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Euthanasia methods, corticosterone and haematocrit levels in Xenopus laevis: evidence for differences in stress?

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Abstract

*Amphibians, like other vertebrates, respond to acute stressors by releasing glucocorticoid steroid hormones that mediate physiological and behavioural responses to stress. Measurement of stress hormones provides a potential means to improve the welfare of laboratory animals. For example, manipulations of laboratory housing and procedures combined with measurement of glucocorticoids may identify which conditions are more stressful to animals. This is important because there is very little experimental evidence to guide best practice for welfare in amphibians and other lower vertebrates. We investigated the effect of different methods of euthanasia on the circulating plasma corticosterone levels in the African clawed frog (*Xenopus laevis*), a model amphibian organism that is frequently used in laboratories. In particular, we investigated the effect of different concentrations and pH of the anaesthetic tricaine methanesulphonate (MS-222). Low concentration and unbuffered (low pH) solutions of MS-222 caused elevated corticosterone levels, but only after the effect of MS-222 treatment on blood fluid volume had been taken into account. The level of disturbance that animals experienced also affected corticosterone levels. Thus, our data suggest that to minimise stress to* X. laevis*, animals should be euthanised after minimal disturbance and in a 3 g L^{-1} MS-222 solution, buffered to pH 7. The potential for the improvement of amphibian welfare using corticosterone measures as a tool is discussed.*

Keywords: *animal welfare, corticosterone, euthanasia, haematocrit, stress,* Xenopus laevis

Introduction

Vertebrates react to unpredictable and deleterious stimuli by mounting stress responses. These evolved responses include physiological, hormonal and behavioural changes that are highly conserved across vertebrate taxa (Romero 2004). A characteristic of the vertebrate stress response is the release of glucocorticoid steroid hormones, and there is a large body of literature describing the neuronal and biochemical mechanisms that are involved in the release of these in response to stressors in mammals and birds. Recent studies have shown that similar mechanisms also occur in amphibians. For example, in the African clawed frog (*Xenopus laevis*) physical stress causes rapid activation of the hypothalamic-pituitary-interrenal (HPI) axis and changes in the central nervous system result in the release of corticosterone (Yao *et al* 2004). These changes in the brain are similar to those seen in higher vertebrates (eg in the rat: Ziegler & Herman 2002). Thus, the amphibian stress response appears to be controlled in broadly the same way as in mammals and birds, and the neurological control of stress and stress hormones is likely to be conserved among vertebrates (Yao *et al* 2004).

Given that corticosterone mediates the behavioural and metabolic stress response in amphibians, there is the potential to investigate the effects of a variety of acute stimuli on amphibian welfare and fitness both in the wild and in the laboratory. However, care needs to be taken to consider both the basal levels of corticosterone in these studies, and other possible causes of elevated corticosterone (Broom & Johnson 2000), such as stimulation of the HPI axis by food intake (eg Crespi *et al* 2004). Nevertheless, several studies indicate that increased acute stress leads to increased corticosterone release in a range of amphibian species. For example, Southern toads (*Bufo terrestris*) exhibit elevated corticosterone levels after transplantation to a polluted habitat (Hopkins *et al* 1997). Male spotted salamanders (*Ambystoma maculatum*) that migrate over man-made surfaces have higher corticosterone levels than those that migrate in natural habitats (Homan *et al* 2003). In the laboratory, *X. laevis* show elevated corticosterone levels after experiencing hyperosmotic stress for 24 h (in 30% artificial sea water), and levels remain above those of control animals for animals maintained in dilute sea water for 2 weeks (Guardabassi *et al* 1991, 1993). Furthermore, *X. laevis* that were exposed to shaking had elevated corticosterone levels compared to control animals, and these levels increased with the time spent receiving the stimulus (up to 6 h of shaking experienced; Yao *et al* 2004).

