The Old School, Brewhouse Hill, Wheathampstead,
Hertfordshire AL4 8AN, UK
www.ufaw.org.uk

Animal Welfare 2021, 30: 145-154
ISSN 0962-7286
doi: 10.7120/09627286.30.2.145

Routine handling does not lead to chronic stress in captive green anole (Anolis carolinensis)

G Borgmans*[†‡], R Palme[§], A Sannen[#], H Vervaecke[#] and R Van Damme[†]

[†] Department of Biology, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium
[‡] Centre for Research and Conservation, Royal Zoological Society of Antwerp, Antwerp, Belgium
[§] Unit of Physiology, Pathophysiology and Experimental Endocrinology, Department of Biomedical Sciences, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria
[#] Ethology & Animal Welfare, Agro- & Biotechnology, HUB-ODISEE University College, Hospitaalstraat 21, 9100 Sint-Niklaas, Belgium
* Contact for correspondence: glenn.borgmans@uantwerpen.be

Abstract

*Routine handling has been shown to affect stress levels in a variety of animal species. This could result in a general decrease in welfare and may confound the results of scientific experiments or observations on captive study animals. In reptiles, there seems to be variation in the effects of handling on stress levels. Furthermore, most studies on reptiles only look at the effect of handling in the short term. In this study we quantified the physiological and behavioural impact of being held, twice daily, for 1 min at a time over a three-week period on the green anole (*Anolis carolinensis*). Measurements were collected at the end of the three-week repeated handling period. Our results showed no effect of repeated handling on body mass, tail-base width, heterophil to lymphocyte ratios (H/L ratios), behaviour and faecal corticosterone metabolite (FCM) levels for both males and females in the experimental treatments ('handled', 'unhandled'). Our study animals did score very highly for several stress-indicating variables in the three weeks preceding the experiments — suggesting that they had experienced considerable stress during capture, transport and temporary housing in the pet store.*

Keywords: *animal welfare, captivity, green anole, handling, reptiles, stress*

Introduction

Routine husbandry practices have been shown to adversely affect captive animals (Balcombe *et al* 2004; Morgan & Tromborg 2007). Even non-invasive manipulation, such as simply holding the animal, or cleaning or moving its cage, can affect the endocrinology, physiology and behaviour of animals. This could result in a general decrease in welfare (Morgan & Tromborg 2007) and may confound the results of scientific experiments or observations on captive study animals (Garner 2005).

Balcombe *et al* (2004) provide an extensive review of the literature on the effect of handling in a variety of animal species (rats, mice, rhesus macaques, hamsters, rabbits, fruit bats and a number of bird species). The routine handling of laboratory animals induced changes in physiological variables, eg serum or plasma concentrations of corticosterone, glucose, growth hormone or prolactin, heart rate, blood pressure and behaviour. Changes from baseline or control measurements typically ranged from 20 to 100% or more and lasted at least 30 min or longer, showing that routine handling could have an effect on any measurements taken after the handling protocol itself. Besides the review by Balcombe *et al* (2004), which focused mainly on mammals and birds, a large body of work exists showing handling to also cause stress in fish (Farbridge & Leatherland 1992; Foo & Lam 1993; Hoffmayer & Parsons 2001; Ramsay *et al* 2009).

Besides this body of work on the effect of handling on stress in mammals, birds and fish, a number of studies have investigated this in reptiles (Table 1). There appears to be variation in the effects of handling on stress levels in reptiles (Table 1). While the majority of studies mainly find an increase in plasma corticosterone in response to handling, a number did not find an effect. Furthermore, almost all of these studies look at the effect of handling in the short term. Analyses of the effects of long-term repeated handling are rare for reptiles which is surprising given the fact that many reptilian species are kept in captivity for an extended period of time for research purposes and thus are frequently subject to routine handling. Therefore, in this study, we investigated the effect of three weeks' repeated handling. While most studies focus on one technique of measuring stress (Table 1), we used an integrative approach to get a broader view of the response to handling.

In this study we quantified the physiological and behavioural impact of handling on the green anole (*Anolis caroli-*

Table 1 Research investigating the effect of handling, restraint and short-term confinement in reptiles.

