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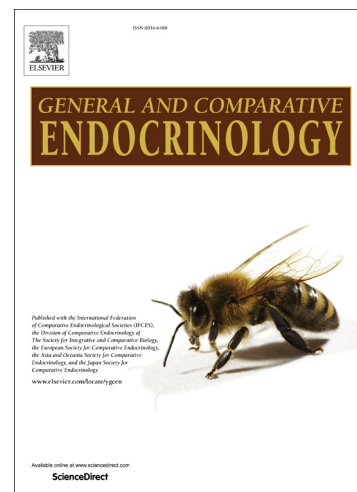
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## Abstract

Understanding the immediate and longer-term effects of transportation and re-housing in a laboratory species is crucial in order to refine the transfer process, enable the optimal introduction of new animals to a novel environment and to provide a sufficient acclimatisation period before usage. Whilst consideration of animal welfare in most model vertebrate species has received attention, little quantitative evidence exists for the optimal care of the common laboratory amphibian *Xenopus laevis*. Techniques for the non-invasive welfare assessment of amphibians are also limited and here a non-invasive physiological assay was developed to investigate the impacts of transportation, transport medium and re-housing on *X. laevis*. First the impacts of transportation and transport medium (water, damp sponge or damp sphagnum moss) were investigated. Transportation caused an increase in water-borne corticosterone regardless of transport medium. Frogs transported in damp sphagnum moss also had a greater decrease in body mass in comparison to frogs not transported, suggesting that this is the least suitable transport medium for *X. laevis*. Next the prolonged impacts of transportation and re-housing were investigated. Frogs were transported between research facilities with different housing protocols. Samples were collected prior to and immediately following transportation, as well as 1 day, 7 days and 35 days after re-housing. Water-borne corticosterone increased following transportation and remained high for at least 7 days, decreasing to baseline levels by 35 days. Body mass decreased following transportation and remained lower than baseline levels across the entire 35 day observation period. These findings suggest the process of transportation and re-housing is stressful in this species. Together these findings have important relevance for both improving animal welfare and ensuring optimal and efficient scientific research.

**Keywords:** Corticosterone; Welfare; Amphibian; Transport; *Xenopus*.

**Highlights:**

- Transportation and re-housing had a physiological impact on *Xenopus laevis* frogs.
- Transport in all mediums caused water-borne corticosterone to increase.
- Decrease in body mass following transportation in sphagnum moss.
- Raised corticosterone following transport and re-housing for at least 7 days.
- Reduced body mass following transport and re-housing for at least 35 days.

## 1 Introduction

High-quality animal welfare is ethically important and ensures physiologically and psychologically healthy animals that can fulfil their original purposes of captivity, e.g. reliable experimental results (Poole, 1997) or the quality of meat (Schwartzkopf-Genswein *et al.*, 2012). Transportation of captive animals, for research, agriculture or conservation purposes, is a significant stressor in a range of species, e.g. mammals (Minka and Ayo, 2007); birds (Leche *et al.*, 2013); fish (Boerrigter *et al.*, 2015); reptiles (Mancera *et al.*, 2014); and amphibians (Narayan and Hero, 2011). Refinement of transportation and re-housing methods can reduce potential negative impacts (Conour *et al.*, 2006) and may lead to shorter acclimatisation periods before animals recover for use (Obernier and Baldwin, 2006). It is therefore crucial to understand the immediate and longer-term effects of animal transportation and re-housing (Grandin, 1997).

Despite their numbers in captivity (for purposes of conservation, education, research or as pets) the welfare of amphibians has generally received less attention than that of other classes of tetrapods, in part due to challenges in interpreting amphibian behaviour (Alworth and Harvey, 2007) and quantifying the hypothalamic-pituitary-adrenal axis (HPA) response. *Xenopus laevis* (Daudin) is a common model organism in scientific research with over 6,000 procedures performed in the UK in 2013 alone (Home Office, 2014). There is, however, little quantitative evidence concerning the optimal care of this species in captivity, particularly in comparison to other model organisms (Reed, 2005). For example, three media are suggested for *X. laevis* transportation: water, damp sphagnum moss, and damp sponge (Green, 2010; Leadley Brown, 1970; Reed, 2005; Swallow *et al.*, 2005), yet whilst these media are likely to provide different experiences in terms of support, vibration, tactility and novelty, there is no evidence as to which might be most suitable for *X. laevis* welfare and all three methods are currently used (survey results from 77 respondents: water (68.8%), moss (40.3%) and sponge (20.8%), multiple answers permitted; Holmes *et al.*, 2015).

Understanding the impacts of transportation and re-housing on *X. laevis* is consequently vital to allow laboratories to better prepare for new arrivals and leave sufficient acclimatisation periods to ensure robust scientific data.

Specific stressors associated with transferring animals between facilities include: the initial capture, handling and confinement; vibration, disruption and conditions during transportation; and the introduction to novel housing, individuals and care protocols on arrival (reviewed in Conour *et al.*, 2006; Santurtun and Phillips, 2015; Swallow *et al.*, 2005). In amphibians physiological and/or behavioural changes indicative of stress have been observed following capture and handling (e.g. Narayan *et al.*, 2012; Ricciardella *et al.*, 2010; Woodley and Lacy, 2010), exposure to water vibrations (Davis and Maerz, 2011), and in response to a novel social environment (Cikanek *et al.*, 2014). The amphibian hypothalamic-pituitary-interrenal axis (HPI, analogous to the mammalian hypothalamic-pituitary adrenal axis; Rollins-Smith, 2001), releases corticosterone when activated and increases in corticosterone have been observed following both infection and physical stressors in amphibians (Gabor *et al.*, 2013b; Yao *et al.*, 2004). In *X. laevis* increases in corticosterone or corticotropin-releasing factor peptide content have been observed following tank agitation (Boorse and Denver, 2004; Glennemeier and Denver, 2002; Yao *et al.*, 2004), as well as after brief handling and exposure to a novel environment (Archard and Goldsmith, 2010). These studies, however, do not represent the full spectrum of disturbance resulting from transportation and re-housing and there was no prolonged monitoring after cessation of the stressors.