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The relationship between stress and corticosterone levels lends itself to the study of laboratory animal welfare. In contrast to birds and mammals, maintenance and welfare guidelines for amphibians are generally vague because there is very little experimental evidence to inform best practice. For example, a wide range of methods are used to euthanise amphibians, including a variety of anaesthetic agents, as well as physical techniques (eg AVMA 2001; Read 2005). In the UK, animal workers involved in the euthanasia of animals used for scientific purposes are directed by British Home Office legislation (Code of Practice for the Humane Killing of Animals under Schedule One to ASPA 1986, Home Office 1987). It sets out legally-acceptable methods of euthanasia for all animals covered by the legislation (vertebrates and *Octopus vulgaris*). However, for amphibians the directions are vague. Unlike other taxa, such as rodents, there has been little scientific investigation into the effects of different euthanasia methods on amphibian welfare, or on the science being carried out during this type of animal use. Schedule One states only that the methods of euthanasia acceptable for amphibians are an "overdose of an anaesthetic using a route and an anaesthetic agent appropriate for the size and species of animal", or "concussion of the brain by striking the cranium" for animals up to 1 kg in weight, followed by "destruction of the brain before the return of consciousness". In general, anaesthetic overdoses are preferred by animal workers because they are easier to carry out, and are perceived as less stressful to the workers themselves (Reilly 2001). Differences in the stress experienced by amphibians culled under the different methods have not been investigated. This means that Schedule One cannot give specific guidance on favoured anaesthetic agents, nor the routes and doses for application.

In the present study, we investigated the effects of different Schedule One euthanasia methods on the levels of circulating plasma corticosterone and haematocrit in *X. laevis*. *X. laevis* are large aquatic anurans, that are widely used in scientific research, being a model amphibian laboratory animal (Gurdon 1996; Gurdon & Hopwood 2000; Read 2005). The most widely-used chemical for the anaesthesia and euthanasia of aquatic amphibians is tricaine methanesulphonate (MS-222; Downes 1995; Read 2005). However, it is not easy to confirm brain death in aquatic vertebrates in anaesthetic solutions, and it is possible that animals may actually die from drowning after loss of consciousness. It is therefore common practice to ensure death after deep loss of consciousness via a physical method, usually double pithing (Read 2005).

It has long been recognised that MS-222 is highly acidic (Ohr 1976a), and that this acidity causes changes in blood physiology (eg in bull frogs [*Rana catesbeiana*] Ohr 1976b) that affects epithelial transport of water and sodium chloride (eg in leopard frogs [*Rana pipiens*] Ohr 1976b), which may in turn induce anaesthesia more slowly than equivalent buffered solutions in both amphibians (Ohr 1976a) and fish (Smit & Hattingh 1979). To test the effect of MS-222 concentration and pH on the stress of *X. laevis* during euthanasia, we used two different concentrations that are widely used for this species, both buffered and unbuffered. We compared corticosterone levels in these four treatments to those in animals from two control groups: animals that were euthanised instantly via brain concussion, and animals that were held isolated in water (to simulate handling and isolation stress in the absence of MS-222) before also being euthanised via brain concussion. Haematocrit was also compared between the six groups of animals, to investigate the effect of the anaesthetic on blood volume.

Materials and methods

Animals

Animals were wild caught, South African, adult female *Xenopus laevis*, ranging in weight from 32.1–97.0 g (57.2 [± 1.6] g). Prior to the experiment, they were housed in 15 mixed sex static tanks, in groups of 9–15 animals, for over a year. Tanks were white, plastic, 50-cm cubes, with secure lids, containing approximately 70 L aged tap water. Each tank was enriched with short lengths of grey plastic drainpipe. Two weeks prior to the experiment, animals were redistributed into 13 single sex tanks of 8–9 females per tank. Males were not used in the experiment. Room temperature was maintained at 22 (± 1)°C, and animals were housed under a 12:12 light:dark cycle (58 W white strip lights, Osram, St Helens, UK). Each week, tanks were cleaned and animals were fed diced ox heart to excess. Animals were not fed in the week prior to use in the experiment, as feeding results in short-term increases in corticosterone levels (Crespi *et al* 2004). All animals used were required to be euthanised for other purposes and were therefore not solely euthanised to provide data for this study. Sample size was therefore determined by the number of animals already assigned to be euthanised.

Euthanasia methods

Six different methods of euthanasia were used to cull six animals per tank. All methods are approved Schedule One methods for amphibians under British Home Office legislation for the humane euthanasia of animals used in scientific research. Four of the treatments involved the use of MS-222 (Pharmaq, Hampshire, UK) to anaesthetise the animals prior to a physical method of brain destruction. Solutions used were 1 g L^{-1} unbuffered, 1 g L^{-1} buffered, 3 g L^{-1} unbuffered and 3 g L^{-1} buffered. Buffering was to pH 7 with sodium hydrogen carbonate ($NaHCO_3$, BDH Chemicals Ltd, Poole, UK). The 1 g L^{-1} MS-222 unbuffered solution was pH 4.3, while 3 g L^{-1} unbuffered was pH 3.5.

