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Abstract: Recently, non-invasive stress endocrinology research has highlighted the impacts of catastrophic environmental stressors, such as increasing environmental temperature and pathogenic disease (chytridiomycosis), on amphibian eco-physiological response. This study used non-invasive stress endocrinology to look at patterns of stress along an altitudinal gradient in an Australian free-living amphibian Mixophyes fasciolatus. Enzyme immuno-assays (EIA's) were performed on baseline urine samples and validated using an adrenocorticotropic hormone (ACTH) challenge. Frogs (n=10) were injected with ACTH after the initial urine sample on day 0 and recapture attempts occurred on days 1, 2, 3,4,6 & 10 post injection. Baseline samples were taken from 1 lowland (60m) and 2 highland sites (660m & 790m) during autumn 2011 in Springbrook National Park (NP) and Currumbin NP in South East Queensland (SEQ), Australia. The ACTH challenge successfully validated EIA's in M. fasciolatus, with a peak response on day 2 and a return to baseline by day 6. A multiple linear regression of baseline corticosterone and altitude found that corticosterone concentrations were significantly higher at highland sites ($p = 0.08 > 0.05$). We hypothesise that the observed increased baseline corticosterone at highland sites is associated with geographic range limits. Whether high baseline corticosterone within high altitude populations are indications of chronic stress or their relation to life-time fitness and survival requires urgent investigation.

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Attn:

The Editor

General and Comparative Endocrinology

Dear Sir/Madam

Please find attached the submission files for our paper entitled "Non-invasive monitoring of stress physiology within free-living highland and lowland populations of native Australian Great Barred Frog (*Mixophyes fasciolatus*)", co-authored by: Graham M.C.; Narayan J.E.; McCallum I.H. and Hero J-M, for submission as a regular article.

This paper provides novel contribution on the research topics of conservation physiology, non-invasive endocrinology and conservation of native Australian frog species along an altitudinal gradient in South East Queensland. We utilized established non-invasive urinary stress hormonal assessment to explore the differences in stress hormone concentrations (corticosterone metabolites) between high and lowland frog populations. We found significant difference in corticosterone levels of frog species between lowland and highland sub-populations. We discuss this novel finding in relation to amphibian stress physiology, geographic range limits and susceptibility to environmental stressors such as the pathogenic disease Chytridiomycosis. Our paper would appeal to the readers of General and Comparative Endocrinology as it successfully integrates non-invasive endocrinology with the ecology of amphibians to provide a deeper understanding of a serious conservation issue. We confirm that this manuscript has not been submitted elsewhere and is not under consideration by another journal. All authors have reviewed the manuscript and agree to its submission in General and Comparative Endocrinology. The authors have no conflicts of interest to announce.

We look forward to hearing back from you at your earliest convenience.

Sincerely

Clara Graham

(BSc Hons)

Highlights:

- Urinary Corticosterone enzyme-immunoassay was validated for male Great Barred Frogs
- First study of stress physiology along an environmental gradient in amphibians
- Significant ($P<0.001$) increase in urinary corticosterone with altitude
- Possible association between baseline stress and geographic range limit
- Stress may increase vulnerability to disease and other environmental stressors at high altitudes

Non-invasive monitoring of stress physiology within free-living highland and lowland populations of native Australian Great Barred Frog (*Mixophyes fasciolatus*)

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Abstract

Recently, non-invasive stress endocrinology research has highlighted the impacts of catastrophic environmental stressors, such as increasing environmental temperature and pathogenic disease (Chytridiomycosis), on amphibian eco-physiological response. This study used non-invasive stress endocrinology to look at patterns of stress along an altitudinal gradient in an Australian free-living amphibian *Mixophyes fasciolatus.* Enzyme immuno-assays (EIA's) were performed on baseline urine samples and validated using an adrenocorticotropic hormone (ACTH) challenge. Frogs (n=10) were injected with ACTH after the initial urine sample on day 0 and recapture attempts occurred on days 1, 2, 3,4,6 & 10 post injection. Baseline samples were taken from 1 lowland (60m) and 2 highland sites (660m & 790m) during autumn 2011 in Springbrook National Park (NP) and Currumbin NP in South East Queensland (SEQ), Australia. The ACTH challenge successfully validated EIA's in *M. fasciolatus*, with a peak response on day 2 and a return to baseline by day 6. A multiple linear regression of baseline corticosterone and altitude found that corticosterone concentrations were significantly higher at highland sites ($p = 0.08 > 0.05$). We hypothesise that the observed increased baseline corticosterone at highland sites is associated with geographic range limits. Whether high baseline corticosterone within high altitude populations are indications of chronic stress or their relation to life-time fitness and survival requires urgent investigation.

