

How much stress do researchers inflict on their study animals? A case study using a scincid lizard, *Eulamprus heatwolei*

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Accepted 17 January 2006

Summary

Research on live vertebrates is regulated by ethics committees, who prohibit ‘excessively stressful’ procedures. That judgment is based on intuition – a notoriously unreliable criterion when dealing with animals phylogenetically distant from humans. To objectively evaluate the stress imposed by research practices, we measured plasma corticosterone levels in lizards (*Eulamprus heatwolei* Wells & Wellington, Scincidae). Some procedures (handling and measuring, toe-clipping for identification, exposure to predator scent) did not induce significant increases in corticosterone levels, suggesting that these stimuli generated relatively little stress. However, other stimuli (testing locomotor speed, microchip implantation, blood sampling, an unfamiliar enclosure, tail autotomy, exposure to a heterospecific lizard) were more stressful, with corticosterone levels increasing only transiently in some treatments (<2 h for tail autotomy), but persisting much longer in others (14 days for microchip implantation). Overall, our data

suggest that the levels of stress induced by routine laboratory procedures are no greater than those often experienced by lizards in nature; but that intuition provides a poor basis for evaluating the levels of stress induced by research. For example, toe-clipping is often criticized and sometimes banned; but our data suggest that this method is actually less stressful than the technique frequently recommended to replace it on ethical grounds (microchip implantation). Toe-clipping also was less stressful than superficially trivial manipulations such as housing the animal in an unfamiliar enclosure. More generally, we urge researchers to seek objective information on the effects of their activities on research subjects, rather than relying upon subjectivity and anthropomorphism in making these evaluations.

Key words: corticosterone, ethics, lizard, *Eulamprus heatwolei*, methods, research.

Introduction

One of the major issues confronting scientists who conduct research on live vertebrates is whether or not their research imposes significant stress on their study organisms. High levels of stress to the organisms can raise two major problems. First, it can introduce confounding factors that will obscure any clear-cut answer to the research questions being addressed (Belliere et al., 2004; Guillette et al., 1995). Second, inflicting high levels of stress on our study organisms is ethically indefensible (Association for the Study of Animal Behaviour, 2003; Office of Technology Assessment, 1986). This latter issue has grown to become a major community concern over recent decades, stimulating the creation of institutional ethics committees to regulate researchers and hence, prevent excessively stressful procedures (Hagelin et al., 2003; Jennings, 1994; National Health and Medical Research Council, 2004; National Research Council, 1996). For example, many ethics committees forbid toe-clipping as a

technique for individual identification, insisting instead on ‘less stressful’ measures such as microchip implantation (Mellor et al., 2004). Approval by such a committee and adherence to accepted ethical codes of conduct are now prerequisites for conducting research in many parts of the world: for example, wildlife authorities may require ethics approvals before providing research permits, and journals may adhere to specific guidelines that outlaw some previously popular research methods (Association for the Study of Animal Behaviour, 2003; Department of Sustainability and Environment, 1975; Fish and Wildlife Division, 2003).

Although judgments about stress thus dictate the kinds of research methods that can be used, the term ‘stress’ itself generally remains poorly defined even in the regulations that guide the activities of ethics committees (e.g. National Health and Medical Research Council, 2004; University of Canterbury Animal Ethics Committee, 2004). Such definitions range from emotive descriptions [e.g. ‘fearful’ or ‘frustrated’] (Petherick,

2005)], to very general statements such as ‘the biological responses an animal exhibits in an attempt to cope with a threat to its homeostasis’ (Carstens and Moberg, 2000). The term ‘stress’ is often used interchangeably with ‘distress’, though the latter is sometimes used to describe a more severe condition.

Despite broad consensus about the need for ethical treatment of research animals, the difficulty in defining stress has discouraged attempts to actually measure the effects of experimental procedures on animal welfare. Therefore, ethics committees generally have to base their decisions on intuition, a notoriously unreliable criterion when dealing with organisms that are phylogenetically remote from ourselves. Ectothermic vertebrates (such as reptiles and amphibians) are of special interest in this respect because they are popular research subjects, but are physiologically and behaviourally so divergent from endothermic vertebrates (such as humans) that it is difficult for us to judge stress in a lizard (for example) by overt behavioural symptoms. How, then, can we proceed to replace subjectivity with objective information? The central problem involves developing a way to measure ‘stress’ and fortunately, existing literature provides a useful and well-understood methodology in this respect. An increase in glucocorticoids is a classic marker of physiological stress in vertebrates (Belluore et al., 2004), with corticosterone being the principal glucocorticoid in reptiles, amphibians, birds, and many rodents (Romero, 2004). Although plasma corticosterone levels can be influenced by a variety of factors including reproductive stage (Woodley and Moore, 2002), and thus elevated levels do not always indicate stress, they can be used reliably for this purpose under controlled conditions when other factors are held constant. Thus, our working definition of stress is ‘the physiological response of an animal to an experimental stimulus, as measured by elevated circulating glucocorticoid levels’. Changes in plasma corticosterone levels have been used to assess the impact of capture, handling, restraint and confinement on reptilian species (Moore et al., 1991; Cree et al., 2003; Cree et al., 2000; Mathies et al., 2001; Kreger and Mench, 1993; Cash et al., 1997), but the amount of stress induced by alternative commonly used research procedures beyond initial capture and housing has not been previously assessed. The present paper describes a study that set out to quantify the ways in which plasma corticosterone levels in lizards are affected by a variety of methods that are commonly used in fields of research such as behavioural ecology and microevolution. For comparison with the levels of stress induced by ‘natural’ events in a lizard’s life, our design included treatments such as tail autotomy, and encountering potentially threatening stimuli such as the scent of a predator or the actual presence of a larger competitor.