Anaesthesia took place in 4 L plastic boxes containing 1.5 L of anaesthetic to a depth of 72 mm, at 22 (± 1)°C. To prevent the reduction of anaesthetic concentration between animals, a fresh MS-222 solution was made up for every animal. Animals were anaesthetised individually. To commence a trial, the tank lid was opened and the animal nearest the centre of the tank was netted and quickly removed. The animal was placed directly into anaesthetic, and a lid put on the plastic box. Care was taken to minimise disturbance to the animal both during capture and while in the anaesthetic.

The length of time that animals were placed in the different MS-222 treatments was determined by preliminary trials on

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Table 1 Time to unconsciousness for adult *X. laevis* placed in different concentrations of MS-222, buffered and unbuffered.

MS-222 solution	Buffer	Time to unconsciousness (min)			n
		Minimum	Maximum	Median	
1 g L^{-1}	yes	6.0	10.0	6.50	6
1 g L^{-1}	no	3.0	8.5	6.25	6
3 g L^{-1}	yes	3.0	3.5	3.00	9
3 g L^{-1}	no	2.5	7.5*	4.50	6

* 7.5 min is an outlier. All other values are in the range 2.5–4.5 min, with median 4.5 min.

other adult South African *X. laevis*, which also needed to be culled. In these trials, animals were placed singly into one of the four types of MS-222 solution and the time taken to become unconscious was recorded. Consciousness was determined by testing the pedal reflex of animals every 30 s after they had ceased movement in the anaesthetic. Data from this preliminary trial are summarised in Table 1. Based on these data (maximum time to unconsciousness plus 1 min), animals in the main experiment were left in MS-222 for 11.0 min in 1 g L^{-1} and 5.5 min in 3 g L^{-1}. After this time, the animals had their pedal reflexes tested. Any animal that still had a pedal reflex was removed from the experiment. It was allowed to remain in MS-222 until unconscious and then euthanised via double pithing. These animals were then replaced in the experiment by another animal, which was placed into a new MS-222 solution. This happened once for an animal placed in 1 g L^{-1} unbuffered MS-222 and three times for animals placed in 3 g L^{-1} unbuffered MS-222.

The final two treatments were controls. The fifth method of euthanasia was a physical method that involved instant brain concussion by holding the animal securely and striking the back of the head against a solid edge. This stunning procedure was carried out immediately after removal of the animal from its tank, and it causes loss of consciousness. Instant brain concussion is the quickest Schedule One method and hence should involve an extremely short period of stress to the animal, most likely insufficient to cause changes in circulating plasma corticosterone levels. The sixth treatment was to house an animal in a container as for the MS-222 treatments, but instead of containing MS-222 it held only 1.5 L water (at 22 [± 1]°C). After 11 min in the container, the animal was removed and culled by instant brain concussion, as described above. This control serves to determine if housing the animal in a small container itself causes elevated plasma corticosterone levels.

Blood sampling and processing

After anaesthesia in MS-222 or brain destruction via stunning, animals were dissected ventrally to reveal the heart, and as large a blood sample as possible up to 1 ml was taken via cardiac puncture. All samples were taken within 2 min of removal from the anaesthetic or stunning. A fresh, heparinised (with H1027, Sigma, UK) 1 ml syringe with a 1/2" 27 gauge needle was used for each animal. Blood was stored in eppendorfs on ice. The needle was removed from the syringe before the blood was emptied into an eppendorf, to prevent lysis of red blood cells. Two haematocrit capillary tubes were immediately filled from the eppendorf, and then spun straight away at 15,000 rpm for 2 min in a microhaematocrit centrifuge (Hawksley, Lancing, UK). After spinning, the length of the sample in the tube was measured, along with the length of the packed cell volume of both red and white blood cells and the length of the plasma. Haematocrit was calculated as the length of the red blood cell volume divided by the total length of each sample. The proportional length of plasma in the samples was calculated similarly. Haematocrit and plasma values were averaged per animal for analysis.