Key words Corticosterone; stress; altitude; Chytridiomycosis*;* Climate; *Mixophyes fasciolatus*

1. Introduction

Conservation physiology is a recently coined field of conservation biology that uses the investigation of physiological systems—such as the hypothalamo-pituitary adrenal (HPA)

axis—to understand how different physical and psychological stressors are affecting the eco-physiological responses of study organisms, giving an organism view of the environmental challenges and conservation threats faced [1]. Non-invasive endocrine techniques, such as reproductive and stress hormone evaluation in excreta (urine or faeces), are integral components of conservation physiology that have been widely used to study reproductive hormonal cycles and physiological stress responses in free-living animals, especially birds and mammals [2, 3]. Over the past decade, there has been considerable interest in measuring stress hormone metabolites (cortisol in mammals and corticosterone in amphibians, reptiles and birds) in urine and/or faeces as an index of the relatedness of both stress and reproductive success in various wild animals [4-6]. In recent years research in amphibian conservation physiology and particularly focus into the stress physiology of free-living and captive frog populations has progressed significantly through the development of non-invasive urinary steroid hormone assays [7-10]. Most recently, non-invasive stress endocrinology research has highlighted the impacts of catastrophic environmental stressors such as increasing environmental temperature and pathogenic disease (chytridiomycosis) on amphibian eco-physiological response [11, 12].

Amphibians are the focus of the present study because in the recent few decades native amphibian populations worldwide have experienced rapid population declines and extinctions. The Global Amphibian Assessment 2004 [13] estimated that 43 % of the approximately 6000 amphibian species worldwide are experiencing population declines with 34 species confirmed extinct, and another 130 species possibly extinct. A number of environmental factors have been proposed to contribute to amphibian declines including: habitat disturbance and fragmentation; pollution; atmospheric change; and the introduction of invasive species (such as cane toad, *Rhinella marina*) [14] and predators [15, 16]. Around

77 Australia and globally, these amphibian declines have been concentrated within protected or
78 pristine mountainous natural habitats, which suggests plausible multi-faceted interactions
79 between environmental stressors—such as extreme spikes and increases in environmental
80 temperature and chytridiomycosis disease— that could lead to increased severity of stressors
81 within these fragile natural environments. Currently, there is a paucity of clear reasoning to
82 explain highland protected area amphibian declines, which has created a major conservation
83 challenge. Intensive field based monitoring of the stress physiology of free-living amphibian
84 populations will be beneficial to understanding the causal factors in these enigmatic declines.
85 Most importantly, non-invasive stress hormonal monitoring can help determine high risk frog
86 populations and provide insights into the physiological tolerance of these fragile populations
87 against unpredictable stressors [17].
88
89 The hypothalamic-pituitary-interrenal (HPI) axis is the major stress endocrine system in
90 amphibians (equivalent to the mammalian HPA axis), which is responsible for physiological,
91 biochemical and behavioural responses to perceived environmental stressors [18]. The HPI
92 axis is responsible for the release of corticosterone, which maintains energy homeostasis
93 during predictable life history states and stressful events [19, 20]. Corticosterone
94 concentrations are known to vary seasonally responding to predictable life history variables,
95 such as changes in temperature, and individually due to the expression of different stress
96 response phenotypes [7, 21]. Corticosterone is also released in response to unpredictable
97 stress events such as capture and captivity [22, 23]. These unpredictable stressors result in a
98 sharp increase in circulating corticosterone and the short-term stress response, which redirects
99 energy away from non-essential functions such as growth and the reproductive and immune
100 systems causing the ‘emergency life-history state’ [24]. While short term suppression of
101 these biological systems is beneficial to the animal, a reoccurring stressor could lead to long

term or chronic elevation of baseline corticosterone concentrations which may have detrimental effects on survival and fitness of the individual [25, 26]. Urinary measurements of stress hormones have the advantage over faecal methods by providing a reduced lag-time for hormone excretion (i.e. relative to blood circulation), simplified processing, and reduced labour and ultimately lower costs [7].