Materials and methods

Study species

Five species of viviparous scincid lizards occur sympatrically and at high densities at Kanangra-Boyd National Park (33°58.207’S, 150°03.346’E; 1200 m above sea level), 160 km west of Sydney, Australia. All species (three within the genus

Egernia and two within *Eulamprus*) are diurnally active heliotherms that inhabit rocky outcrops among the eucalypt forest at this site. They occupy refuges that are similar in most abiotic characteristics (Langkilde et al., 2003), with strong, highly aggressive competition among these species for the warmest refuge sites (Langkilde and Shine, 2004). The current study focuses on one of these species, *Eulamprus heatwolei* (Wells & Wellington, Scincidae). As one of the smallest of the five taxa (approximately 85 mm snout–vent length *SVL*) (Cogger, 2000), *E. heatwolei* is often excluded from preferred refuges by more dominant species (Langkilde and Shine, 2004). *Eulamprus heatwolei* is insectivorous, and females give birth to a litter of one to eight offspring per year in late summer (Greer, 1989). Tail autotomy is common in natural populations (83.5% of adult *E. heatwolei* captured from Kanangra-Boyd National Park as part of a separate study had previously autotomised their tails, $N=85$; T.L. and R.S., unpublished data). Previous research has shown that *E. heatwolei* exhibit elevated plasma corticosterone levels in response to prolonged handling (Langkilde et al., 2005).

Collection and husbandry of animals

To obtain natural baseline corticosterone levels, blood samples were obtained from 10 male and 10 female *E. heatwolei* immediately upon capture from the field at Kanangra-Boyd National Park, in the first 2 weeks of November 2004. In this and all other cases, we took a 70 μ l blood sample from the post-orbital sinus using a 75 μ l heparinized capillary tube. For the experimental component of this study, we captured 250 adult *E. heatwolei* (100 males and 150 females) by hand from Kanangra-Boyd National Park in the first 2 weeks of November 2004. This species breeds synchronously, and at this time of year all lizards are just starting to mate, and females are commencing ovulation (T.L. and R.S., unpublished data). All lizards were sexed upon capture and abdominally palpated to determine the number of follicles, and whether or not they had ovulated. They were then placed into individual canvas bags, transferred to the University of Sydney, and individually housed in plastic containers (20×12×10 cm; L×W×D) with a paper substrate, a water dish, and a shelter. The room was maintained at 16°C, and heat for thermoregulation was provided by strips of heating tape (reaching 40°C) running under one end of each container on a 9 h on:15 h off cycle. The overhead fluorescent lights in the room were set on a 12 h on:12 h off cycle, mimicking field conditions. Water was provided *ad libitum*, and lizards were fed four crickets twice weekly. Lizards were maintained under these conditions for 14 days before the experiment started, as previous research suggests that lizards adapt to captivity (with corticosterone levels returning to baseline levels) within this time (Jones and Bell, 2004; Manzo et al., 1994).

Experimental design

(1) *How long after exposure to a stressor should a blood sample be obtained?*

To obtain a reliable estimate of the physiological stress caused by a particular treatment, a blood sample must be taken

after plasma corticosterone concentrations have increased, but before they decrease again. To assess the timing of changes in plasma corticosterone after the application of a stressor, we assigned 50 female *E. heatwolei* to one of five treatments ($N=10$ females in each group). Forty females (from groups 1–4) were exposed to a potential stressor by obtaining a blood sample and then chasing them around the inside of an empty container ($60 \times 33 \times 20$ cm; $L \times W \times D$) for 30 s before returning them to their enclosures. We obtained blood samples from the females assigned to the first treatment group 5 min after the initiation of the stressor, after 30 min for the second treatment group, after 1 h for the third group, and after 2 h for females in the fourth group. Females assigned to the fifth treatment acted as controls; they were not disturbed in any way, and a blood sample was obtained immediately upon capture from the enclosure. Results from this experiment determined the timing of sampling in the subsequent experiments.

(2) How does treatment affect plasma corticosterone levels?

Lizards were randomly assigned to one of 10 treatments ($N=10$ males and 10 females per treatment), designed to mimic common laboratory research techniques and natural events, as well as appropriate controls. The treatments were as follows.

(i) Control – lizards remained undisturbed in their enclosures.