Species	Stressor	Effect	Reference
Turtles			
Lepidochelys kempii	Restrained upside down, sampled at 30 and 60 min	Increased plasma corticosterone	Gregory & Schmid (2001)
		Increased plasma glucose	
Terrapene carolina triunguis	Handled for 15 min	Increased FCM	Rittenhouse *et al* (2005)
Trachemys scripta elegans	Held in a 19 l plastic bucket, sampled at 30 and 60 min	Increased plasma corticosterone	Cash *et al* (1997)
Lizards			
Amblyrhynchus cristatus	30 min capture and restraint	Increased plasma corticosterone	Romero & Wikelski (2002)
Eulamprus heatwolei	Handled for physiological measurements (time unspecified)	No increased plasma corticosterone	Langkilde & Shine (2006)
Hoplodactylus maculatus	Captured and held in cloth bag for 2.5 h	No change in female corticosterone	Girling & Cree (1995)
	Captured and held in cloth bag, sampled at 4 and 24 h	Increased plasma corticosterone	Cree *et al* (2003)
		No change in male testosterone	
Iguana iguana	Held in hand while gently rocking/speaking for 1 min	Increased heart rate	Cabanac & Cabanac (2000)
	Handled for 5 min over an 8-day period	Increased FCM	Kalliokoski *et al* (2012)
Pogona barbata	Captured and held in cloth bag, sampled at 3.5 and 24 h	No increased plasma corticosterone	Cree *et al* (2000)
		No increased plasma progesterone	
	Captured and held in cloth bag, sampled at 3.5 h	No change in male testosterone	
Sphenodon punctatus	Restrained in a bag for 3 h	Increased plasma corticosterone	Tyrrell & Cree (1998)
Tiliqua scincoides	Gentle handling, manual/container restrained for 10 min	No increased plasma corticosterone	Kreger & Mench (1993)
		No increased H/L ratio	
		No increased active behaviour	
Urosaurus ornatus	Restrained in a bag for 4 h	Increased plasma corticosterone	Moore *et al* (1991)
		Decreased plasma testosterone	
	Kept in individual cages for 3 weeks	Increased plasma corticosterone	
		Decreased plasma testosterone	
	Restrained in a bag for 10 min	Increased plasma corticosterone	Woodley *et al* (2002)
Snakes			
Boiga irregularis	1 night spent in a trap	Increased plasma corticosterone	Mathies *et al* (2001)
	1 night spent in a trap, 10 min in a bag	Increased plasma corticosterone	
	1 night spent in a trap, 2 h in a bag	Increased plasma corticosterone	
Crotalus atrox	Grabbed with snake-grabber every 5 s for 5 min	Increased plasma corticosterone	Schuett *et al* (2004)
Python regius	Gentle handling and manual restraint	No increased plasma corticosterone	Kreger & Mench (1993)
		No increased H/L ratio	
		No increased active behaviour	
	Container restraint	Increased plasma corticosterone	
		No increased H/L ratio	
		No increased active behaviour	
Thamnophis sirtalis parietalis	Restrained in a bag for 4 h	Increased plasma corticosterone	Moore *et al* (2000)
		Decreased plasma testosterone	
Crocodiles			
Alligator mississippiensis	Handled for bleeding (time unspecified)	Increased plasma corticosterone	Lance & Lauren (1984)
	8 h restraint in a zinc box	Increased plasma corticosterone	Lance & Elsey (1999)
		Increased plasma glucose	

nensis). This species is commonly kept as a pet, especially in North America, and used as a model species for scientific studies in a laboratory setting across a range of research fields, including behavioural biology, physiology, and morphology (eg Waters *et al* 2005; Merchant *et al* 2008; Montuelle *et al* 2008; Stellar & White 2010). It is therefore surprising that the effect of handling has not been investigated in this species. We hypothesised that a high frequency of handling would lead to an increase in stress and concordant changes in behavioural and physiological indices.

Materials and methods

Study animals and housing

All procedures were carried out with the approval of the University of Antwerp's ethical committee for animal experiments (Ethische Commissie Dierproeven, ECD, file nr 2013-70). Thirty-three adult *A carolinensis* (19 males, 14 females) were obtained from a licensed commercial supplier in Belgium. The animals had been caught in the field in Florida, USA, less than one week prior to being sent by air to Belgium. In the laboratory, lizards were placed into individual glass terraria (40 × 30 × 70 cm; length × width × height). Lizards were housed individually to facilitate the identification of faecal samples. Light bulbs (45 W) were placed at the top of the cages, providing a shallow thermogradient within the cages, ie air temperatures between 20°C at the bottom of the cages and 30°C directly under the lamp at the top. Lights were switched on during daytime (0600–2000h) to create a diurnal rhythm. The maximum temperature of 30°C falls within the range of mean preferred temperature (MPT) of *A carolinensis* (Licht 1968) and corresponds to the mean body temperatures measured in the field (Lailvaux & Irschick 2007). Relative humidity was monitored using a hygrometer (TH50 hygrometer, Hama, Germany) and maintained at around 60% by misting the terraria daily. The walls of adjacent cages were lined with white paper precluding visual contact between individual lizards. The bottom of the cages were covered with white paper towels to aid in the detection and collection of faecal pellets. Each cage contained a diagonally placed wooden perch with a diameter of 2 cm — the preferred perch diameter for *A carolinensis* (Gilman & Irschick 2013) — and two banana leaves (average size around 20 × 10 cm; length × width) under which lizards could hide. Animals were provided with *ad libitum* water and fed twice a week with common house crickets (*Acheta domesticus*) and once a week with wax moth larvae (*Galleria mellonella*). Once a week crickets were dusted with an ultrafine calcium carbonate supplement containing vitamin D3 (Repti Calcium, Zoo Med Europe, USA).