The aims of the present study were to validate non-invasive urinary corticosterone enzyme-immunoassay in free-living native Australian wet forest amphibian species, the Great Barred frog, *Mixophyes fasciolatus*. We performed a biological validation, through adrenocorticotropic hormone (ACTH) challenge, which is crucial to demonstrate that hormonal measures accurately reflect physiological events of interest. This approach also clarifies the excretory lag-time between stimulation of an endocrine gland and the appearance of its hormonal metabolites in excreta [7]. Secondly, we assessed baseline levels of urinary corticosterone metabolites in adult male *M. fasciolatus* from two highland and one lowland sub-population in South East Queensland. *M. fasciolatus* is an Australian ground-dwelling frog, which is a non-declining amphibian species under the International Union for Conservation of Nature (IUCN) Red List, and is distributed at all altitudes along creeks and streams from the mid-coast of South East Queensland (SEQ) to the Northern New South Wales coast, Australia [27]. We used *M. fasciolatus* for the current study because it is easily accessible and its altitudinal distribution provides a good opportunity to investigate the stress physiology of a mountainous frog population. We proposed the hypothesis that baseline urinary corticosterone concentrations will vary between lowland and highland sites and stress hormonal titres may be correlated with body-condition. The outcomes of this study will provide a crucial window of opportunity for investigating stress physiology of congener

126 species in future such the Giant Barred Frog (*M. iteratus*) and Fleayi's Barred Frog (*M.*
127 *fleayi*) that are considered endangered under the IUCN Red List.
128
129 **2. Methods**
130
131 *2.1 Field sites*
132 Free living adult male individuals of Great Barred Frog (*Mixophyes fasciolatus*) were used in
133 this study because they vocalise and are therefore more readily located than their female
134 counterparts. Frogs were sampled at three sites in South East Queensland (SEQ), Australia
135 and sampling took place at night-time during the autumn months of March, April and May in
136 2011. The three study sites consisted of one low land site at 60 m (27°58'36.77"S,
137 153°16'36.09"E) and two high land sites at 592 m and 790 m (28°11'19.20"S
138 153°16'10.22"E, 28°13'40.58"S 153°16'19.26"E). These sites were set up in Springbrook
139 National Park (NP) and Currumbin NP in SEQ. The vegetation in these areas consists of sub-
140 tropical to temperate rainforests with increasing levels of habitat disturbance through
141 urbanisation at lower altitudes. Sampling transects were adjacent to permanent water bodies
142 at all three sites.
143
144 *2.2 Frog capture, urine sampling protocols and morphometrics*
145 Individuals were located using spotlighting of eye shine and call playback. Call playback
146 stimulates calling behaviour and can be used to locate individuals, particularly when not
147 sitting in the open. Precautionary handling methods were used when sampling to prevent any
148 transmission of the pathogenic chytrid fungus [11]. Frogs were captured in sterile plastic
149 freezer bags and sterile un-powered latex gloves changed between captures, following an
150 official handling protocol [7].

Urine samples were obtained from each frog to measure baseline urinary corticosterone metabolites (hereafter termed as urinary corticosterone) based on the established methods of [7]. Most recently, a urinary corticosterone enzyme-immunoassay (EIA) was validated for another native Australian frog, the Stony Creek Frog (*Litoria wilcoxii*) [11]. Urinary steroid metabolites evaluation is non-invasive and superior to traditional blood plasma methods as demonstrated by several recent studies using non-native cane toads (*Rhinella marina*) and several threatened native amphibians [7, 22, 28]. Correlated patterns of change in blood plasma and urinary corticosterone concentrations have recently been demonstrated in cane toads (*Rhinella marina*) during capture and handling stress (Narayan et al. unpublished data). Samples were taken immediately after capture by gently inserting the sterile tip of a 200 µl pipette (2-3 mm) into the frog's cloaca. Urine was collected effortlessly as the frog's tended to urinate immediately via the micturition reflex. Urine samples representative of baseline corticosterone metabolites were taken from frogs during a similar time period without repetition, to minimise the effect of reproductive state or handling stress on corticosterone concentrations.

Snout-vent length (SVL) and body-weight (g) measurements were taken for each frog and body condition was calculated using Fulton's index ($K=M/L^3$) [29]. The frogs were then toe clipped using the methods of Hero [30] for identification and to prevent pseudo-replication.

2.3 Biological validation of urinary corticosterone enzyme-immunoassay

The urinary corticosterone enzyme-immunoassay (EIA) for adult male *M. fasciolatus* was biologically validated using an adrenocorticotropic hormone (ACTH) challenge. A standard dose of ACTH was administered using the established methods of [7]. Injections were administered at night-time on day 0 and were given to individual frogs near the coelomic