Immediately after dissection and blood sampling, all animals were exsanguinated via removal of the heart, and then double pithed to ensure total brain destruction. Within 1 h of sampling, all blood stored on ice was spun down (Eppendorf centrifuge, Hamburg, Germany) at 14,500 rpm for 5 min, to separate plasma from blood cells. Plasma was then drawn off and stored in separate eppendorfs, which were frozen at –20°C until needed for corticosterone analysis.

Corticosterone measurements

Corticosterone concentrations were measured by radioimmunoassay following a modification of the method described by Wingfield *et al* (1992) and Maddocks *et al* (2001). Plasma samples (40 µL aliquots) were extracted in diethyl ether after addition of 2,000 cpm of tritiated corticosterone ([1,2,6,7-^{3}H], corticosterone, Amersham Biosciences, UK) to estimate recovery efficiency during extraction. Evaporated extracts were reconstituted in 550 µL assay diluent. One hundred µL aliquots of reconstituted extract were then mixed with 750 µL scintillant (Ultima Gold, Packard BioSciences, Groningen, The Netherlands), and counted in a scintillation counter (Wallac 1211 Rackbeta, Turku, Finland) to calculate percentage recovery. Two 200 µL aliquots of each extract (each containing 14.6 µL extracted plasma) were assayed using an anti-corticosterone antiserum (code B3-163, Esoterix Inc,

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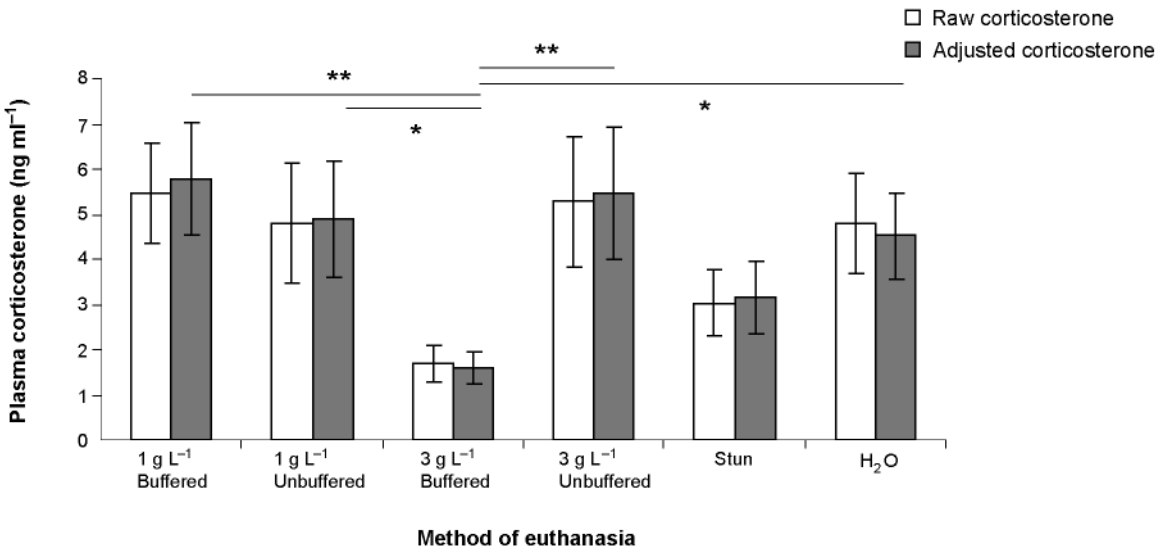
Figure 1



Effect of euthanasia method on the corticosterone levels of *X. laevis*. Data are presented as unmanipulated levels, and as levels adjusted to take into account the effect of euthanasia method on haematocrit values. Values shown are untransformed mean (± SE). Euthanasia methods were 1 g L^{-1} Buffered (1 g L^{-1} MS-222 solution, buffered to pH 7), 1 g L^{-1} Unbuffered (1 g L^{-1} unbuffered MS-222 solution), 3 g L^{-1} Buffered (3 g L^{-1} MS-222 solution, buffered to pH 7), 3 g L^{-1} Unbuffered (3 g L^{-1} unbuffered MS-222 solution), Stun (stunning by instant brain concussion), H_2O Stun (stunning after being housed in 1.5 L water for 11 min). Lines indicate significant differences for the adjusted corticosterone levels, using simple contrasts at * $P < 0.05$, ** $P < 0.01$.