Amphibian hormones, such as corticosterone, have traditionally been quantified using invasive measures such as cardiac puncture (e.g. Crespi *et al.*, 2015; Mosconi *et al.*, 2006) or whole body homogenisation (e.g. Thurmond *et al.*, 1986). These methods present ethical problems and may mask experimental results by inducing additional stress (Palme, 2012). Sampling of urine, saliva or faeces can reduce invasiveness; however these practices may still involve prolonged handling or cloacal penetration (reviewed in Narayan, 2013) and remain problematic for fully aquatic species. Research into fish welfare has led to the extraction of cortisol from the surrounding water, a much less intrusive method (Scott and Ellis, 2007; Scott *et al.*, 2008; Scott *et al.*, 2001), and this technique has had some application in amphibians (Gabor *et al.*, 2013a; Gabor *et al.*, 2013b; Gabor *et al.*, 2015; Gabor *et al.*, 2016). It has been suggested that trends in glucocorticoids should be supported with other physiological measures (Breuner *et al.*, 2013; Dawkins, 2006; Otovic and Hutchinson, 2015), and reductions in body mass resulting from transportation have been noted in terrestrial animals (reviewed in Schwartzkopf-Genswein *et al.*, 2012). The use of changes in body mass as an indicator of welfare has received less attention in amphibians,

however in Ocoee salamanders (*Desmognathus ocoee*) chronic handling stress produced a decrease in body mass (Bliley and Woodley, 2012).

Here a water-borne corticosterone assay was developed and validated for *X. laevis* and combined with measures of body mass in order to investigate the effects of transportation, transport medium and re-housing in this species. To investigate the impacts of transportation and transport medium *X. laevis* were transported in water, sponge or sphagnum moss and the changes in water-borne corticosterone and body mass compared against frogs that were similarly sampled but not transported. Whilst body condition index scores have often been used in comparisons with hormone data, body mass was selected here to avoid the disturbance that obtaining snout-vent length in this fully aquatic species requires. This was from both a welfare perspective and to ensure sampling method didn't impact on hormone results. It was expected that transportation might result in an increase in corticosterone and a decrease in body mass but no prediction was made regarding the impact of transport medium. In the second experiment *X. laevis* were transported between facilities and re-housed in a novel physical, social and feeding environment as might be experienced by frogs during stock animal purchasing. Water-borne corticosterone and body mass were monitored across 5 weeks to investigate the longer-term impacts of transportation and re-housing.

## 2 Materials and Methods

### 2.1 Subjects and Housing

Subjects were wild-type *Xenopus laevis*, purchased from the European Xenopus Resource Centre (EXRC, University of Portsmouth) and housed at the University of Chester. Housing parameters were selected from Reed (2005), Green (2010) and A. Jafkins at EXRC (personal communication). *X. laevis* were housed in same-sex groups of five individuals per glass tank (584 mm x 431 mm x 305 mm, Clearseal), in dechlorinated mains water at a depth of 140 mm with a temperature range 20-23 °C (air temperature 23-25 °C). Water quality was maintained by Hamburg Matten-style biological filtration and partial water changes (~30 %) three times a week. Water quality was checked weekly for pH (6.4-7.6), nitrates (<20 mg/l), nitrites (<5 mg/l) and ammonia (<0.5 mg/l) using a dipstick water testing kit (King British). *X. laevis* were housed under a 12:12 light:dark cycle and fed 2.3 mm Royale Horizon Trout Pellets (Skretting) *ad libitum* three times a week. Home tanks were provided with black PVC tubing (140 mm x 118 mm x 50 mm, Floplast) and terracotta pots as environmental enrichment (Green, 2010; Reed, 2005). Photographs of *X. laevis* markings allowed for individual identification as patterns remain constant throughout life (Reed, 2005).

## 2.2 Effect of Transportation and Transport Medium

To investigate the impact of transportation and transport medium *X. laevis* (n = 3 males and n = 3 females in each transport group) were transported by car in white buckets (249 mm x 326 mm (h x dia), Taylor Davis). Frogs were transported in buckets containing 3 individuals of the same sex. The buckets contained either dechlorinated water (~7 litres, 30% of overall container as advised in Swallow *et al.*, 2005), damp cubes of sponge (25 mm<sup>3</sup>, soaked overnight in ~7 litres of dechlorinated water) or damp rehydrated sphagnum moss (Lucky Reptile SM-100, one 100 g brick soaked overnight in ~7 litres of dechlorinated water per bucket).

The transportation distance was 166 miles and took approximately 4 hours. Frogs were weighed and sampled for corticosterone 20 hours prior to transportation and immediately following transportation. Hormone sampling was as described in Section 2.4 and was performed at 15:00 hrs on both sampling occasions since *X. laevis* plasma corticosterone may vary during the light cycle (Crespi *et al.*, 2004). A control group of frogs were sampled at the same time and in the same manner but not transported (n = 12, 5 females and 7 males).