177 cavity (away from any vital organs), using a thin 25-gauge needle and 1 ml syringe. ACTH
178 dose for each frog (n = 10) was 0.446 mg ACTH g^{-1} bodyweight (Sigma Chemical Co., A-
179 0298) in 100 µL saline vehicle (0.9% NaCl) using a 1-mL sterile syringe. The method of
180 delivery of the ACTH injection was identical to those used for *L. wilcoxii* [11] and *R. marina*
181 *[*8]. A urine sample was collected from each male frog prior to injection (day 0) and on
182 successful recapture, on days 1, 2, 3, 4, 6 and 10 post ACTH injection.
183
184 *2.4 Corticosterone enzyme-immunoassay*
185 A urinary corticosterone enzyme-immunoassay (EIA) was adapted from [7] to quantify
186 urinary corticosterone concentrations in adult male *M. fasciolatus.* The corticosterone EIA
187 used a polyclonal anti-corticosterone antiserum (CJM06, UC Davis California) that measures
188 conjugated corticosterone metabolites. The anti-corticosterone antiserum was diluted 1: 45
189 000, horseradish peroxidise-conjugated corticosterone label diluted 1: 120 000 and
190 corticosterone standards (1.56–400 $pgwell^{-1}$). Cross reactivity of the CJM06 anti-
191 corticosterone antiserum was 100 % with corticosterone, 14.25 % with desoxycorticosterone
192 and 0.9 % with tetrahydrocorticosterone (C. J. Munro, *pers. comm*). Dilution rates for *M.*
193 *fasciolatus* urine were based on the concentration of pooled samples that resulted in 50 %
194 binding on the parallelism curve [31] (Fig. 1 A and 1B), which was 1: 2 for urinary
195 corticosterone metabolites in the baseline samples and 1: 4 for the ACTH challenged urine
196 samples. The recovery of standard corticosterone added to frog urine was expressed as a
197 linear regression formula (y = mx + b where, y = concentration of hormone observed, x =
198 concentration of hormone expected, m = slope of the line) and the multiple correlation
199 coefficient is squared to produce the coefficient of determination (r^2) [7]. The recovery of
200 corticosterone added to frog urine samples was 93 % as demonstrated by this linear
201 regression equation: $y = 0.9368x - 0.2431$, $r^2 = 0.9988$ (n = 6). Intra- (within) and inter-

(between) assay coefficients of variation (CV) were determined from high- (~70 %) and low- (~30 %) binding internal controls run on all assays. Intra-assay CV were 7.4 % and 2.6 % for low and high-percentage-bound controls, and inter-assay CV were 15.4 % and 14.3 % for low- and high-percentage-bound controls respectively. The assay sensitivity was calculated as the value 2 standard deviations from the mean response of the blank (zero binding) samples and was 2.54 $\pm$ 0.74 pg/well (n = 8). All urinary corticosterone concentrations were corrected using creatinine (Cr) concentration to control for any variably in water content in frog urine as per established methods [7]. Urinary corticosterone concentrations were presented as pg corticosterone metabolites/ µg Cr.

2.5 Statistical analysis

Statistical analysis was performed using the statistical package lme4 [32] in the statistical programming environment R [33]. The biological validation of the glucocorticoid assay was analysed using a Linear Mixed Effects model (LME), and included repeated measures. The response variable was urinary corticosterone, the fixed effect was time (day) and the random effect was the individual frog. Although each frog was recaptured throughout the 6 re-capture attempts, not all frogs were recaptured on each sampling event and this resulted in an uneven number of samples per frog. This unevenness in number of observations per individual can create biased estimates of model parameters and their variance; it is therefore useful to use maximum likelihood estimates when analysing differences in y variable values across time [34]. A Monte Carlo Markov Chain analysis (MCMC) is a Bayesian analysis capable of producing maximum likelihoods for LME models and was used in our analysis to generate iterations of the LME model. These iterations were used to estimate the mean urinary corticosterone response and upper and lower 95 % highest posterior density intervals (parameter dispersion) for each sampling day during the ACTH challenge. Baseline (day 0)

was held constant and used to estimate difference intervals for each day. If highest posterior density intervals were clear of the day 0 mean the difference was significant. Baseline urinary corticosterone levels were analysed using a multiple linear regression, with altitude and body condition as the explanatory variables. When interacting variables were not significant the analysis was rerun as a linear regression between corticosterone and altitude. Before analysis the data was transformed using a base 10 logarithm on corticosterone, to account for the higher variation at higher altitudes.