CA, USA) and [1,2,6,7-^{3}H], corticosterone label (Amersham Biosciences, UK). Corticosterone concentrations were corrected for recovery efficiency (range of recovery efficiencies = 63–86%) and expressed as ng ml^{-1}. The assay ran with a bound:free ratio of 0.4, 50% binding was at 196 pg per tube, and the assay detection limit for 14.6 µL extracted plasma was 0.5 ng ml^{-1}.

Statistical analysis

Statistical analyses were carried out using SPSS v12.0. Time to unconsciousness was not measured as a continuous variable, and was hence analysed using Mann-Whitney *U*-tests. Corticosterone data were square-root transformed to reduce skew and achieve normality of residuals from analyses. Corticosterone data were analysed using ANCOVA, with transformed corticosterone levels as the dependent variable, euthanasia treatment as the independent variable, and the order in which animals were removed from tanks as a covariate. Haematocrit data are proportions, and hence were arcsine square-root transformed prior to analysis. These data were analysed using ANOVA with transformed haematocrit levels as the dependent variable and euthanasia treatment as the independent variable. To incorporate the effects of haematocrit on corticosterone levels in circulating blood plasma, untransformed corticosterone levels were individually adjusted for every animal, via multiplication with a conversion factor. The conversion factors were individually calculated for each animal as the proportion of plasma in the blood of that animal, divided by the mean proportion of plasma in the samples for control animals killed after immediate brain concussion. These adjusted corticosterone values were then square-root transformed prior to analysis with ANCOVA.

Ethical note

This experiment was carried out with the approval of the University of Bristol Ethical Review Process, under University Investigation Number UB/07/001. It did not require a British Home Office license. However, all personnel involved in euthanising the *X. laevis* used in this experiment were fully trained to do so and had attended Home Office training for personal licenses.

Results

The preliminary trials show that adult *X. laevis* became unconscious significantly faster in the 3 g L^{-1} than in 1 g L^{-1} MS-222 treatments ($U = 20.0$, n = 27, $P < 0.001$), but that there was no significant difference in time to unconsciousness between buffered and unbuffered MS-222 treatments ($U = 73.0$, n = 27, $P = 0.427$; Table 1).

In the experimental trials, there was no significant effect of euthanasia treatment on plasma corticosterone values (although $F_{5, 71} = 2.32$, $P = 0.052$). Figure 1 shows that there is a trend for animals in the buffered 3 g L^{-1} MS-222 treatment to have lower circulating levels of plasma corticosterone than animals in the other MS-222 treatments, or the treatment where animals are stunned after 11 min in water. Order of removal from tanks had a significant effect on corticosterone levels ($F_{1, 71} = 4.81$, $P = 0.032$; Figure 2). *X. laevis* that were taken from a tank later in the

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Figure 2

Effect of order of removal from tanks on the corticosterone levels of *X. laevis*. Data are presented as the untransformed means (± SE) of the unmanipulated hormone levels.