## 2.3 Effect of Transportation and Re-housing

To investigate the effects of transportation and re-housing on *X. laevis* the necessary transportation of frogs from our supplier (EXRC, Portsmouth) to our own facilities (University of Chester) was utilised. *X. laevis* (n = 16, 8 males and 8 females) were transported by car in buckets containing water as before (Section 2.2) in single-sex groups of 5 (along with other frogs not being tested). The transportation distance was 254 miles, with the journey taking approximately 5.5 hours. Frogs were weighed and sampled for corticosterone 18 hours prior to transportation (Pre -1 day), immediately following transportation (Post 0 days) and then Post 1 day, 7 days and 35 days following transportation. All sampling was performed as described in Section 2.4 and at the same time of day on each sampling occasion (15:30 hrs).

At EXRC *X. laevis* were housed in single-sex groups (5-10 adult females, 10-15 adult males) in a recirculating housing system (Tecniplast) and fed 2.3 mm Royale Horizon Trout Pellets *ad libitum* daily. Following transportation frogs were housed individually (water depth 140 mm, 254 mm x 203 mm x 208 mm, Clearseal) until 8 days following transportation, whereupon they were housed in single-sex groups of 5 individuals in home tanks as described above (Section 2.1). This initial difference in housing following transportation was designed to simulate the change in physical, social and feeding environment that *X. laevis* may encounter on being moved to a new facility.



#### 2.4 Hormone Sampling

Free corticosterone (hereafter referred to as just corticosterone) release rates of *X. laevis* were obtained non-invasively by extracting hormones from the surrounding water (method modified from Ellis *et al.*, 2004). Individual *X. laevis* were placed into sampling tanks (210 mm x 130 mm x 140 mm; Hagen) containing 1000 ml deionized water (Labwater 1, Purite) for 1 hour and then returned to home tanks. A sampling time of 1 hour was selected following pilot studies examining a range of sampling times. One hour allowed for sufficient levels of corticosterone to be quantified whilst minimising frog disturbance. Powder-free vinyl gloves (Nutouch New Generation) were worn throughout sampling procedure and during the subsequent immunoassay in order to prevent cross-contamination and protect amphibian skin. Frogs were weighed following sampling to minimise disturbance prior to sampling as body mass was not found to change over the course of the sampling hour (pilot study,  $n = 15$ ,  $z = -0.63$ ,  $p = 0.53$ , Wilcoxon signed ranks test). Between uses sampling tanks were washed (Anigene HLD4ND Disinfectant (Medimark Scientific, Kent, UK) then with 70% ethanol) and rinsed with water.

Following collection the water samples were vacuum filtered (in order to remove particles that might clog solid phase extraction cartridges) through filter paper (pore size = 11 $\mu$ m, Fisherbrand) and then through cellulose nitrate filter paper (pore size = 0.45  $\mu$ m, Sartorius). Water samples were then pumped through activated solid phase extraction cartridges (previously primed with 5 ml HPLC-grade 100 % methanol and 5 ml distilled water, Sep-pak<sup>®</sup> Plus C18, Waters Ltd., U.K.) at 25 ml/min (Ellis *et al.*, 2004). Cartridges were washed of impurities with 5ml distilled water (Ellis *et al.*, 2004) and stored at -20 °C until elution. Corticosteroids were eluted from the cartridges (at 20 °C) into borosilicate glass tubes (16 mm x 100 mm, Fisherbrand, Thermo Fisher Scientific) using 4 ml ethyl acetate. The ethyl acetate was evaporated under nitrogen at 37 °C and samples re-suspended in 500  $\mu$ l EIA PBS buffer (0.1 g BSA in 100 ml 0.1 M PBS. PBS = 5.42 g NaH<sub>2</sub>PO<sub>4</sub>H<sub>2</sub>O, 8.66 g Na<sub>2</sub>HPO<sub>4</sub> (anhydrous), 8.7 g NaCl, 1000 ml d.H<sub>2</sub>O, pH 7). Samples were vortexed (Multi-Reax, Heidolph) at 1600 rpm for 20 minutes and stored at -20 °C until required.

The impact of sampling water volume was verified by sampling different volumes of pooled *X. laevis* water samples and comparing the resulting levels of corticosterone. Frogs ( $n = 15$ ) were housed individually (254 mm x 203 mm x 208 mm, Clearseal) in 5 L water. After 1 hour frogs were removed, samples of 250 ml, 500 ml and 1000 ml were taken from each tank and corticosterone extracted as described above. No difference was found in the amount of corticosterone extracted from 250 ml, 500 ml or 1000 ml once differences in volume were accounted for ( $F_{(2,28)} = 2.399$ ,  $p = 0.11$ ; repeated measures GLM). For all future sample

collections frogs were housed in 1000 ml of water and the entire sample was used during the extraction process.