(ii) Blood sampling – a 70 μ l blood sample was collected from the post-orbital sinus using a 75 μ l heparinized capillary tube.

(iii) Handling/measuring – animals were handled to obtain measurements of size (snout–vent length and tail length) and mass.

(iv) Predator scent – a paper towel impregnated with the scent of a lizard-eating snake from Kanangra-Boyd (red-bellied black snake, *Pseudechis porphyriacus*; towel kept in snake cage for 4 days) was placed into the lizard's enclosure for 1 h.

(v) Toe-clipping – the distal one-third of each of three toes was clipped using sharp scissors, with no more than one toe clipped from each foot and never clipping the fourth (longest) toe.

(vi) Microchip insertion – a 12×2 mm cylindrical microchip (PIT tag, Biomark, Inc., Boise, Idaho, USA) was inserted under the skin just anterior to the rear leg using the sterile syringe applicator supplied with the tags, and the small incision sealed with medical grade suture glue.

(vii) Tail autotomy – the lizard was induced to autotomise its tail by grasping the tail 10 mm from the cloaca with a pair of forceps.

(viii) Unfamiliar enclosure – the lizard was placed into a $60 \times 33 \times 20$ cm ($L \times W \times D$) lidded container supplied with two plastic shelters and a water container for 1 h.

(ix) Testing locomotor speed – the lizard was sprinted four times down a 1 m race-track, using a soft paintbrush to encourage it to run.

(x) Exposure to heterospecific lizard – the lizard was transferred to an unfamiliar enclosure (as described above) containing a potentially aggressive heterospecific lizard, *E. saxatilis* (Langkilde and Shine, 2004), for 1 h.

All lizards were returned to their home enclosures upon completion of the treatments. Blood samples were obtained 1 h after the initiation of each treatment. Reproductive stage can affect circulating corticosterone (Woodley and Moore, 2002), however there was no significant difference between treatments in the number of follicles females possessed ($F_{[1,98]}=0.03$, $P=0.87$), or whether or not they had been ovulated ($\chi^2=10.76$, d.f.=9, $P=0.29$).

(3) How do a lizard's sex and size influence its response?

To enable us to determine whether a lizard's sex and/or size influences its plasma corticosterone levels, or its response to treatments, we measured SVL and mass of each animal. Sex was determined by hemipene eversion upon capture.

(4) How long does a response last, and do lizards acclimate to the stressor?

Ethical treatment of research animals requires us to evaluate the duration of any stress response as well as its peak intensity: all else being equal, a treatment causing a transitory response is preferable to one that causes corticosterone levels to remain elevated for days or weeks. We monitored the plasma corticosterone concentrations of *E. heatwolei* exposed to three treatments: tail autotomy ($N=8$ males and 8 females), toe-clipping ($N=5$ males and 5 females), and microchip implantation ($N=10$ males and 10 females), as well as controls ($N=8$ males and 8 females), by obtaining additional blood samples from these animals at two intervals (after 2 h and after 14 days) following initiation of the treatment. The number of animals differed among treatment groups for logistical reasons.

Animals may be able to acclimate to a repeated or prolonged stressor, such that plasma corticosterone levels fall despite continued exposure to stress. In such a case, plasma corticosterone levels may provide a poor indicator of actual stress levels experienced by the study organism. However, such acclimated animals may respond more strongly than naïve controls if they are subsequently exposed to a new or additional stressor (Romero, 2004). To test whether any observed decrease in corticosterone levels after 14 days was due to a 'real' reduction in stress (*vs* acclimation to a persistent stressor), we exposed these animals to an additional stressor and obtained a final blood sample 1 h later to measure plasma corticosterone. The blood sample obtained 14 days after the initiation of the original treatment, in combination with chasing the individual around an empty container ($60 \times 33 \times 20$ cm; $L \times W \times D$) for 30 s, acted as the 'novel' stressor for this part of the study. All lizards were placed back into their familiar enclosures after we applied the stressor (i.e. before we obtained the blood sample).

(5) Do time of day and/or handling duration affect the stress response?

Plasma corticosterone levels may vary significantly over a 24-h period (Chan and Callard, 1972; Dauphin-Villernant and Xavier, 1987; Jones and Bell, 2004) (but see Tyrrell and Cree, 1998), and be confounded by the stress caused by handling the

animal and obtaining the blood sample. Thus, we recorded the time of day that each sample was obtained, as well as the total duration of handling from first disturbance until the completion of blood sampling.

(6) *Is there a simpler way to measure the stress response?*

Measuring plasma corticosterone levels is invasive, time-consuming, and costly, so it would be useful to identify a simpler alternative. We assessed the validity of one alternative potential measure of 'stress': breathing rate. Exposure to external stimuli typically elicits increased respiratory rates in lizards, and these are easily quantified by counting lateral expansions and contractions of the chest cavity (Avery, 1993; Langkilde and Shine, 2005). We recorded the number of breaths per 30 s for each lizard immediately after the initiation (in cases where treatments were applied for 1 h, e.g. unfamiliar enclosure) or completion of each treatment. These data allow us to assess the relationship (if any) between breathing rate and plasma corticosterone level, and to compare the patterns of response revealed by these two measures.