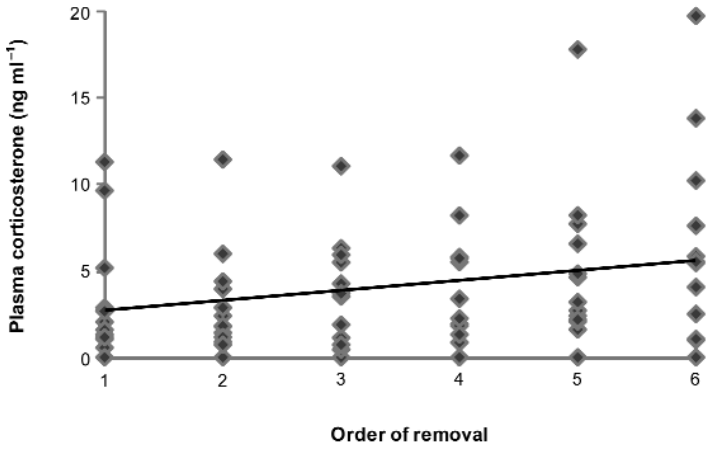
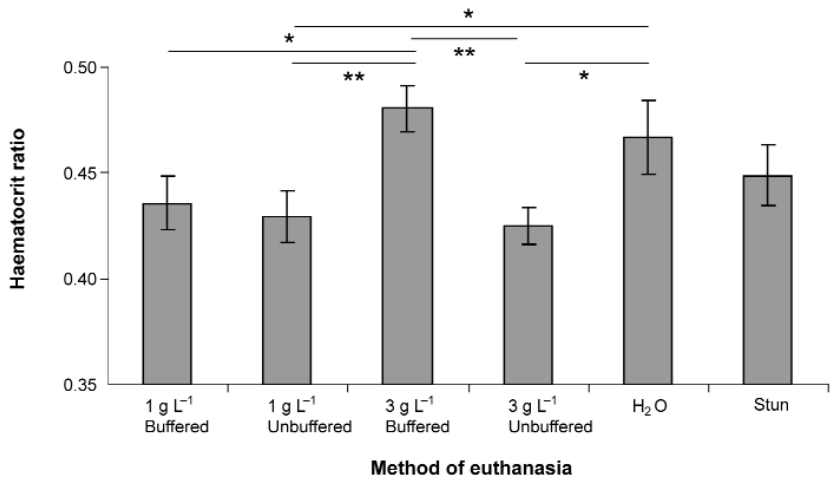


Figure 3

Effect of euthanasia method on the haematocrit levels of *X. laevis*. Values shown are untransformed means (± SE). For descriptions of the methods of euthanasia see Figure 1 legend and *Materials and methods*. Lines indicate significant differences using simple contrasts at the * $P < 0.05$, ** $P < 0.01$.



sequence of removal for euthanasia had significantly higher corticosterone levels.

Haematocrit ratios differed significantly between treatments ($F_{5, 71} = 2.79$, $P = 0.023$; Figure 3). Simple *post hoc* contrasts show that haematocrit was significantly higher for animals in the 3 g L^{-1} buffered treatment than in any of the other three MS-222 treatments (1 g L^{-1} buffered, contrast = 0.05, $P = 0.008$; 1 g L^{-1} unbuffered, contrast = 0.05, $P = 0.020$; 3 g L^{-1} unbuffered, contrast = 0.06, $P = 0.004$), but was not significantly different from either of the control treatments (stun, contrast = 0.03, $P = 0.095$; stun after being held in water, contrast = 0.01, $P = 0.462$). In addition, animals in both the 1 g L^{-1} unbuffered (contrast = –0.04, $P = 0.046$) and the 3 g L^{-1} unbuffered (contrast = –0.04, $P = 0.026$) MS-222 treatments had significantly lower haematocrit values than those in the treatment where animals were held in water for 11 min before stunning. All other comparisons between treatments were not significant (all $P > 0.098$). Thus, animals that experienced the 3 g L^{-1} buffered MS-222 had lower levels of fluid in their blood compared to 1 g L^{-1} buffered, 1 g L^{-1} unbuffered and 3 g L^{-1} unbuffered MS-222 treatments, but did not differ significantly from either of the control treatments. The fluid levels in the control treatments did not differ significantly, but fluid levels in the treatment where animals were stunned after being held in water were significantly lower than those in both of the unbuffered MS-222 treatments.

Adjusting corticosterone values to account for changes in the proportion of plasma in blood caused corticosterone levels to increase for animals in both the 1 g L^{-1} MS-222 treatments, the 3 g L^{-1} unbuffered MS-222 treatment and the control treatment where animals were euthanised via instant brain concussion. In contrast, corticosterone levels decreased for

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Table 2 Effect of haematocrit levels on corticosterone values of *X. laevis* euthanised using different Schedule One methods. Results of paired sample *t*-tests are shown, each with df =12. Significant effects are shown in bold.

Euthanasia treatment	*t*-test	*P*-value
1 g L^{-1} buffered	1.55	0.147
1 g L^{-1} unbuffered	1.12	0.287
3 g L^{-1} buffered	–2.15	0.053
3 g L^{-1} unbuffered	2.73	**0.018**
Instant brain concussion	0.64	0.533
Brain concussion after 11 min in water	–1.01	0.333

animals in the 3 g L^{-1} buffered MS-222 treatment and the control treatment where animals were euthanised via brain concussion after being held in water (Figure 1). However, the difference was only significant for the 3 g L^{-1} unbuffered MS-222 treatment (and approached significance for the 3 g L^{-1} buffered MS-222 treatment; Table 2).

