally, our findings demonstrate that the VIE tagging method induces a stress response to the same magnitude as simply capturing and handling a toad – an activity which is required at a minimum for the successful implementation of a mark and recapture method.

A corticosterone stress response will be apparent in any interference with animals in the wild, no matter which technique is used (Schmidt & Schwarzkopf, 2010). We have demonstrated that the VIE method keeps this stress response to a minimum, highlighting its applicability as a humane marking technique for amphibians. This is especially apparent when comparing the VIE method to the traditional toe-clipping method, which has been demonstrated to elicit a higher stress response (Narayan et al., 2011). Furthermore, previous studies have demonstrated that toe-clipping has a plethora of detrimental impacts other than prompting a stress response (i.e. reduced jumping ability, locomotion, and ability to adapt to foraging conditions), not only for frogs but a range of taxa including lizards, geckos, and salamanders (Davis & Ovaska, 2001; Schmidt & Schwarzkopf, 2010; Guimarães et al., 2014). Conversely, the VIE method has been concluded to be a safe, effective, and convenient tagging technique for most applicable species with little to no detrimental impacts (Woods & James, 2003; Sapsford et al., 2015). Thus, considering the previous literature surrounding the 'VIE vs toe-clipping' techniques and our findings for the VIE method, there is little doubt that VIE is currently the most appropriate technique for the mark and recapture of frogs.

To improve animal welfare, we must first improve our technology. We need to direct our attention away from invasive mark-recapture methods, as well as hormone measuring methods, which cause unnecessary stress and detrimental impacts on animals. The advancements that VIE provides can greatly improve the success of research involving mark and recapture techniques whilst also minimising stress responses. This study has provided key findings to make the use of VIE tagging, as well as UCM testing a common practice.

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