Obtaining and analyzing blood samples

A total of 456 blood samples were obtained during the course of this experiment. Although standardizing the time of day that all these samples were obtained would have minimized the potential effect of diel variations in corticosterone levels, it would have dramatically lengthened the duration of this study, thus introducing variation due to factors such as time of year and reproductive stage [factors that can have a significant impact on corticosterone levels (Woodley and Moore, 2002)]. Thus, all blood samples were taken (from the postorbital sinus, as described above) during the animals' normal activity period (0900–1700 h), when levels are least variable (Jones and Bell, 2004), with trials timed so that lizards within each treatment were evenly

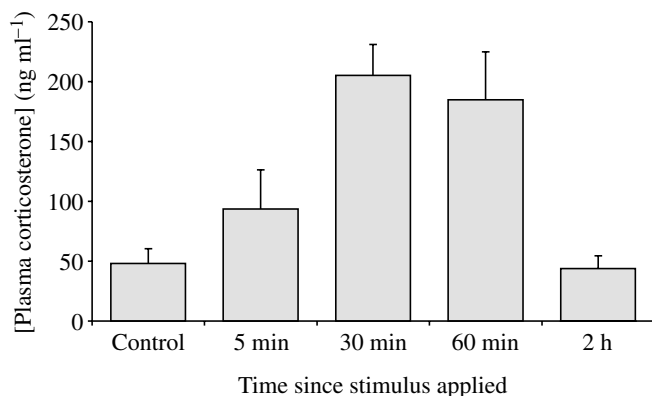


Fig. 1. The time course of change in plasma corticosterone levels of 40 female water skinks (*Eulamprus heatwolei*) following exposure to a stressful stimulus (having a blood sample taken, then being chased around an enclosure for 30 s), and 10 'control' females that were undisturbed prior to the blood sample being obtained. Each sample is based on data from 10 females, so that each animal provided only a single sample for analysis. Values are means \pm s.e.m.

sampled over this period. In addition, the time of day that blood samples were obtained was recorded, and included as a factor in the analyses (see above).

Samples were centrifuged, and plasma stored at -80°C until assayed. Corticosterone was measured by enzyme immunoassay using OCTEIA Corticosterone HS Kits [Immunodiagnostic Systems (IDS) Ltd., Boldon, Tyne and Wear, UK]. The assay was validated by parallelism of a pooled sample of *Eulamprus heatwolei* plasma with the standard curve when serially diluted. All samples had detectable levels of corticosterone, intra-assay coefficient of variation (CV) was 0.019%, and inter-assay CV was 0.17%. Data were analyzed using Statview 5.0 (SAS Institute Inc. 1998) following log transformation to meet the assumptions of parametric testing.

Results

(1) *How long after exposure to a stressor should a blood sample be obtained?*

Plasma corticosterone concentrations increased with time after the stressor was applied (two-factor ANOVA with time since application as the factor and corticosterone concentration as the dependent variable: $F_{[3,24]}=7.29$, $P=0.001$; Fig. 1). Fisher's PLSD *post-hoc* tests reveal that plasma corticosterone concentrations had not increased significantly when tested 5 min after the stimulus was applied, but were significantly elevated 30 and 60 min after the stressor was applied, and had recovered to background levels after 2 h (Fig. 1). Although there may be sex differences in the timing of the stress response in this species, these data provided a general guideline, and in all subsequent experiments we obtained blood samples 1 h after the application of a stressor to ensure sufficient time for plasma corticosterone levels to increase.

(2) *How does treatment affect plasma corticosterone levels?*

Treatment significantly affected plasma corticosterone levels [two-factor analysis of covariance (ANCOVA) with treatment and sex as the factors, SVL as the covariate, and plasma corticosterone levels 1 h post stimulus as the dependent variable: treatment $F_{[9,188]}=11.57$, $P<0.0001$, all interactions non-significant; Fig. 2A]. Fisher's PLSD *post-hoc* tests identified four significantly different levels of plasma corticosterone among our test animals (i.e. no statistically significant differences *within* any of these groups, but each group different from the others). The first group (A; lowest plasma corticosterone levels) contained the field animals. The second group (B; slightly above field levels) comprised the laboratory control animals together with those that were handled and measured, those that were exposed to predator scent, and those that were toe-clipped. The third group (C; high levels of plasma corticosterone) comprised animals that had been implanted with microchips, had blood samples taken, had autotomised their tails, had been placed into an unfamiliar enclosure, or had been tested for locomotor speed. The final