After this adjustment of corticosterone values to account for the effect of changes in the proportion of plasma in the blood, corticosterone values varied significantly with euthanasia treatment ($F_{5, 71} = 2.63$, $P = 0.031$; Figure 1). Simple contrasts show that the corticosterone values for animals in the 3 g L^{-1} buffered MS-222 treatment were significantly lower than those in the 1 g L^{-1} buffered (contrast = 0.87, $P = 0.008$) and unbuffered (contrast = 0.83, $P = 0.010$) MS-222 treatments, as well as the 3 g L^{-1} unbuffered MS-222 treatment (contrast = 0.95, $P = 0.004$) and the control treatment, where animals were stunned after being held in water (contrast = 0.72, $P = 0.024$). Corticosterone values did not differ significantly between the 3 g L^{-1} buffered treatment and the instant stunning control treatment (contrast = 0.41, $P = 0.197$). In addition, there was no significant difference in corticosterone values between the two control treatments (contrast = 0.32, $P = 0.320$). All other contrasts were also not significantly different from one another (all $P \leq 0.094$). As for analysis of unadjusted plasma corticosterone values, order of removal from tanks had a significant positive effect on adjusted corticosterone values ($F_{1, 71} = 4.94$, $P = 0.030$).

Discussion

Method of euthanasia affected the level of circulating plasma corticosterone in adult female *Xenopus laevis*. Specifically, corticosterone levels were lower in animals that were culled in a buffered 3 g L^{-1} MS-222 solution than those that were culled in unbuffered or lower concentrations of MS-222. These results suggest that both the concentration of the anaesthetic and the pH of anaesthetic solution affect corticosterone levels. We know that corticosterone levels were elevated in the 3 g L^{-1} unbuffered and both of the 1 g L^{-1} MS-222 treatments (rather than suppressed in the 3 g L^{-1} buffered treatment) because the corticosterone levels of animals in the 3 g L^{-1} buffered treatment were not significantly different from those in the treatment where animals were stunned instantly. Furthermore, the levels of corticosterone in the 3 g L^{-1} buffered treatment were similar to those of unstressed control *X. laevis* in an experiment where animals were physically shaken to induce a stress response, causing both changes in corticotrophin-releasing hormone neuronal physiology in the brain and elevated corticosterone levels in blood plasma (Yao *et al* 2004). If we assume that lower corticosterone levels do indicate lower stress, then we can conclude that a buffered 3 g L^{-1} solution is the best of the MS-222 solutions tested here to euthanise *X. laevis.*

As expected, animals in the stronger MS-222 solutions lost consciousness faster. This is a standard effect that has been reported in fish (eg Smit & Hattingh 1979), and other amphibians (eg frogs: Ohr 1976a; salamanders: Lowe 2004). However, some of these studies (eg Lowe 2004) also report that a lower pH at set MS-222 concentrations resulted in a longer time to loss of consciousness and Ohr (1976b) demonstrates that it is the acidity of unbuffered MS-222 that injures frog skin, causing delayed uptake of the anaesthetic. We found no evidence of this in our data, although the sample sizes used in our preliminary study of time to loss of consciousness were low. Nevertheless, our data show that the welfare benefit of increasing MS-222 concentration to reduce the time to unconsciousness is offset by the decreasing pH in unbuffered solutions.

Levels of corticosterone were also higher in the treatment where animals were held in water before instant stunning than in the 3 g L^{-1} buffered MS-222 treatment (although levels were not significantly different between the two stunning treatments). This suggests that the increase in corticosterone in the other MS-222 treatments may be at least partly due to the stress of disturbance and isolation, in addition to the stress of being in the anaesthetic solution. Certainly, the fact that corticosterone levels were positively related to the order in which animals were removed from tanks suggests that disturbance (in this case simply the lifting of the tank lid and removal of other animals from the tank) is enough to induce elevated corticosterone levels. This effect is also reported in the cichlid fish (*Haplochromis burtoni*) (Fox *et al* 1997). The effect of disturbance and daily maintenance regimes on stress is an important welfare consideration in laboratory animals (Morgan & Tromborg 2007), and deserves further attention.