3. Results

3.1 Biological validation

A complete biological validation of the urinary corticosterone enzyme-immunoassay for adult male *M. fasciolatus* was demonstrated via the ACTH challenge. The MCMC analysis showed urinary corticosterone concentrations were significantly different to baseline (day 0) concentrations on days 1 through to 4 after the ACTH challenge (Fig. 2). Mean urinary corticosterone concentrations increased from day 1, with a peak excretion of corticosterone after ACTH injection occurring at day 2, and returning to baseline by days 6. Urinary corticosterone concentrations were not significantly different to baseline (day 0) on days 6 and 10 indicating that urinary corticosterone had returned to baseline levels. Mean urinary corticosterone concentration on day 2 post ACTH challenge was 125.6 pg/µg Cr with a lower posterity density interval of 82.73 and an upper posterity density interval of 115.28 pg/µg Cr, and individual peak responses ranging from 124.95 to 139.36 pg/µg Cr. The mean baseline urinary corticosterone concentration over the period of the ACTH challenge was 26.54 pg/µg Cr, with a lower posterity density interval of 15.12 and an upper posterity density interval of 37.9 pg/µg Cr. Individual baseline corticosterone concentrations (day 0) ranged from 17.47 to 37.63 pg/µg Cr (N=10).

3.2 Baseline urinary corticosterone of frogs between lowland and highland sub-populations

The multiple linear regression showed a significant relationship between urinary corticosterone and altitude (coefficient =0.0236, S.E. = 0.003899, p = 2.05e-07 < 0.05), and a weak non-significant relationship with body condition (coefficient= -93.8, S.E. = 52.6, p = 0.08 > 0.05). Linear regression of baseline corticosterone concentrations versus altitude showed a significantly positive relationship between these two factors (coefficient = 1.27, SE=3.38e-02, P=2e-16<0.05; Fig. 3). The mean baseline corticosterone concentration for the lowland frog population was 20.77 pg/µg Cr with a coefficient of variance (CV) of 28 %. The mean baseline corticosterone concentration for highland frogs was 23.88 pg/µg Cr with a CV of 19% at the 660m site and 38.49 pg/µg Cr with a CV of 26% for the 790m site.

4. Discussion

This study used the urinary corticosterone enzyme immunoassay (EIA) methods of [7] and biologically validated urinary corticosterone metabolite EIAs for the first time in adult male *Mixophyes fasciolatus*. The adrenocorticotropic hormone (ACTH) challenge demonstrated a clear raise and return to baseline profile of urinary corticosterone in male *M. fasciolatus*. We also investigated baseline urinary corticosterone in male frogs across an environmental gradient (one lowland and two highland sub-populations) and mean baseline urinary corticosterone concentrations were significantly higher for the two highland sub-populations in comparison to the lowland sub-population.

The pattern of change in urinary corticosterone post ACTH response is similar to those shown earlier for the cane toad (*Rhinella marina*), which showed a mean peak increase on day two [8], and the Fijian Ground Frog (*Platymantis vitiana*) which showed a mean peak

277 increase on day one [7]. Furthermore, [11] earlier demonstrated urinary corticosterone
278 concentrations of male Stony Creek frogs (*Litoria wilcoxii*) increased within 1–2 days after
279 ACTH challenge and returned to baseline levels within 3 days post-ACTH injection. The
280 ACTH challenge confirms that the urinary corticosterone EIA is measuring the steroidal
281 metabolite of interest (urinary corticosterone) and it also explains the excretory lag-time
282 between ACTH injections and appearance of corticosterone metabolites in frog urine. As
283 shown, the excretory lag-time of corticosterone excretion in response to a maximal
284 experimental stressor (ACTH injection) was two days and urinary corticosterone
285 concentrations decreased thereafter, returning to baseline (day 0) levels within six days post
286 ACTH injection. This return to baseline in urinary corticosterone highlights the role of the
287 negative feedback loop of the hypothalamo-pituitary interrenal (HPI) axis, most likely via
288 binding of circulating corticosterone by binding globulins and the hypothalamus decreasing
289 corticotrophin-releasing hormone (CRH) secretion, resulting in decreased ACTH secretion by
290 the pituitary [35].
291
292 This is first ever comparison of stress hormonal titres within free-living amphibian
293 populations from lowland and highland sub-populations. Both highland populations had
294 significantly higher baseline urinary corticosterone in comparison to their lowland
295 counterparts. One possible explanation for the increased baseline corticosterone levels in
296 highland *M. fasciolatus* populations is that frog populations at higher altitudes are living in an
297 environment that is approaching their eco-physiological limit. Hence the high baseline
298 corticosterone may be a result of increased environmental stress over numerous decades.
299 Recently, [36] highlighted that glucocorticoid hormones such as corticosterone play an
300 important role in the geographic range limits of free-living species by overwhelming key
301 physiological systems (such as immune system response, reproductive hormonal system,