## 2.5 Enzyme Immunoassay

An enzyme immunoassay (EIA) was modified from Smith and French (1997) for *X. laevis*. Checkerboard assays were used to optimise the EIA and the standard curve was calibrated by running rows of 1:2 and 1:5 dilutions of corticosterone resulting in a 1:2 serial dilution from 30,000 pg/ml to 58.59 pg/ml. To run the EIA, the antibody (Rabbit CJM006, produced against corticosterone-3-CMO-BSA (Steraloids, Wilton, New Hampshire; Q1559-000), as described in Watson *et al.* (2013)) was diluted 1:16,000 in 0.05 M carbonate buffer (1.59 g  $\text{Na}_2\text{CO}_3$ , 2.93 g  $\text{NaHCO}_3$ , 1000 ml d. $\text{H}_2\text{O}$ , pH 9.6), loaded 50  $\mu\text{l}$ /well onto a 96-well Maxisorp Nunc-Immuno microtitre plate (Thermo-Fisher Scientific, UK) and incubated overnight (4 °C). After incubation plates were allowed to reach 20 °C and then washed four times with 1:5 diluted ELISA wash buffer (40 g NaCl, 1 g KCl, 1.2 g  $\text{KH}_2\text{PO}_4$ , 7.2 g  $\text{Na}_2\text{HPO}_4$ , 1000 ml d. $\text{H}_2\text{O}$ , 2.5 ml Tween 20, pH 7). Plates were loaded with 50  $\mu\text{l}$ /well EIA PBS buffer, followed by either 50  $\mu\text{l}$ /well corticosterone standard (C2505 Sigma-Aldrich, Dorset, UK) or 50  $\mu\text{l}$ /well *X. laevis* sample (diluted 1:2 in EIA buffer), and then loaded immediately with 50  $\mu\text{l}$ /well of horseradish peroxidase conjugate (1:40,000 in EIA buffer; Sigma-Aldrich, St. Louis, MO; P8375) and incubated for 3 hours in darkness following Watson *et al.* (2013). Following incubation plates were washed and 100  $\mu\text{l}$ /well EIA substrate added (12.5 ml Citrate buffer (9.61 g citric acid (anhydrous), 1000 ml d. $\text{H}_2\text{O}$ , pH 4), 125  $\mu\text{l}$  EIA ABTS (0.329 g 2,2'-azino-di(3-ethylbenzthiazoline-6-sulphonic acid), 15 ml d. $\text{H}_2\text{O}$ , pH 6), 40  $\mu\text{l}$   $\text{H}_2\text{O}_2$  (2 % w/v, 500  $\mu\text{l}$   $\text{H}_2\text{O}_2$ , 7.5 ml d. $\text{H}_2\text{O}$ )). Plates were left to incubate in darkness until  $B_0$  wells reached an absorbance of 1.0 and then read at 405 nm on a microplate reader (MRX II, Dynex Technologies) using the program Revelation (Version 4.22). Samples and qualitative controls were run in quadruplicate, samples from the same individual were run on the same plate and samples re-run together if coefficients of variance (CV) were above 5 %.

The enzyme immunoassay was validated for water-borne hormones using a pooled sample from 10 female and 5 male non-experimental *X. laevis* (Grotjan and Keel, 1996). To determine specificity the slope of the standard curve (standards diluted in a halving serial dilution) was checked for parallelism against the slope of halving dilutions of a pooled sample (neat to 1/512 dilution in EIA PBS buffer) using an ANCOVA (two repeats, each with two *X. laevis* slopes and one standard slope). No significant differences were found in the slopes of the standards compared to the serially diluted samples suggesting that the *X. laevis* samples behave in the same manner as the standards (Plate 1:  $F_{(2,34)} = 1.66$ ,  $p = 0.21$ ; Plate 2:  $F_{(2,29)} = 1.80$ ,  $p = 0.18$ ; Figure 1). To determine assay accuracy, pooled sample (1:4



dilution in EIA PBS buffer, dilution selected from consultation of specificity test results) was combined with individual standards (ratio 1:1) and observed and expected corticosterone readings compared using linear regression. The mean percentage recovery of *X. laevis* corticosterone was  $108.06 \pm 5.62$  % ( $F_{(1,19)} = 964.26$ ,  $p < 0.001$ ). Two pooled controls were included on each plate to determine higher (neat sample) and lower (1:4 dilution in EIA PBS buffer) intra- and inter-assay CV (precision). Intra-assay CVs were HQC: 1.59 %, LQC: 3.44 %; inter-assay CVs were HQC: 10.62 %, LQC: 11.23 %. The sensitivity of the assay was 117 pg/ml. Assay corticosterone recovery was assessed by spiking 500 ml samples (5 *X. laevis* samples, 3 empty water samples) with 5000 pg corticosterone and these were compared to unspiked replicates. Recovery of the spiked *X. laevis* and empty samples was  $90.18 \pm 3.78$  %.

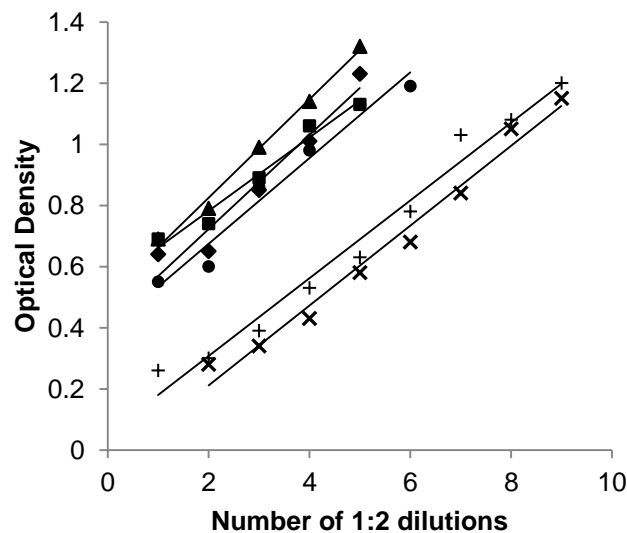


Figure 1: Parallelism (specificity) of serial halving dilutions of pooled *X. laevis* water-borne corticosterone compared against standard corticosterone curves repeated across 2 plates. Plate 1: standard 1 (+), sample 1 (▲), sample 2 (●). Plate 2: standard 2 (X), sample 3 (■), sample 4 (◆).

## 2.6 Statistical Analyses

All statistical analysis was carried out using SPSS version 21.0. There was no correlation between corticosterone and *X. laevis* body mass (Pearson's correlations: females:  $r = 0.12$ ,  $p = 0.78$ ; 8 males:  $r = 0.40$ ,  $p = 0.33$ ), hence corticosterone release rate was expressed as pg/hr (having accounted for re-suspension volume).

### 2.6.1 Effect of Transportation and Transport Medium

The change in water-borne corticosterone and body mass before and after transportation was calculated. Three samples were spilled during collection and so were removed from the

analysis (1 male and 1 female sample in the water transport group and 1 female sample from the no transport group).