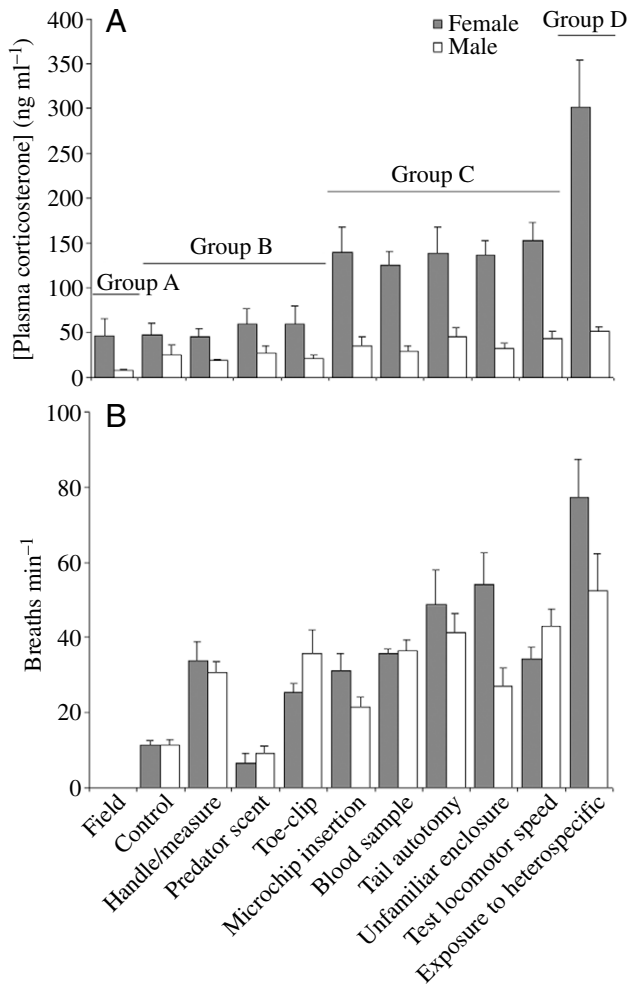


Fig. 2. Effects of routine laboratory procedures and simulated 'natural' stressful events on (A) plasma corticosterone levels and (B) breathing rates, of water skinks (*Eulamprus heatwolei*). Stimuli are described in the text. Groups A–D in A are significantly different from one another, and are discussed in the text. Blood samples were taken 1 h following exposure to the potentially stressful stimulus. Each sample consists of 20 animals (10 males and 10 females), with data shown separately for male and female lizards. Values are means \pm s.e.m.

group (D; very high levels of plasma corticosterone) contained the lizards that had been exposed to aggressive heterospecific animals.

(3) How do a lizard's sex and size influence its response?

Plasma corticosterone levels consistently were at least threefold higher in female lizards than in males (two-factor ANCOVA with treatment and sex as the factors, SVL as the covariate, and plasma corticosterone levels 1 h post stimulus as the dependent variable: sex $F_{[1,188]}=139.46$, $P<0.0001$, all interactions non-significant; Fig. 2A). In contrast, a lizard's body size did not influence its plasma corticosterone levels (two-factor ANCOVA with treatment and sex as the factors, SVL as the covariate, and plasma corticosterone levels 1 h post

stimulus as the dependent variable: SVL $F_{[1,188]}=0.99$, $P=0.32$, all interactions non-significant).

(4) How long does a response last, and do lizards acclimate to the stressor?

The duration of increased corticosterone levels following exposure to a stressor depended on the lizard's sex as well as the procedure to which the animal had been exposed (two-factor repeated measures ANOVA with treatment and sex as the factors, and corticosterone levels at 1 h, 2 h and 14 days as the repeated dependent variable: treatment $F_{[3,54]}=4.61$, $P=0.006$, sex $F_{[1,54]}=53.49$, $P<0.0001$, corticosterone levels over time $F_{[2,108]}=30.27$, $P<0.0001$, corticosterone levels over time \times sex $F_{[2,108]}=3.89$, $P=0.02$, all other interactions non-significant; see Fig. 3). Plasma corticosterone levels in females increased 1 h after tail autotomy, but fell back to control levels within 2 h and remained low 14 days later (Fig. 3A). In contrast, insertion of a microchip also caused a rise in plasma corticosterone levels of females within 1 h, but this higher level was still evident not only after 2 h, but also after 14 days (compared to controls; Fig. 3A). Finally, toe-clipping did not cause any significant increase in plasma corticosterone levels of female lizards (either at 1 h, 2 h or 14 days; Fig. 3A). In contrast to females, male lizards showed no significant increase in plasma corticosterone levels in response to any of the three treatments (compared to the control) over any of our test periods (Fig. 3B).