302 metabolic functions) as well as basic life-history processes (such as reproductive behaviour,
303 locomotion, foraging) in suboptimal environments where energy demands are too great to be
304 modulated. A review of *M. fasciolatus* habitat requirements found that they have an
305 altitudinal distribution from 40 to 820m [27], with an altitude of 790m; our highest site is
306 approaching the edge of *M. fasciolatus's* geographical distribution. Chronic stress could
307 lead to depression of the reproductive system [18] and interestingly amphibians at high
308 altitudes often have less frequent breeding seasons with a decreased breeding length, smaller
309 clutch sizes and a longer development time [37, 38]. It has been shown that *M. fasciolatus*
310 sub-populations at high altitudes have decreased breeding season [38], which could possibly
311 be explained by corticosterone induced redistribution of important life-history decisions such
312 as reproduction.
313
314 Previous studies of stress physiology in vertebrates living at the edge of their
315 geographical/biological limitations however have shown equivocal results. Song wrens
316 (*Cyphorhinus phaeocephalus*) in lowland tropical forest of Honduras in western Ecuador
317 showed increased basal corticosterone and lower body condition at the edge of their range
318 limit [36]. However, western fence lizards (*Sceloporus occidentalis*) in the western United
319 States showed an increase in the stress hormonal response to standard capture and handling
320 with no change in body condition or baseline corticosterone at the edge of their range limits
321 [39]. Despite differences in their physiological symptoms, both organisms showed signs of
322 stress towards the edge of their geographic range, and the observed differences in the stress
323 response may be a result of different adaptive measures [18, 21, 40]. Although our study
324 showed no significant difference in body condition (fitness indicator) between highland and
325 lowland sites, the increased baseline stress hormone concentrations indicates that stress is
326 present. It would therefore be useful in the future to use more encompassing fitness

indicators, while looking at stress along an altitudinal gradient. A study of metabolic depression as a fitness indicator of stress has strongly shown that a salamander showed significant metabolic suppression at its geographic range limit [41].

There are currently two major hypotheses used to explain the effects of increased baseline stress in free living animals, the Cort-fitness hypothesis and the Cort-adaption (or Cort-activity) hypothesis [21, 42]. The Cort-fitness hypothesis is common throughout conservation physiology literature and predicts that fitness is negatively correlated with increasing basal corticosterone concentrations. The Cort-adaption hypothesis predicts that reproductive success is positively correlated with increased corticosterone. Neither hypothesis is all-encompassing and it is likely that the effect of corticosterone on fitness and or reproduction is multifaceted and situation dependent [18, 21]. Here, we hypothesise that corticosterone levels increase towards the edge of species distributions, until the organism can no longer deal with the levels of stress present and are no longer able to persist. We also hypothesise that animals at the edge of their range limits would be more vulnerable to further unpredictable stressors, such as disease invasion. We are currently conducting further field investigations to assess the relationships between pathogenic disease prevalence (chytridiomycosis) and physiological stress in free-living lowland and highland populations of *M. fasciolatus*.

This study has demonstrated that the use of conservation physiology and in particular non-invasive stress endocrinology is important to gaining a holistic understanding of the factors driving patterns of high altitude amphibian declines in Australia and globally. A large number of high altitude amphibian declines have been associated with the pathogenic amphibian disease chytridiomycosis. [11] demonstrated a positive correlation between

infection with the pathogenic chytrid fungus (*Bd*) and elevated baseline corticosterone in free-living populations of *L. wilcoxii*. If highland amphibian populations are experiencing decreased fitness as a result of increased baseline corticosterone, stress may be a key casual factor in declines. Higher baseline urinary corticosterone metabolites within high elevation frog populations, highlights possible physiological preparedness against unpredictable stressors such as *Bd* and climate variability. Whether high baseline corticosterone within high altitude populations are indications of chronic stress or their relation to life-time fitness and survival requires urgent investigation.

5. Conclusions

This is the first study demonstrating the ecological application of conservation physiology in investigating patterns of stress within a free-living amphibian at different altitudes. Conservation physiology offers in-depth insight into conservation problems by giving an organism view on how environmental variables can affect health and survival. This study has successfully validated EIA's in *M fasciolatus* and found elevated baseline corticosterone concentration in highland *M. fasciolatus* populations. This pattern of elevated baseline corticosterone in highland populations may be an extremely important variable when attempting to understand the causal factors in enigmatic, highland, protected area amphibian declines. The repetition of the study in declining or at risk species may prove to be very revealing. Future research would best be directed towards the assessment of the relationship between elevated corticosterone and fitness in laboratory and field situations. Future research into the dynamics of pathogens such as *Bd* and stress in amphibians should be undertaken with more repeated sample sizes, during the high disease prevalence season of spring, and attempt to link *Bd* prevalence and intensity at different altitudes with patterns of stress. Furthermore, under controlled laboratory settings, the effect of different environmental

variables on the stress axis and immune system responses of amphibians should be studied. Overall, physiological investigation of species range limits would also offer great insight into factors driving species distributions and would be applicable to many fields of conservation biology and ecological physiology.