A multivariate general linear model (MGLM) was used to investigate the impact of transport medium, with change in corticosterone and body mass as dependent variables and transport treatment group (no transport, water, sponge and moss) and sex as between-subject factors. Follow up GLMs were then used to investigate the impact of transport treatment group on the separate measures (corticosterone change and body mass) and post-hoc comparisons between all four transport groups were performed using Gabriel's pairwise comparison (as group sizes were different).

### 2.6.2 *Effect of Transportation and Re-housing*

The effect of transportation and re-housing on corticosterone and body mass was analysed using a repeated measures MGLM with sampling time points of corticosterone and body mass as within-subject factors and sex as a between-subjects factor. Follow up repeated measures GLMs were used to further investigate the effects of transportation and within-subject planned contrasts compared the baseline samples (prior to transportation) against each subsequent sample.

### 2.7 *Ethics*

All work was carried out in consultation with the UK Home Office, in accordance with University of Chester Research Guidelines and under approval from the University of Chester Faculty Research Ethics Committee (FREC ref no 986/14/CH/BS 25 Nov 2014).

## 3 **Results**

### 3.1 *Effect of Transportation and Transport Medium*

There was a significant effect of treatment group (no transport, water, sponge, moss transportation;  $F_{(6,38)} = 3.44$ ,  $p = 0.008$ ; Figure 2), which when analysed further showed treatment group differences for both corticosterone change ( $F_{(3,19)} = 6.05$ ,  $p = 0.005$ ) and body mass change ( $F_{(3,19)} = 4.29$ ,  $p = 0.02$ ). Post-hoc pairwise comparisons (Gabriel's) revealed that all three transport groups showed an increase in corticosterone compared to the non-transport group (water  $p = 0.04$ , sponge  $p = 0.01$ , moss  $p = 0.004$ ; Figure 2a), whilst the moss treatment group had a greater decrease in body mass compared to the no transport group ( $p = 0.003$ ; Figure 2b). There was no interaction between transport method and sex ( $F_{(6,38)} = 0.63$ ,  $p = 0.70$ ), however there was an overall difference between the sexes ( $F_{(2,18)} = 7.20$ ,  $p = 0.005$ ), apparent in body mass change ( $F_{(1,19)} = 15.16$ ,  $p = 0.001$ ) but not corticosterone change ( $F_{(1,19)} = 1.25$ ,  $p = 0.27$ ).

### 3.2 Effect of Transportation and Re-housing

Overall there was a significant effect of transportation and re-housing on *X. laevis* ( $F_{(8,7)} = 52.98$ ,  $p < 0.001$ ; Figure 3). There was a significant difference between males and females ( $F_{(2,13)} = 63.85$ ,  $p < 0.001$ ), however there was no interaction between sampling time and sex ( $F_{(8,7)} = 1.48$ ,  $p = 0.31$ ).

Further investigation revealed that water-borne corticosterone release rates changed following transportation ( $F_{(4,56)} = 9.94$ ,  $p < 0.001$ ; Figure 3a), and release rates were higher 0, 1 and 7 days following transportation (all planned contrasts  $p < 0.05$ ). By 35 days after transportation there was no difference compared to baseline samples ( $p = 0.41$ ). No difference in water-borne corticosterone release rates were observed between the sexes ( $F_{(1,14)} = 3.01$ ,  $p = 0.11$ ), however there was a significant interaction between time and sex ( $F_{(4,56)} = 3.93$ ,  $p = 0.007$ ). Planned contrasts revealed a significant interaction when comparing male and female scores between baseline corticosterone and 7 days following transportation ( $F_{(1,14)} = 5.84$ ,  $p = 0.03$ ). No other interaction planned contrasts were significant (all  $p > 0.05$ ).

*X. laevis* body mass decreased as a result of transportation ( $F_{(4,56)} = 46.81$ ,  $p < 0.001$ ; Figure 3b) with body mass at each time point following transportation being lower than pre-transportation baseline measurements (all planned contrasts  $p < 0.05$ ). Females had a greater body mass than males ( $F_{(1,14)} = 137.21$ ,  $p < 0.001$ ) and there was no significant interaction between time and sex ( $F_{(4,56)} = 2.33$ ,  $p = 0.07$ ).