Experimental design

The lizards remained under the conditions described above for the three weeks following their arrival at the laboratory. This time interval will hereafter be referred to as the 'acclimatisation' period. The data from this period are also utilised in two other experiments (Borgmans *et al* 2018, 2019). At the end of this period, measurements were carried out as described below after which animals were randomly assigned to one of two groups. Individuals allocated to the 'handling first' group (ten males, seven females) were taken from their cages twice a day and restrained in hand for 1 min. The hand remained completely stationary with the animal kept in the same hand throughout. This was done for the next three weeks and the animals subsequently received the 'unhandled' treatment which was identical to the 'acclimatisation' period. Individuals of the 'handling second' group (eleven males, six females) received the reversed order of treatment. This cross-over design allowed testing for an effect of order of treatments. Cages were set up in such a way to ensure that unhandled animals were unable to see conspecifics being handled by experimenters and precautions were taken to ensure complete silence when experimenters had to enter the experimental room to implement various tasks (eg feeding, handling, spraying). Enclosures were not cleaned during experimental treatments, only between them. This all sought to minimise the 'background level' of stress which could have affected measurements.

Measurements

All measurements were carried out in the last seven days of each three-week period ('acclimatisation', first treatment, second treatment). If day 1 is the first day of a three-week period, then faecal samples were collected on days 14–16 and behavioural observations made on day 19. Blood samples were collected on day 20 and the morphological measurements taken on day 21. Sample collection took place over multiple days, so as not to overstress the animals by carrying them all out on the same day. The specific order of measurements was chosen to avoid a possible carry-over effect from previous measurements.

Morphometrics

Tail width (measured at the base of the tail and considered an indicator of fat deposition and, hence, condition and snout-vent length (SVL) (Avery 1974; Bauwens 1985; Donoghue *et al* 1998; Vervust *et al* 2008) were measured using digital calipers (smallest increment = 0.1 mm, Absolute, Digimatic, Mitotoyo, USA) while body mass was recorded with an electronic balance (smallest increment = 0.01 g, Scout pro, Ohaus, USA). Tail-width measurements were corrected for SVL by using the residuals from a linear regression of tail width against SVL in the analysis.

Heterophil to lymphocyte (H/L) ratio

Heterophils and lymphocytes are both types of white blood cell that play a role in the immune system. Heterophils (the equivalent of neuterophils in mammals and amphibians) form part of the innate immune system, while lymphocytes are part of the acquired immune system. High heterophils to lymphocyte ratios in blood samples are considered an indication of high glucocorticoid and stress levels in all vertebrate taxa (for a review, see Davis & Maerz 2008), including reptiles (Saad & El Ridi 1988; Morici *et al* 1997; Lance & Elsey 1999; Case *et al* 2005; Chen *et al* 2007; Borgmans *et al* 2018).

Table 2 Ethogram behavioural observations.

Behaviour	Definition
Sitting	Time spent remaining stationary
Hiding	Time spent remaining stationary while (partially) remaining hidden from sight
Basking	Time spent remaining stationary while being positioned directly under the heat lamp
Climbing	Time spent climbing on any non-horizontal structure, eg walls of the cages, wooden bar, leaves
Walking	Time spent moving on horizontal structures
Foraging	Time spent actively hunting for food items (crickets or mealworms)
Licking	Time spent drinking water
Head movement	Number of times individuals move their head laterally from one stationary position to another
Dewlap extension	Number of times individuals (partially) extend their dewlap (often combined with push-ups and head nods)
Push-up	Number of time individuals perform a push-up with two (or all) of their legs (often combined with head nods and dewlap extensions)
Head nod	Number of times individuals move their head vertically (often combined with push-ups and dewlap extensions)

Blood (max 60 µl) was obtained from the post-orbital sinus by inserting a capillary tube (75 mm; 60 µl) between the eye and the eyelid (MacLean *et al* 1973). The use of post-orbital sinus sampling has been shown to cause acute stress, leading to a number of long-term effects in rodents (Balcombe *et al* 2004). Collecting blood from the post-orbital sinus also induces an acute stress response in lizards and plasma corticosterone concentrations were found to return to baseline levels after 2 h (Langkilde & Shine 2006). Our laboratory has extensive experience using this technique on lizards and no animals suffered long-term negative effects or died from this treatment. Blood smears were made following Walberg (2001). Air-dried smears were fixed in 90% ethanol for 15 min and stained with three-step staining (Hemacolour®, Merck Millipore, Germany). The numbers of heterophils and lymphocytes visible in ten fields (magnification: 40 × 10, field size: 0.2 × 0.2 mm, WILD Heerbrugg M20, Switzerland) were counted and used to calculate H/L ratios.

Faecal corticosterone metabolites (FCM)

The traditional technique of measuring plasma levels of corticosterone (CORT) to assess physiological stress in vertebrates has been criticised because acute rises in CORT-levels, associated with blood sampling, may mask more subtle variation in CORT-levels due to mild, prolonged stress. Instead, faecal corticosterone metabolites (FCM) can be measured with minimal disturbance to the animal and may reflect average stress over longer time-periods (Möstl & Palme 2002; Palme *et al* 2005). Details on the use and validation of FCM can be found in the literature review by Palme (2019). This alternative technique has recently been used in a variety of vertebrates, including reptiles (Rittenhouse *et al* 2005; Kalliokoski *et al* 2012).