6. Acknowledgments

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Figure Legends

Figure 1: Parallelism of pooled urine samples of *Mixophyes fasciolatus* against corticosterone standard curve. B/TB is the % of binding over total binding. The recommended 50 % binding occurred when samples were diluted 1:2 and 1:4 for the ACTH (a) and baseline pool (b) respectively. The pooled urine sample shows parallelism with the standard curve, which validates that the hormone measured in the urine sample, is immunologically similar to the corticosterone standard.

Figure 2: Corticosterone levels of 10 frogs over 10 days following ACTH challenge. Levels are shown relative to the mean baseline urinary corticosterone on day 0 (26.52 pg/µg Cr). The error bars show the mean effect derived from the mixed effects model, together with 95% confidence intervals. Different plotting symbols show the response relative to the overall mean baseline for each individual frog (these have been offset by 0.2 days for clarity).

Figure 3: The simple linear regression of log of corticosterone concentration (pg/µg Cr) against altitude. The Regression line (log Corticosterone =1.26 + 0.00034 x Altitude) shows a significant positive relationship between corticosterone concentrations and altitude (P=4.61e-07, n=50, r^2= 0.457).

Figures 1

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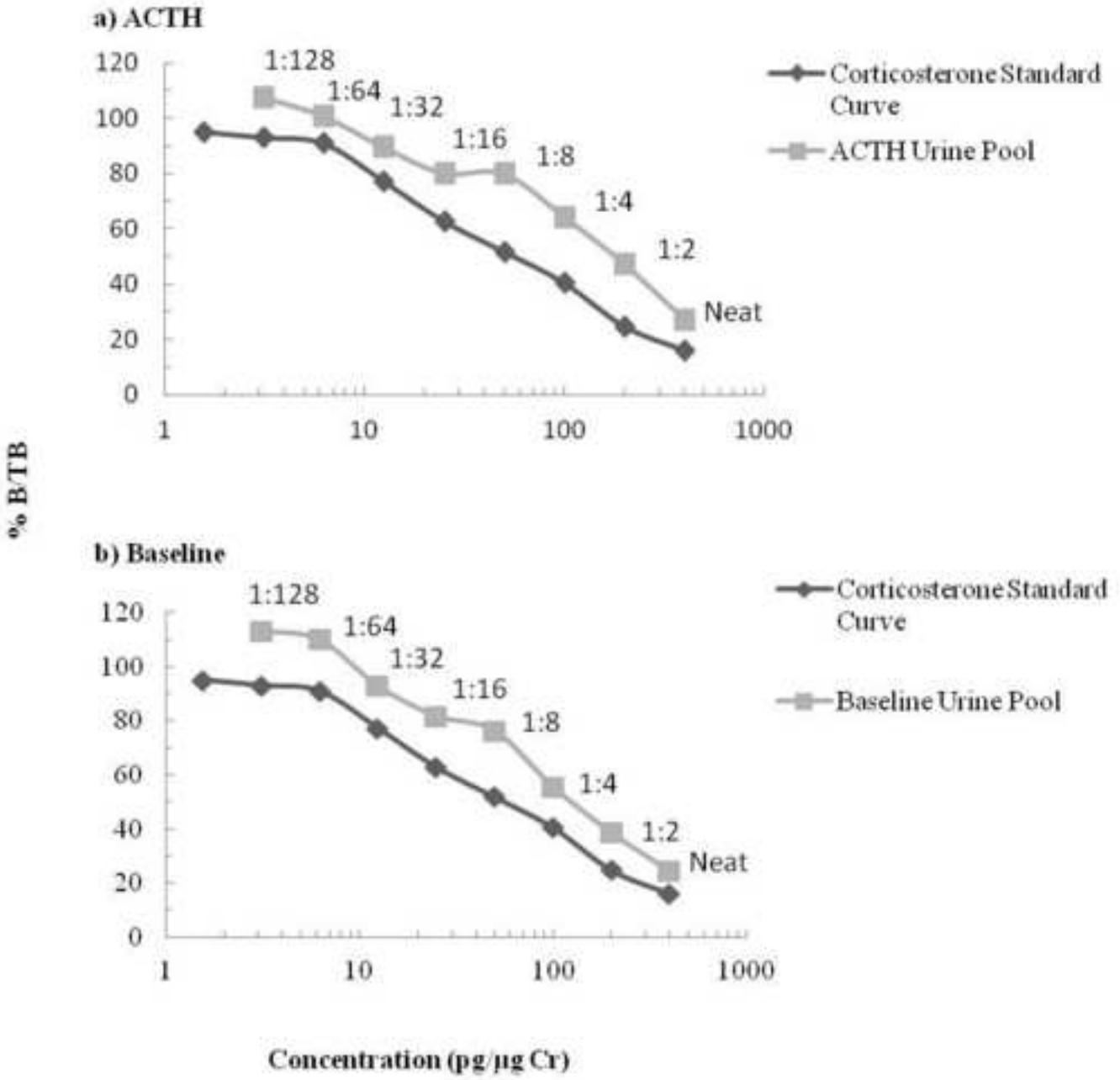