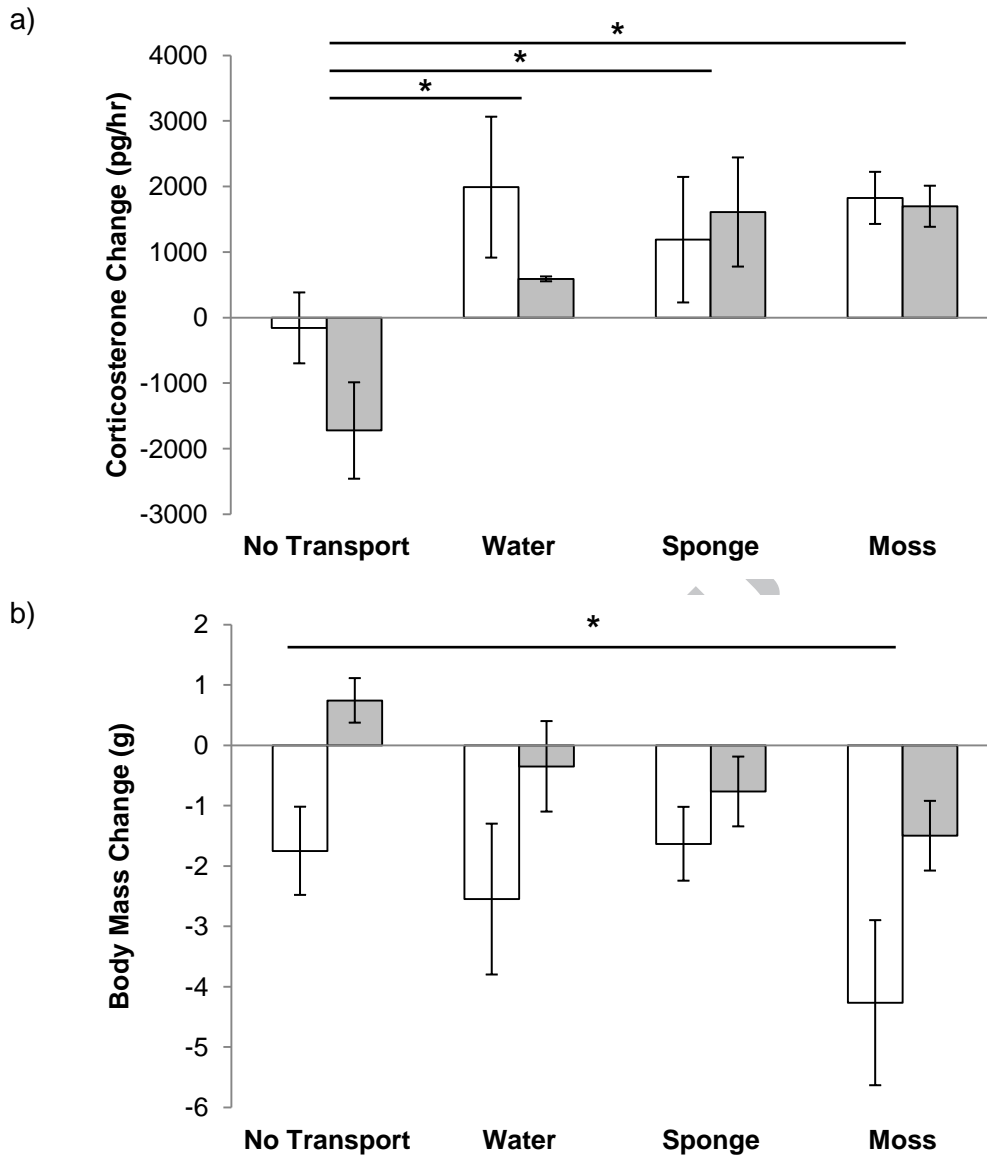


Figure 2: Effects of transportation and method on female (white bars) and male (grey bars) *X. laevis*, measured via (a) change in water-borne corticosterone and (b) change in body mass before and after transportation in water, sponge or moss or no transportation (mean  $\pm$  standard error). An increase in corticosterone was observed in all transport groups compared to non-transported frogs, whilst the moss treatment resulted in a significantly greater decrease in body mass compared to *X. laevis* not transported (differences between treatment groups indicated by \*).