Cages were checked three times a day (0900, 1200 and 1500h) for three days and samples collected when available. Faecal pellets were collected from the lizards' home cages using tweezers and stored in small plastic bags before being frozen at –21°C immediately after collection. Tweezers were cleaned with 90% ethanol between consecutive collections to reduce contamination. When an individual had multiple samples within a treatment the data were weighted by number of samples and total faecal weight. When two pellets from the same individual collected on the same day weighed less than 10 mg (Sartorius CPA223S, Sartorius, Germany), they were pooled. A minimum of 10 mg sample is required for accurate steroid measurement (R Palme, personal observation 2014). To extract FCM, 0.5 ml of a 60% methanol solution (60:40, methanol:water) was added to each sample (Palme *et al* 2013). Samples were then mixed for 2 min using a vortex and centrifuged (at 5,000 rpm) for 5 min. An aliquot of 0.1 ml from each mixture was stored at –21°C until analysis. Extracts were analysed using a 5α-pregnane-3ß,11ß,21-triol-20-one enzyme immunoassay (EIA). The results are expressed per g of dry faeces. The results of an earlier validation experiment, reported in Borgmans *et al* (2018), showed this EIA to be most suitable for measuring FCM levels in *A carolinensis*. Details of the EIA, including cross-reactions of the antibody, were described by Touma *et al* (2003).

Behavioural observations

The behaviour of the lizards in their home cage was observed from a 3 m distance using continuous focal animal sampling with observation software (JWatcher v1.0; Blumstein *et al* 2006). All observations were carried out live by the same observer (GB). Since one observer carried out all experiments the observer was not blinded for the analyses of the behaviour. The duration of the following behaviours was noted over a 10-min observation period for each individual (Table 2 and see Appendix [supplementary material to papers published in *Animal Welfare*: https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material]): 'sitting', 'hiding',

'basking', 'walking', 'climbing', 'foraging', 'licking', 'wiping.' In addition, the number of lateral head movements, dewlap extensions, push-ups, and head nods were recorded. All observations were carried out between 0900 and 1700h, when the lizards were fully active (G Borgmans, personal observation 2014). The order of the observations was randomised within this active period.

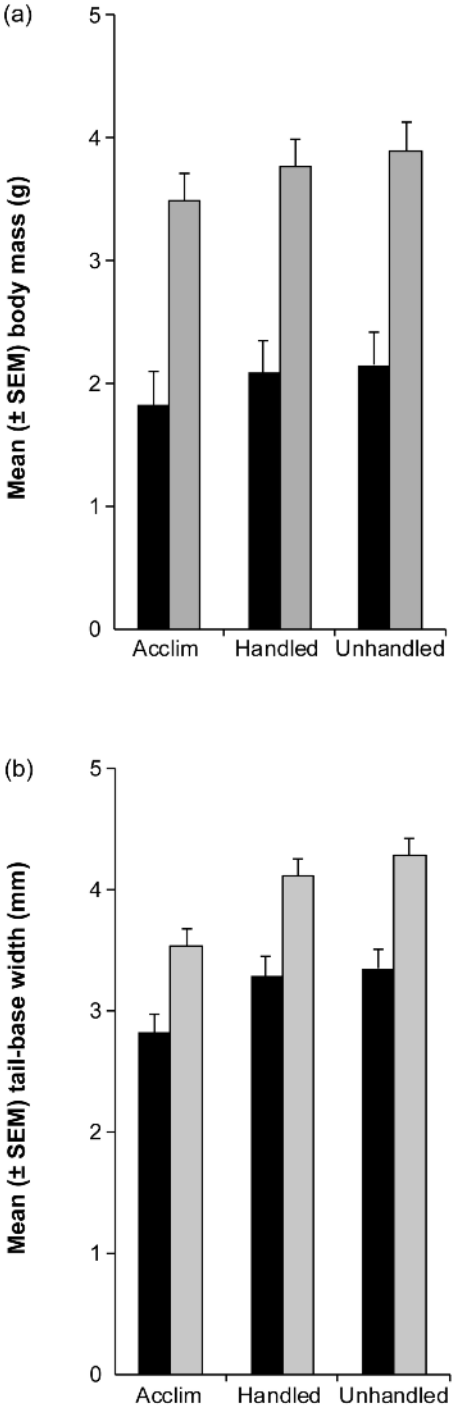
Colour

Spectrophotometry was also initially included as a measure for stress since body colour has been shown to change in response to different stressors in reptiles (Summers & Greenberg 1994). However, our measurements yielded inconclusive results and we remain to be convinced of the effectiveness of using body colour as a tool for measuring chronic stress in *A carolinensis* lizards. Therefore, information on body colour measurements will not be included in this manuscript. The full methods and results of these can be found in the Appendix (https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material).