Did the reduction in plasma corticosterone levels after 14 days reflect acclimation to the stimulus, thus masking continued stress? If so, we would expect corticosterone levels of lizards subjected to the most stressful treatments to decrease over time, but then increase strongly if the animal was exposed to an additional stressor. In contrast, lizards subjected to less stressful treatments should show lower responses to a subsequent stressor. In fact, we found the opposite pattern. When we applied an additional stressor 14 days after the initial treatment, plasma corticosterone levels increased in all treatments, but did so most markedly in animals that had initially been exposed to relatively minor stress (i.e. had experienced an initial treatment that induced only minor elevation of plasma corticosterone levels). Thus, the final plasma corticosterone levels were similar across all treatments, and not significantly different from the control treatment (Fig. 3). In fact, the only treatment that generated a pattern suggesting acclimation (i.e. high but then decreasing corticosterone levels over time) was tail autotomy in females (see above). Animals from this treatment exhibited a low level of response to the additional stressor (Fig. 3A), arguing against acclimation as an explanation for the decrease through time in plasma corticosterone levels in this treatment. Finally, although corticosterone levels increased in males as well as females in response to the additional stressor, females again exhibited higher concentrations than did males (two-factor repeated measures ANOVA with treatment and sex as the factors, and corticosterone levels before and after stimulus applied at 14 days as the repeated dependent variable:

treatment $F_{[3,54]}=1.12$, $P=0.35$, sex $F_{[1,54]}=58.06$, $P<0.0001$, corticosterone levels before and after stimulus $F_{[1,54]}=236.35$, $P<0.0001$, corticosterone levels before and after stimulus \times treatment $F_{[3,54]}=3.62$, $P=0.002$, all other interactions non-significant; Fig. 3).

(5) Do time of day and/or handling duration affect the stress response?

The time of day that blood samples were obtained significantly influenced our results (two-factor ANCOVA with treatment and sex as the factors, minutes since midnight as the covariate, and corticosterone levels 1 h after stimulus as the dependent variable: minutes since midnight $F_{[1,169]}=2.12$, $P=0.15$, treatment \times minutes since midnight $F_{[9,169]}=2.19$, $P=0.03$, all other interactions non-significant). However, a closer inspection of individual regressions reveal that this significant interaction effect was due to a single treatment; the response to tail autotomy was affected by time of day, with marginally higher plasma corticosterone levels recorded later in the day (regression: $F_{[1,19]}=4.54$, $P=0.047$). Following Bonferroni correction ($\alpha=0.005$), this result was no longer statistically significant. The duration of handling required to take a blood sample did not significantly influence corticosterone levels (two-factor ANCOVA with treatment and sex as the factors, handling time as the covariate, and corticosterone levels 1 h after application of the stressor as dependent variable: duration of handling $F_{[1,186]}=0.10$, $P=0.76$, all interactions non-significant).

(6) Is there a simpler way to measure the stress response?

At first sight, our results are encouraging for the idea that breathing rates may offer a simple, cost-effective alternative to measuring plasma corticosterone levels. The breathing rate of a lizard immediately after application of a stressor was positively correlated with the animal's plasma corticosterone levels 1-h post stimulus (correlation z-test on data from both sexes combined: $r=0.36$, d.f.=183, $P<0.0001$; Fig. 4). However, the correlation between breathing rate and plasma corticosterone levels 1-h post stimulus (1) was low (explaining only 13% of total variation in corticosterone levels); and (2) differed between the sexes, with males showing lower corticosterone levels than did females with the same breathing rate (1-factor ANCOVA with sex as the factor, breathing rate as the covariate and corticosterone levels as the dependent variable: sex $F_{[1,181]}=102.11$, $P<0.0001$, breathing rate $F_{[1,181]}=34.17$, $P<0.0001$, all interactions non-significant; Fig. 4). In addition, (3) patterns of response to stimuli suggested by breathing rate differ from those suggested by the corticosterone data. For example, the sex disparity seen for corticosterone levels was also seen for breathing rates in response to some stimuli, but not others (2 factor ANOVA with sex and treatment as the factors and breaths per minute as the dependent variable: treatment $F_{[9,164]}=30.80$, $P<0.0001$,

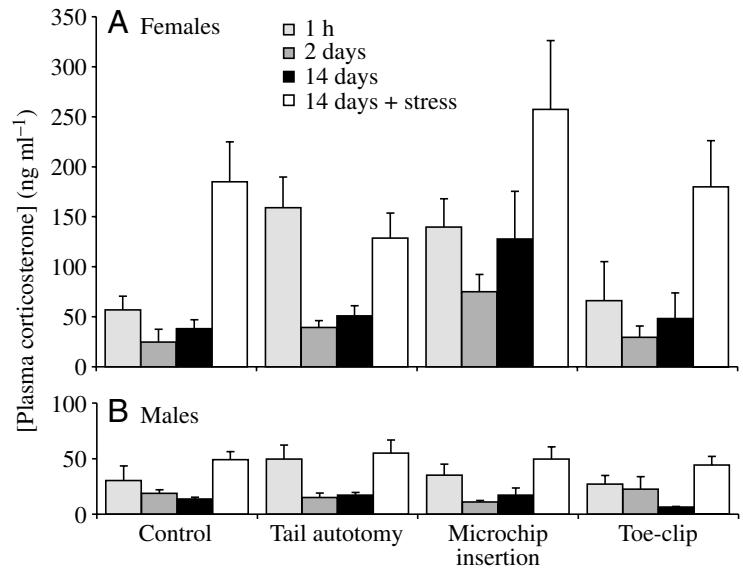


Fig. 3. Effects of four treatments on plasma corticosterone levels of (A) female and (B) male water skinks (*Eulamprus heatwolei*) at 1 h, 2 h and 14 days since initiation of the respective treatments, and in response to the application of a 'novel' stressor (having a blood sample taken, then being chased around an enclosure for 30 s) 14 days after the initial treatment initiation. Sample sizes for treatments were: control, $N=16$; tail autotomy, $N=16$; microchip insertion, $N=20$; toe-clip, $N=10$, and samples consist of equal numbers of males and female lizards. Values are means \pm s.e.m.