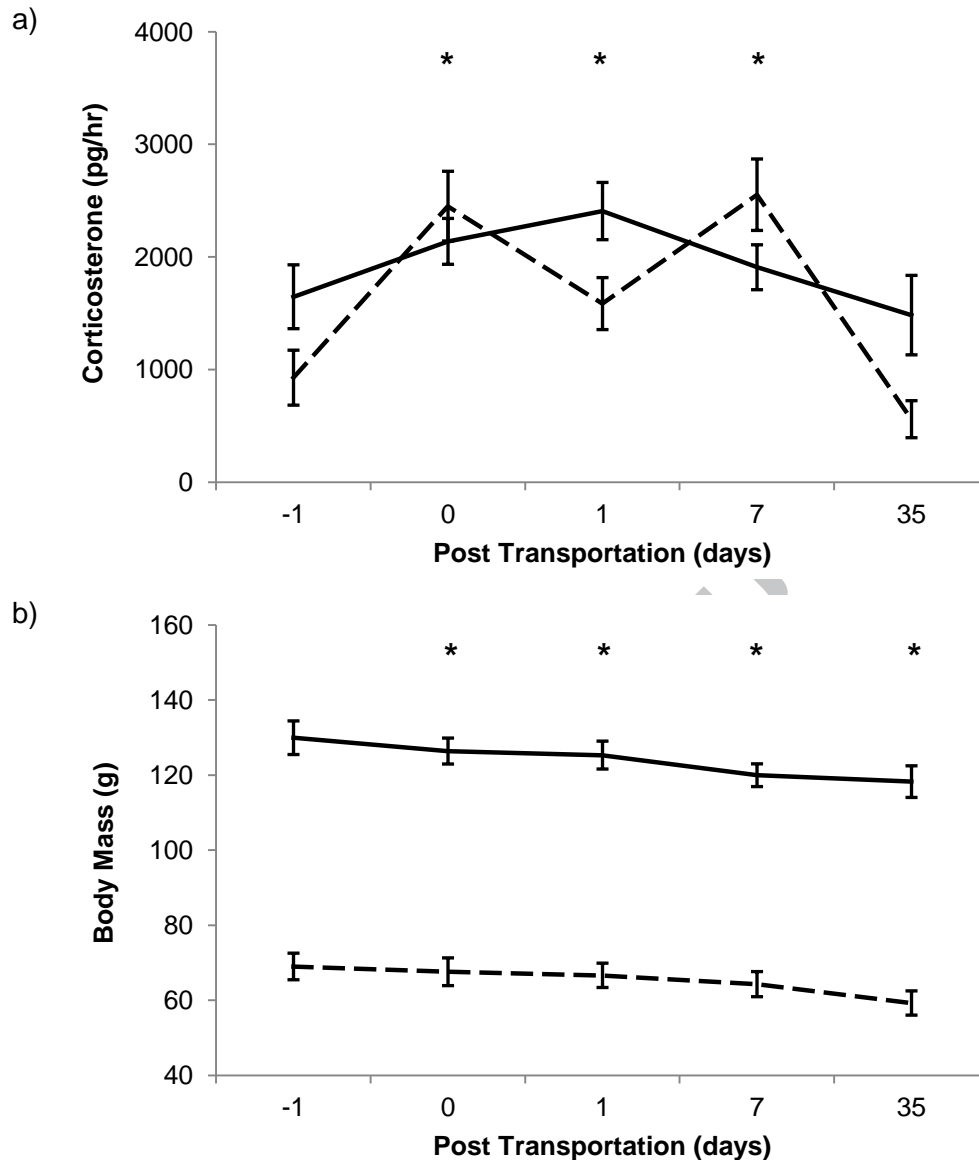


Figure 3: Effects of transportation and re-housing on (a) water-borne corticosterone release rates and (b) body mass on female (solid lines) and male (dashed lines) *X. laevis* over time (mean  $\pm$  standard error). Transportation and re-housing resulted in an increase in corticosterone and a decrease in body mass (significant differences between sampling time points and baseline levels (-1 days) indicated by \*).

#### 4 Discussion

Understanding the immediate and longer-term impacts of transportation and re-housing in a laboratory species is crucial in order to refine the transportation process, enable the optimal introduction of new animals to a novel environment and to provide a sufficient acclimatisation period before usage (Obernier and Baldwin, 2006). In line with transportation studies in mammals, birds and fish (e.g. Boerrigter *et al.*, 2015; Leche *et al.*, 2013; Minka and Ayo, 2007; Schwartzkopf-Genswein *et al.*, 2012) water-borne corticosterone release rates of *X. laevis* increased and body mass decreased following transportation.

The activation of the *X. laevis* HPI axis (as seen by the subsequent increase in corticosterone) is in keeping with responses observed in controlled laboratory agitation studies (Boorse and Denver, 2004; Glennemeier and Denver, 2002; Yao *et al.*, 2004). Similar increases in corticosterone have been observed in other amphibians in response to capture and handling (Narayan *et al.*, 2012; Ricciardella *et al.*, 2010; Woodley and Lacy, 2010), agitation (Davis and Maerz, 2011) and novel social environment (Cikanek *et al.*, 2014) – all components of the transportation and re-housing process.

Shrinkage (i.e. a loss in body mass) has been noted as an effect of transportation of terrestrial animals (reviewed in Schwartzkopf-Genswein *et al.*, 2012), however the results presented here provide the first evidence of transportation stress causing an immediate change in body mass in an aquatic species. Possible explanations for this include high energy expenditure due to constant spatial adjustment, limited access to food, waste purging, and motion sickness (Santurtun and Phillips, 2015; Schwartzkopf-Genswein *et al.*, 2012). The increased energetic expenditure due to the activation of the HPI axis itself may also have contributed to shrinkage.

Adult female *X. laevis* were often over twice the body mass of adult males (females  $134.26 \pm 5.52$  g, males  $64.76 \pm 1.85$  g), a likely explanation for the inter-sex body mass differences observed here. Inter-sexual differences in corticosterone response have previously been observed in other amphibians (Janin *et al.*, 2011; Woodley and Lacy, 2010), as well as *X. laevis* (Holmes *et al.*, 2016). With their larger bulk females may be more resilient to stressors or other environmental changes. Amphibian responses to stress may also be dependent on reproductive season (Ricciardella *et al.*, 2010), although this is unlikely here where seasonal cues were controlled in the laboratory environment.

#### 4.1 Transport medium

The three transportation mediums tested here (water, damp sponge and damp sphagnum moss) provide different levels of support, vibration, tactility and novelty. Both sponge and sphagnum moss are novel substrates and present degrees of tactility and abrasion, however they might limit the impact of vibration caused by the transportation process. Water, whilst a familiar medium, may result in greater levels of vibration and consequently be energetically expensive due to frequent body position adjustments (although transportation in plastic bags filled 30% of total bag volume might help to limit this; Swallow *et al.*, 2005).

Whilst the results presented here don't provide a conclusive best practice transport medium, the significant decrease in body mass suggests that damp sphagnum moss might be the least suitable medium for transporting *X. laevis*. Sphagnum moss is a novel non-aquatic



environment, may be abrasive against sensitive amphibian skin and has been known to result in mite infestations when used as a transport medium (Ford *et al.*, 2004).

Temperature is an important aspect of *X. laevis* transportation (Reed, 2005; Swallow *et al.*, 2005). Exposure to high temperatures can cause damage to oocytes ((Sive *et al.*, 2000) referenced in (Reed, 2005)) and affect survival upon arrival of a closely related species, *X. tropicalis* (Kurabayashi *et al.*, 2014). It would therefore be useful to know the insulating properties of water and damp sponge during transportation.

#### 4.2 Transportation and re-housing

Prolonged impacts on *X. laevis* were observed following transportation and re-housing. Whilst the differences in housing conditions may appear extreme they were designed to represent the large differences in housing protocols between facilities (Holmes *et al.*, 2015; Major and Wassersug, 1998).

Water-borne corticosterone remained higher than baseline levels for up to 7 days following transportation and re-housing. In wild-caught amphibians raised corticosterone following capture has been observed for up to 5 days (*Rhinella marina*; Narayan *et al.*, 2011), 8 days (*Eurycea nana*; Gabor *et al.*, 2016) and 15 days (*Platymantis vitiana*; Narayan and Hero, 2011). Here we present the first evidence in captive amphibians of a prolonged increase in corticosterone following transportation and re-housing between laboratory environments, suggesting that the process may be stressful in laboratory reared amphibians as well as wild-caught individuals.