Statistical analysis

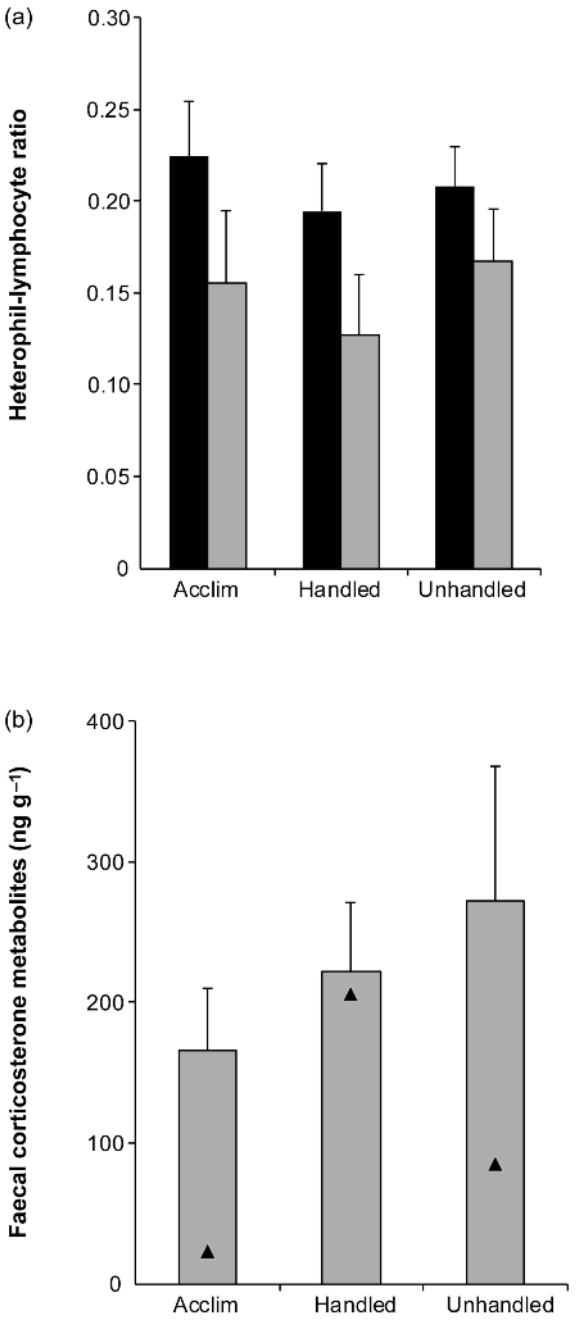
Statistical analyses were carried out using SPSS (IBM SPSS statistics v22). All measured variables were analysed for effects of treatment, sex and for an interaction effect between treatments and sex. When a significant difference was found between sexes, measurements were analysed separately for males and females. Assumption of normality was tested with a Shapiro-Wilk test. H/L ratio and FCM data were $\log_{10}$-transformed to ensure normality. When Mauchly's test of sphericity was violated, a Greenhouse-Geisser correction was applied to the corresponding degrees of freedom. One-way repeated measures ANOVAs (rmANOVA) with treatment as a within-subject factor and order (in which order animals received different treatments as previously explained in *Experimental design*) as a between-subject factor were used to test for differences between the treatments for body mass, the residuals from a linear regression of tail width against SVL (as a corrected value for the tail width measurements, see previously in *Morphometrics*), H/L ratio and FCM level. No significant effect of order or the interaction between order and treatment was found. Therefore, data were lumped to increase sample size. Whenever a repeated measures ANOVA found a statistical difference, a *post hoc* analysis with Bonferroni adjustment was carried out to investigate pair-wise differences. Analysis of H/L ratio and FCM levels had a reduction in sample size. H/L ratio had a sample size of n = 18 for males and n = 11 for females as blood sampling at the end of the 'acclimatisation' period was unsuccessful for one male and three females. FCM levels had a greater reduction in sample size due to the technical limitation of the EIA analysis. Sample size of FCM levels was reduced to n = 8 for males and n = 1 for females. Most of the behavioural variables (Table 1; Appendix [https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material]) did not occur during the observations, the only behaviours to be observed were 'walking', 'climbing', number of head movements and 'yawning.' The last was only observed in < 4% of observations and is not a stress-related behaviour. Therefore, only total time spent moving (combination of 'walking' and

Figure 1



Showing (a) mean (± SEM) body mass (n = 19 for males and n = 14 for females) and (b) mean (± SEM) tail width (n = 19 for males and n = 14 for females) for *Anolis carolinensis* lizards in the 'acclimatisation' (acclim) period, the 'handled' and 'unhandled' treatments. Females (black bars) and males (grey bars).

Figure 2



Showing (a) mean (± SEM) heterophil-lymphocyte ratios (H/L; n = 18 for males and n = 11 for females) and (b) faecal corticosterone metabolite levels (FCM; n = 8 for males and n = 1 for females) in *Anolis carolinensis* lizards during the 'acclimatisation' (acclim) period, and in the 'handled' and 'unhandled' treatments. Females (black bars) and males (grey bars). The one female in the FCM graph (b) is represented by the black triangles.