Figures 2

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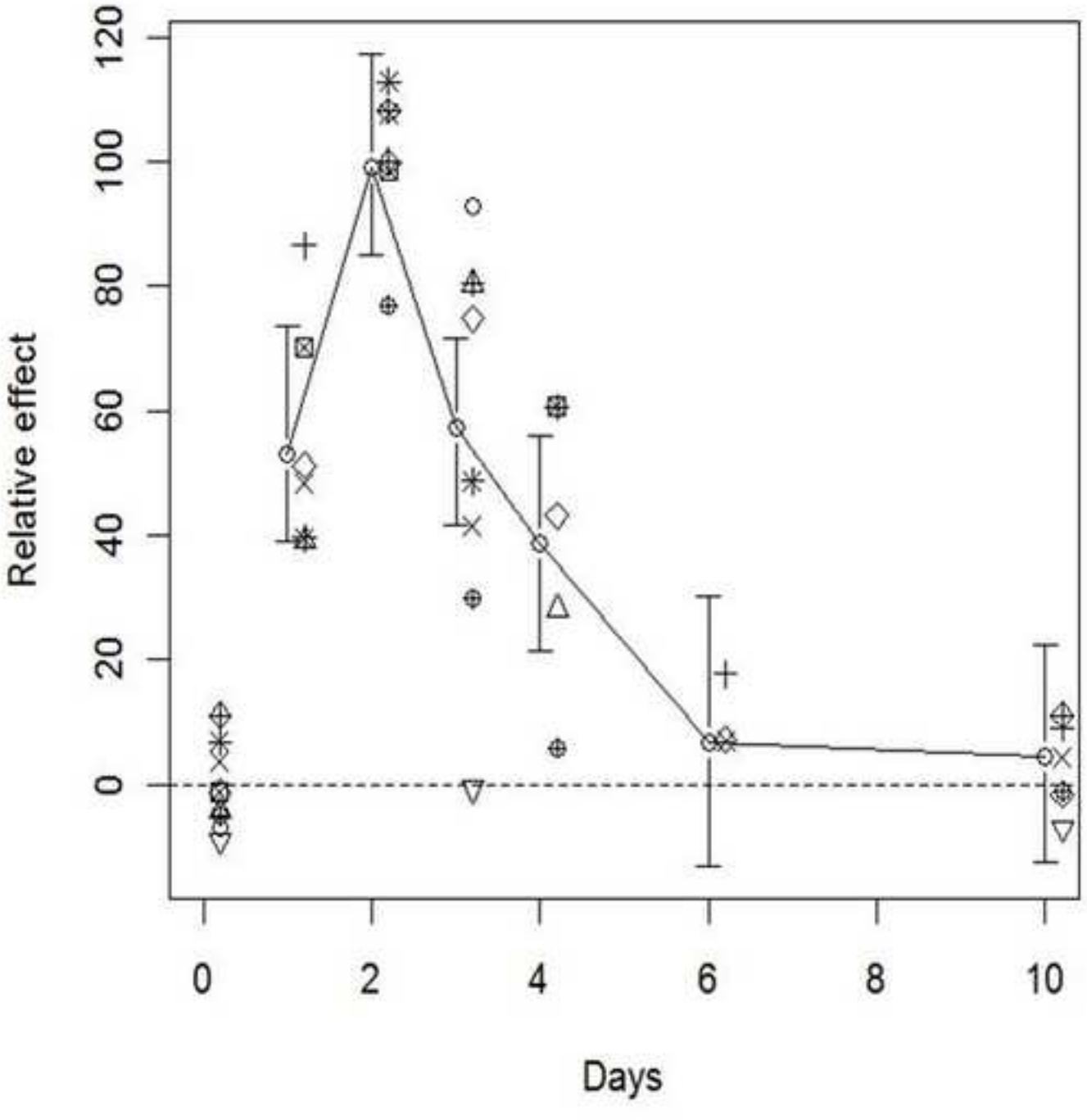

Figures 3

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