Body mass remained lower than baseline levels for 35 days following transportation, indicating that transportation and re-housing in a novel environment may have lasting consequences on the health and welfare of *X. laevis*. Stress has been shown to decrease feeding behaviour in every vertebrate group (reviewed in Carr, 2002). In amphibians, individual aspects of transportation have resulted in reduced feeding behaviour in handled female Ocoee salamanders (*Desmognathus ocoee*; Bliley and Woodley, 2012) and Common toads (*Bufo bufo*) subjected to centrifugal motion (Lychakov, 2012).

A change in feeding regime is also a potential stressor (Conour *et al.*, 2006; Swallow *et al.*, 2005) and here, although food type remained the same and frogs were still able to feed *ad libitum* at feeding times, feeding frequency decreased from 5 to 3 times per week. Whilst this might explain the decrease in body mass observed here, body mass did subsequently return to and exceed baseline levels for all frogs involved, suggesting that feeding frequency wasn't the sole cause of shrinkage. Facilities receiving *X. laevis* may help to minimise shrinkage loss by gradually changing feeding schedules until arriving at the desired routine.

### 4.3 Hormone quantification

The protocols described here are a novel non-invasive method of assessing changes in corticosterone in this fully aquatic species (adapted from Ellis *et al.*, 2004), and the increase in corticosterone following a predicted stressor provides a valuable biological validation. In fish, cortisol is excreted directly into the water through the gills (Ellis *et al.*, 2005). The specific source of water-borne corticosterone in amphibians remains unknown (Gabor *et al.*, 2013a; Gabor *et al.*, 2013b; Gabor *et al.*, 2015; Gabor *et al.*, 2016), however, possibilities include skin secretions, urine, faeces and saliva (Gabor *et al.*, 2013a).

Studies quantifying hormones should take measures to avoid detecting hormonal changes caused by the sampling process itself. Results from pilot studies suggest that this was not the case here as significant rises in water-borne corticosterone were not observed across three consecutive 1 hour sample collections ( $p < 0.05$ ). Whilst the protocols described entail a degree of invasiveness (due to brief handling and transfer to a novel environment during sampling), the process is likely to be less disruptive than other methods currently used in this species (Boorse and Denver, 2004; Glennemeier and Denver, 2002; Yao *et al.*, 2004) and is more practical for aquatic species. Low solute levels in deionized water may result in the disruption of *X. laevis* osmolality and cause swelling, however this was not observed here and no change in body mass was detected before and after sampling (Section 2.4).

### 4.4 Impacts

Stress can negatively affect or even inhibit reproduction (Moore and Jessop, 2003; Orchinik, 1998; Wingfield *et al.*, 1998) and *X. laevis* ovary mass positively correlates with body mass (Archard, 2012). Since oocyte production is a fundamental aspect of research involving *X. laevis* (Schultz and Dawson, 2003), efforts to reduce the negative effects of transportation and re-housing are crucial, particularly in light of the prolonged decrease in body mass observed here. Tank colour is also important for *X. laevis* welfare (Holmes *et al.*, 2016) and container colour may therefore warrant consideration when transporting this species.

Comparison with other studies of *X. laevis* investigating differences in corticosterone is hampered by experimental differences in frog development stage (tadpole, juvenile and adult) and sample type/collection (whole body, blood, plasma, serum and even inference from CRF peptide). Such destructive sampling techniques mean that repeated sample collection from the same individual is impossible, whilst the process of destructive sample collection itself is likely to be additionally stressful. However, calculation of X-fold differences in corticosterone allows limited comparison between studies. Tadpoles shaken for up to 8 hours show between a 1.5-fold increase and 5-fold increase (Boorse and Denver, 2004; Glennemeier and Denver, 2002), whilst tadpoles given an ACTH challenge showed a 5-fold

increase (Glennemeier and Denver, 2002). Juvenile *X. laevis* show a 4.3-fold larger level of plasma corticosterone after 3 hours of shaking and a 20-fold increase after 6 hours (Yao *et al.*, 2004). In adults a stressful method of euthanasia can result in approximately a 1.8-fold higher levels of plasma corticosterone in comparison with a more welfare appropriate method (Archard and Goldsmith, 2010), whilst adult frogs removed from water for 2 weeks showed levels of serum corticosterone 2.3-fold times greater than control frogs kept in water for the duration (Guardabassi *et al.*, 1988). Here the increase in water-borne corticosterone observed ranged between 1.3-fold and 2.6-fold increases, whilst a study using the same methodology to compare tank background colour found a 1.3-fold increase when adults were housed in tanks with a white compared to a black background (Holmes *et al.*, 2016). Comparison of X-fold changes therefore suggests that the effect of transportation observed in this study is not insubstantial, given noted differences in experimental methodology, especially considering how destructive sampling might have influenced resulting levels of corticosterone in the earlier studies.

The findings presented here are also important when considering the welfare and transportation of other aquatic amphibian model species such as *X. tropicalis* (Jafkins *et al.*, 2012) or Budgett's frog (*Lepidobatrachus laevis*; Amin *et al.*, 2015). Finally, many amphibian species are the subjects of conservation programs that involve translocation and reintroduction. It is important, therefore, to consider the impacts of transportation under these circumstances in order to maximise conservation success (Teixeira *et al.*, 2007).

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**Title:** Effects of transportation, transport medium and re-housing on *Xenopus laevis* (Daudin)

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**Highlights:**

- Transportation and re-housing had a physiological impact on *Xenopus laevis* frogs..
- Transport in all mediums caused water-borne corticosterone to increase.
- Decrease in body mass following transportation in sphagnum moss.
- Raised corticosterone following transport and re-housing for at least 7 days.
- Reduced body mass following transport and re-housing for at least 35 days.