'climbing') and number of head movements were analysed using generalised linear model (GzLM). Total time spent moving was modelled with a linear distribution and identity as the link function. Number of head movements was modelled with a Poisson distribution and log as the link function.

Results

Morphometrics

Lizards weighed more in both the 'handled' and 'unhandled' treatment periods compared to the initial 'acclimatisation' period (Figure 1[a], rmANOVA, $F_{2,62}$ = 19.14; $P < 0.001$). The difference in body mass was similar for males and females (rmANOVA, sex × treatment interaction effect: $F_{2,62} = 0.703$; $P = 0.499$). Body masses of lizards at the end of the 'handled' and 'unhandled' treatments did not differ significantly (*post hoc* test: $P = 0.27$). Males did have an overall higher body mass (ANOVA, $F_{1,31} = 25.62$; $P < 0.001$).

Tail width (corrected for SVL) exhibited a similar effect of treatment (Figure 1[b], rmANOVA, $F_{2,62}$ = 29.93; $P < 0.001$), with high values in the 'handled' and 'unhandled' treatments compared with the 'acclimatisation' period. The change was similar for males and females (rmANOVA, sex × treatment interaction effect: $F_{2,62} = 1.57$; $P = 0.26$). The difference between the 'handled' and 'unhandled' treatments was not significant ($P = 1$). Overall, males had wider tail bases than females (ANOVA, $F_{1,31} = 4.65$; $P < 0.05$).

Physiology

The H/L ratios did not differ among the treatments (Figure 2[a], rmANOVA, $F_{2,54} = 2.04$; $P = 0.140$). Females tended to have somewhat higher ratios than males, but this difference was not significant (rmANOVA, sex × treatment effect: $F_{2,54} = 0.064$; $P = 0.938$; ANOVA, sex-effect: $F_{1,27} = 2.98$; $P = 0.096$).

There was no effect of treatment on FCM levels (Figure 2[b], rmANOVA, $F_{2,14} = 1.07$; $P = 0.180$).

Behaviour

The total time spent moving (walking and climbing) did not vary between treatments (GzLM, interaction-effect: Wald $\chi^2_2 = 1.89$; $P = 0.389$; Figure 3[a]) nor between sexes (GzLM, sex × treatment interaction effect: Wald $\chi^2_2 = 3.69$; $P = 0.158$, sex-effect: Wald $\chi^2_2 = 1.48$; $P = 0.224$).

Head movements did show a significant difference between treatments (GzLM, treatment effect: Wald $\chi^2_2 = 10.11$; $P < 0.01$), but not between sexes (GzLM, sex × treatment interaction effect: Wald $\chi^2_2 = 1.3$; $P = 0.52$, sex-effect: Wald $\chi^2_2 = 1.17$; $P = 0.279$). Only the 'unhandled' and the 'handled' treatments differed significantly ($P < 0.01$), where males and females had a lower number of head movements in the 'handled' treatment (Figure 3[b]).

Discussion

Our results show no effect of repeated handling on a series of variables that have been connected to stress levels. Individuals of *A carolinensis* that were handled twice a day for three weeks ('handled' treatment) exhibited comparable changes in general condition (as indicated by body mass and tail-base width), skin reflectance, leukocyte profiles (H/L ratios) and levels of FCM as did individuals that were not handled during the three weeks ('unhandled' treatment). So,

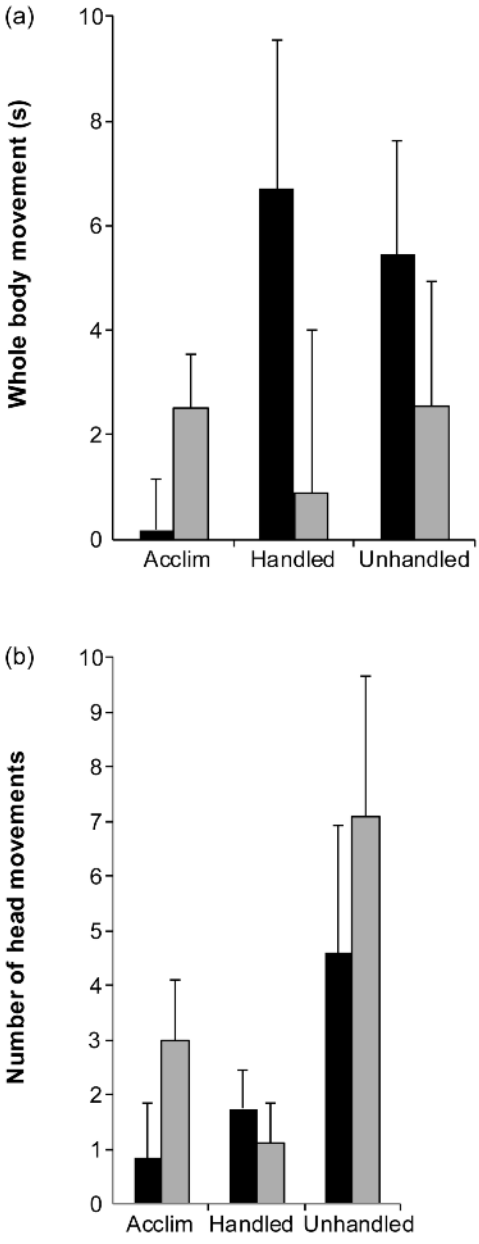
our results suggest that three weeks of repeated handling did not cause an increase in stress in *A carolinensis* lizards. A possible explanation is that the stressor we used was not strong enough to elicit a response. We have to acknowledge that we did not separately test whether our treatment caused an acute stress response. However, most research and basic animal care protocols attempt to minimise handling time. Especially, for example, when using plasma corticosterone as a measurement. A handling time of 1 min seems relevant in the context of basic animal care and scientific experimentation, where animals are required to be caught often (eg transport between enclosures during experiments) or held for specific treatments or measurements. The study by Cabanac and Cabanac (2000) used a 1 min handling period as a stressor and found an increased heart rate as a response, indicating that this treatment elicits an acute response. Furthermore, bouts of tonic immobility were often observed (G Borgmans, personal observation 2014) after animals were handled, which is a long-known response behaviour to an acute stressor in *A carolinensis* lizards (Edson & Gallup 1972; Hennig & Dunlap 1978). Assuming that our handling did cause an acute stress response, the most likely possibility seems to be that the handling treatment did not have any long-term effects. As such, our results cannot be extrapolated to longer handling times or to the fact that some research protocols cause added stress (eg drawing blood) and future research investigating these factors should be carried out.