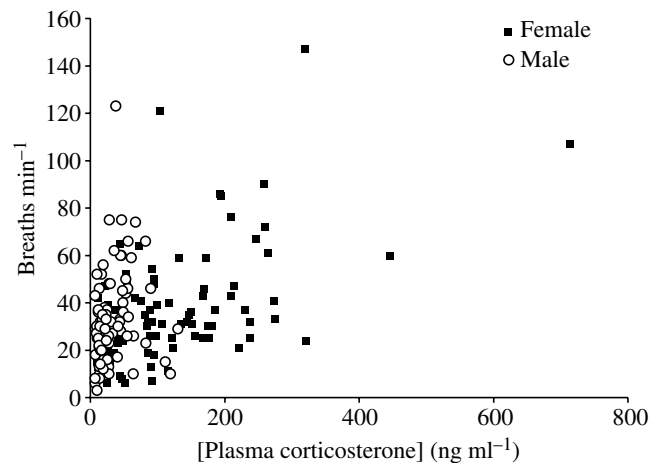


Fig. 4. The relationship between two potential measures of 'stress' in water skinks exposed to a wide variety of potentially stressful stimuli. The two measures were (1) breathing rate at the time the stimulus was applied, and (2) plasma corticosterone level 1 h later. See text for details; each point represents data for a single lizard. Data are shown separately for males and females.

sex $F_{[1,164]}=0.80$, $P=0.37$, treatment \times sex $F_{[9,164]}=2.70$, $P=0.006$, all other interactions non-significant; Fig. 2B). Similarly, although treatment significantly affected breathing rate as well as corticosterone levels, the nature of those differences (e.g. which treatments generated the greatest

stress?) differed substantially between the two measures (Fig. 2A,B).

Discussion

Our study has three major results. (1) Validation of methodology. Because plasma corticosterone responds rapidly and consistently in lizards exposed to research procedures, it can offer a straightforward index of the degree of physiological stress imposed by those procedures compared to the levels experienced by free-ranging animals. (2) Degree of stress induced by alternative methods. Intuition (the primary criterion currently used to evaluate research-induced stress) appears to offer a very unreliable guide in this respect, because some procedures that are generally viewed as stressful (e.g. toe-clipping) did not significantly elevate corticosterone levels, whereas others generally viewed as benign (e.g. being placed into an empty cage) induced a striking increase in plasma corticosterone. (3) Sex differences in plasma corticosterone levels and in stress responses. Female lizards generally had corticosterone levels at least three times greater than males, and responded more sensitively to experimental treatments. Below, we discuss each of these issues in turn.

Validation of methodology

Perhaps the most surprising aspect of our results is that a study such as this has not been conducted previously. Given that researchers often complain about excessive regulation of their endeavours, and the arbitrary nature of decisions by non-expert members of ethics committees, we might have expected researchers to gather objective data to support their cases about the merits of alternative research protocols. Our study is encouraging in this sense, in that it suggests that a widely accepted and easily assayed measure of physiological stress in vertebrates (plasma glucocorticoid levels) can offer a straightforward index of the effects of experimental procedures on animal welfare. The most significant caveat to this finding involves the meaning of the term 'stress'. Because this term has been defined in so many different ways, a critic could argue that plasma glucocorticoid levels do not provide a valid operational measure of the kind of stress that they perceive as important in ethical issues. The only solution to this criticism, so far as we can see, is a wider debate on the meaning of 'stress' within the context of animal-based research.

Unfortunately, the most obvious shortcut method that we attempted – recording breathing rates of our study animals – appears less informative in this respect than plasma corticosterone levels. Respiration rates offer a very sensitive assay: indeed, lizards often increase ventilation rates simply when the observer approaches them. However, the rates drop back to baseline levels rapidly also. Hence, the stress associated with an experimental manipulation may be too small to elevate plasma corticosteroid levels even if it induces a transitory increase in respiration rate (as was the case for handling, in the present study). Researchers might be able to use the easily gathered data on breathing rates to make

comparisons among methods, sexes or species when initially selecting study systems, but we caution that the resulting data may bear little relationship to underlying levels of plasma corticosterone.