Differences in the effect of euthanasia method on plasma corticosterone described above were only significant once the effect of MS-222 on blood fluid levels was taken into consideration. However, given that MS-222 affects haematocrit ratios in the way that it does, it is important to note that this variation should be taken into account whether or not the treatment effect was significantly different from the controls. Haematocrit ratios were higher in the buffered 3 g L^{-1} MS-222 treatment than in any of the other three MS-222 treatments, suggesting that both the pH and the concentration of the anaesthetic (which causes different times to unconsciousness) are important. Due to

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the lack of difference between haematocrit ratios in the 3 g L^{-1} buffered treatment and both control treatments, it is clear that the change results from a higher proportion of fluid volume in the blood in the other three MS-222 treatments, rather than an increase in red blood cell volume in the 3 g L^{-1} MS-222 treatment (which is certainly less likely, given the timescale of the trials). Furthermore, the fluid is likely to have come from outside the blood, rather than, for example, from lysis of the red blood cells because plasma remained clear in these treatments.

This effect of MS-222 on haematocrit ratios is known to be one of a range of effects on fish blood parameters, which include changes in plasma glucose and lactate (Molinero & Gonzalez 1995). Studies on fish show that decreases in haematocrit in response to MS-222 appears likely to be caused by the effects of increasing acidity, (eg Witters *et al* 1990; MacAvoy & Zaepfel 1997). Frangioni *et al* (1997) state that MS-222 should not be used for any study of haematology or respiration. However, given that MS-222 remains the anaesthetic and euthanasia agent of choice for *X. laevis*, future studies should quantify changes and take them into account where possible, as we have here. As a minimum, the concentration, buffering and exposure time of animals to MS-222 should be clearly recorded so that comparisons can be accurately drawn between studies.

Considering that MS-222 at low levels is stressful to *X. laevis*, more consideration needs to be made of the best anaesthetic agent for this species, as well as for other aquatic vertebrates. Currently, the irony exists that anaesthesia, which is supposed to reduce stress in laboratory animals during maintenance and experiments, may actually be increasing animal stress, and thus negatively affecting welfare in some instances. For example, MS-222 is used at low levels to reduce stress during transport in fish, but caused elevated cortisol levels in rainbow trout (*Salmo gairdneri*), indicating that it is in itself stressful (Barton & Peter 1982). In fact, MS-222 exacerbated the cortisol response of gilthead seabream (*Sparus aurata*) to confinement (Molinero & Gonzalez 1995), and black sea bass (*Centropristis striata*) to a handling stressor (King *et al* 2005).

Conclusion and animal welfare implications

The results of the current study indicate that chemical euthanasia of *X. laevis* using MS-222 should use 3 g L^{-1} concentration, buffered to neutral pH. This combination appears to minimise stress, as measured by levels of circulating plasma corticosterone, compared to others tested here. It should therefore reduce suffering, and improve the welfare (Broom & Johnson 2000) of *X. laevis* undergoing euthanasia. However, this concentration is too strong to be used as an anaesthetic, so further investigation of how to mitigate, or provide alternatives for, the stressful effects of MS-222 at lower concentrations are needed. Furthermore, although females are the primary sex to be utilised in laboratories, which is why we used them in the current study, it is important to note that the effect of MS-222 on male *Xenopus* may not simply be as a function of their smaller size. Finally, it is very difficult to take blood samples from *X. laevis* without euthanising the animals (but see Boutilier & Shelton 1986; Hopkins *et al* 1997). Measuring glucocorticoid levels provides a good opportunity to measure stress responses in this and other species of vertebrates, and hence provides an important tool in monitoring and improving welfare. However, future studies would ideally work towards a non-invasive measure of corticosterone production in *X. laevis*, as has been recently developed and validated for cortisol in fish, eg rainbow trout (*Oncorhynchus mykiss*) using samples taken from water they are housed in (reviewed in Scott & Ellis 2007). This non-invasive method would allow the stress response of *X. laevis* to a large range of laboratory and maintenance procedures to be investigated, without sacrificing animals. Long-term monitoring of corticosterone levels by this method may also provide a way of predicting disease outbreaks, which can currently present a large problem to *Xenopus* users.

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