Another possible explanation for our results is that there was a difference in the stress variables between the 'handled' and 'unhandled' treatments but that it was undetectable using our measurements. However, this seems unlikely, given the fact that differences were found between the 'acclimatisation' period and the 'handled' and 'unhandled' treatments. Both body mass and tail width were found to be lower in the 'acclimatisation' period, indicating more stress. No differences in FCM levels and behaviour were found between the 'acclimatisation' and experimental situations. Although FCM was presumed to be a 'gold standard' to which the other variables could be compared, the results did not show the same variation as was found for the morphometrics (body mass and tail width). This difference might be explained by the fact that due to technical limitations, the sample size of the FCM analysis was greatly reduced. This small sample size could have resulted in inaccurate results for the FCM measurements. This issue is explained in more detail in Borgmans *et al* (2018).

Behavioural observations revealed no difference in time spent moving among the treatments. Head movements did show some differences. Both males and females moved their heads most in the 'unhandled' treatment. Males moved their heads least in the 'handled' treatment while females moved their heads least in the 'acclimatisation' period. The results from the behavioural observations did not show the same variation as for the other variables.

Our results showed that animals had higher stress values for some of the measured variables in the 'acclimatisation' period, which leads us to conclude that animals experienced

Figure 3



Showing (a) mean (± SEM) time spent moving (walking or climbing; n = 19 for males and n = 14 for females) and (b) number of head movements (n = 19 for males and n = 14 for females) exhibited by *Anolis carolinensis* lizards during the 'acclimatisation' (acclim) period, and in the 'handled' and 'unhandled' treatments. Females (black bars) and males (grey bars).

such high stress during transport and/or stay at the commercial supplier that an effect could still be measured at the end of this period. This is all the more remarkable when we take into account that measurements in the 'acclimatisation' period were taken near the end of the three-week stay in our standard set-up, which should be more than adequate to acclimatise to the effect of transport and stay at the supplier. A one-week acclimatisation period is generally considered sufficient to undo the adverse effects of transport and time

spent with the supplier when animals are kept in good health (G Borgmans, personal observation 2014). This was surprising as this period was originally intended to be a baseline with which our experimental situations could be compared, and it was identical to the 'unhandled' treatment.

Even when we take into account that the high levels of stress found in the 'acclimatisation' period are probably an artefact resulting from the situation animals were in prior to our experiments, the fact still remains that no differences were found between the 'unhandled' and 'handled' treatments. These finding are in accordance with those of Kreger and Mench (1993), Cree *et al* (2000) and Langkilde and Shine (2006) who found no effect of handling on stress levels. However, all of the studies finding no effect of handling carried out short-term investigations. The only reptile study looking into longer term effects of handling was by Moore *et al* (1991). This study investigated the effect of individual housing on ornate tree lizards (*Urosaurus ornatus*) over a three-week period and found an increase in plasma corticosterone. This result, however, is difficult to compare with our results since the stressor used is less relevant for *A carolinensis* as males are almost always housed individually due to their territoriality. Overall, our results suggest that *A carolinensis* do not experience a negative long-term effect of handling of the magnitude used in this study. This result is valuable not only for animals used in scientific research, but also for those kept at a commercial supplier or as a pet.

Animal welfare implications

The major findings of this study are that routine handling, in the form that it was performed in this study, does not have an effect on stress in *A carolinensis*. This, however, does not mean that other factors do not play a role in improving a captive situation or in avoiding an increase in stress. Different factors (for example, providing a thermal gradient, appropriate humidity, appropriate social grouping or providing live prey) probably play an important role in the welfare of captive lizards.