Degree of stress induced by alternative methods

It is encouraging to see that lizards experienced less stress as a result of many of our experimental procedures than they did from biologically realistic situations encountered in the field. However, the fact that the degree of stress induced by a specific procedure was not intuitively obvious means that many current regulations about research procedures may be based on false premises. For example, the oft-criticized marking technique of toe-clipping did not induce a major rise in corticosterone levels, whereas lizards were affected to a much greater degree by an alternative and supposedly less stressful method of implantation of a microchip, and by superficially trivial manipulations such as housing in an unfamiliar enclosure. In addition, physiological stress caused by microchip insertion lasted for at least 14 days, in contrast to the animal's rapid recovery from tail autotomy. Implanted microchips may cause constant irritation, impede movement, and occasionally migrate around under the skin or into the body cavity (Roark and Dorcas, 2000). In contrast, the much larger wound caused by tail autotomy rapidly heals, presumably reflecting effective selection over many generations to deal with this frequently occurring 'natural' event.

It is possible to reconcile most of our results with other information on the study species, even though we were unable to predict those response patterns *a priori*. For example, the lack of response to predator scent compared to the dramatic response to the presence of a larger competitor species (*Egernia saxatilis*) accords well with prior behavioural observations (Langkilde and Shine, 2005). Similarly, the lack of impact of handling time is unsurprising, given that (1) all animals had a total duration of handling (from disturbance to completion of blood sampling) of <147 s, and (2) based on plasma corticosterone levels, simply handling an animal was not particularly stressful. Finally, we observed none of the temporal variation in plasma corticosterone levels reported for other species (Chan and Callard, 1972; Dauphin-Villernant and Xavier, 1987), but blood samples were obtained only during the lizards' active period, when levels are least variable (Jones and Bell, 2004). Although post-hoc rationalization of our results is thus possible, we doubt that the average animal ethics panel (nor, indeed, a group of expert researchers familiar with the study species) would have predicted our results *a priori*. Hence, using intuition as a basis for decisions about the degree of stress induced by alternative practices is fraught with error.

Sex differences in plasma corticosterone levels and in stress responses

Why did our female lizards have corticosterone levels so much higher than those of males, and respond more sensitively to experimental treatments? Although published data are

scarce, previous studies on reptiles generally have reported the opposite situation: higher plasma corticosterone levels in males than in females (Else et al., 1990; Lance et al., 2001; Mathies et al., 2001). Higher levels of plasma corticosterone may enhance organismal fitness by facilitating access to energy stores, and thus enhance the animal's ability to escape rapidly from a predator, battle with conspecifics, etc. (Lance et al., 2001). Activities such as mate-searching, courtship, mating and male–male combat may require elevated levels of energy expenditure, thus favouring increased plasma corticosterone levels in males rather than females (Lance et al., 2001). Additionally, high levels of corticosterone in pregnant females of a viviparous species (such as *Eulamprus heatwolei*) might have negative effects on the developing offspring (Cree et al., 2003). Neither of these ideas accords with our own data, in that it was females not males that exhibited high levels of plasma corticosterone; and many of the females that we used were pregnant during the trials. Our analyses revealed no effect of pregnancy on plasma corticosterone levels (T.L. and R.S., unpublished data). Nonetheless, our study animals are not unique in females exhibiting higher corticosterone levels than males. Corticosterone is also the major glucocorticoid in several mammalian species including rodents, and in these groups, females typically exhibit higher levels of plasma corticosterone than do males (Handa et al., 1991; Jones et al., 2005). This situation has been interpreted as a proximate effect of sex hormones on hypothalamic–pituitary–adrenal (HPA) function: estrogen enhances the response of the HPA axis to stress, whereas testosterone suppresses it (Greenberg and Wingfield, 1987; Handa et al., 1991; Jones et al., 2005).

In summary, our data suggest that direct measurement of plasma corticosterone levels offers a useful technique for quantifying the impact of research techniques on study organisms. In the viviparous lizards we studied, the magnitude and duration of elevation in plasma corticosterone levels differed strongly between the sexes and was influenced by experimental procedures. The range of protocols that we applied to our lizards encompassed many of the routine procedures used by behavioural ecologists, and in general the animals were affected no more strongly by these experimental manipulations than they were by simulated 'natural' events (such as autotomy and encountering a heterospecific lizard). However, the relative impact of different techniques often diverged from our *a priori* expectations. When selecting our research methods to minimize stress on study organisms, we might be better advised to ask the animals directly (via techniques such as quantifying their plasma corticosterone levels) rather than relying on our own intuition as to what constitutes a 'stressful' procedure.

We thank Tara Brennan and the Bone and Skin Research Group at the University of Sydney for providing access to their microplate reader, and S. van Barneveld, M. Wall, C. Avolio, D. Allsop and M. Elphick for assistance. The research presented here adhered to UFAW Handbook on the Care and Management of Laboratory Animals, the legal requirements

of Australia, and the ethics guidelines for the University of Sydney, New South Wales, Australia. Animal collection was authorized by New South Wales Scientific Purposes Permit S10702. This study was funded by the Australian Research Council (to R.S.) and the Australian Society of Herpetologists (to T.L.).

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