Conclusion

Our results suggest more stress in the 'acclimatisation' period, probably an artefact from the situation in which animals were maintained prior to the onset of experiments. When this is taken into account, our results seem to suggest that there is no effect of being held, twice daily, for 1 min at a time over a three-week period on *A carolinensis*.

Acknowledgements

The authors would like to thank R Van Damme, J Stevens, Z Pereboom, A Sannen and H Vervaecke for creating the REPTAM project of which this paper is a part. Further thanks go to J Scholliers and J Mertens for their assistance in the practical work. The research that yielded these results, was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment through the contract [RT 13/2 REPTAM 1].

References

Avery R 1974 Storage lipids in the lizard *Lacerta vivipara*: a quantitative study. *Journal of Zoology* 173: 419-425. https://doi.org/10.1111/j.1469-7998.1974.tb04124.x

Balcombe JP, Barnard ND and Sandusky C 2004 Laboratory routines cause animal stress. *Journal of the American Association for Laboratory Animal Science 43*(6): 42-51

Bauwens D 1985 *Demografische kenmerken en aantalsdynamiek in een populatie van de levendbarende hagedis (*Lacerta vivipara*)*. Unpublished doctoral dissertation, Universitaire Instelling Antwerp, Antwerp, Belgium. [Title translation: Demographical characteristics and population dynamics in a population of viviparous lizards (*Lacerta vivipara*)]

Blumstein D, Evans C and Daniel J 2006 *JWatcher 1.0*. http://www.jwatcher.ucla.edu

Borgmans G, Palme R, Sannen A, Vervaecke H and Van Damme R 2018 The effect of environmental provisioning on stress levels in captive green anole (*Anolis carolinensis*). *Animal Welfare 27*: 35-46. https://doi.org/10.7120/09627286.27.1.035

Borgmans G, Palme R, Sannen A, Vervaecke H and Van Damme R 2019 The effect of cage size on stress levels in captive green anole (*Anolis carolinensis*). *Animal Welfare 28*: 455-464. https://doi.org/10.7120/09627286.28.4.455

Cabanac A and Cabanac M 2000 Heart rate response to gentle handling of frog and lizard. *Behavioural Processes 52(2)*: 89-95. https://doi.org/10.1016/S0376-6357(00)00108-X

Case BC, Lewbart GA and Doerr PD 2005 The physiological and behavioural impacts of and preference for an enriched environment in the eastern box turtle (*Terrapene carolina carolina*). *Applied Animal Behaviour Science 92(4)*: 353-365. https://doi.org/10.1016/j.applanim.2004.11.011

Cash WB, Holberton RL and Knight SS 1997 Corticosterone secretion in response to capture and handling in free-living red-eared slider turtles. *General and Comparative Endocrinology, 108*: 427-433. https://doi.org/10.1006/gcen.1997.6999

Chen X, Niu C and Pu L 2007 Effects of stocking density on growth and non-specific immune responses in juvenile soft-shelled turtle (*Pelodiscus sinensis*). *Aquaculture Research 38*: 1380-1386. https://doi.org/10.1111/j.1365-2109.2007.01813.x

Cree A, Amey AP and Whittier JM 2000 Lack of consistent hormonal responses to capture during the breeding season of the bearded dragon (*Pogona barbata*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 126*: 275-285. https://doi.org/10.1016/S1095-6433(00)00210-5

Cree A, Tyrrell CL, Preest MR, Thorburn D and Guillette LJ 2003 Protecting embryos from stress: corticosterone effects and the corticosterone response to capture and confinement during pregnancy in a live-bearing lizard (*Hoplodactylus maculatus*). *General and Comparative Endocrinology 134*: 316-329. https://doi.org/10.1016/S0016-6480(03)00282-X

Cuthill IC, Bennett ATD, Partridge JC and Maier EJ 1999 Plumage reflectance and the objective assessment of avian sexual dichromatism. *American Naturalist 153*: 183-200. https://doi.org/10.1086/303160

Davis AK and Maerz JC 2008 Sex-related differences in hematological stress indices of breeding paedomorphic mole salamanders. *Journal of Herpetology 42*: 197-201. https://doi.org/10.1670/06-277R2.1

Donoghue S, Vidal J and Kronfeld D 1998 Growth and morphometrics of green iguanas (*Iguana iguana*) fed four levels of dietary protein. *The Journal of Nutrition 128*: 2587-2589. https://doi.org/10.1093/jn/128.12.2587S

Edson PH and Gallup GG 1972 Tonic immobility as a fear response in lizards *Anolis carolinensis*. *Psychonomic Science 26*: 27-28. https://doi.org/10.3758/BF03337874

Endler JA 1990 On the measurement and classification of colour in studies of animal colour patterns. *Biological Journal of the Linnean Society 41*: 315-352. https://doi.org/10.1111/j.1095-8312.1990.tb00839.x

Farbridge K and Leatherland J 1992 Plasma growth hormone levels in fed and fasted rainbow trout (*Oncorhynchus mykiss*) are decreased following handling stress. *Fish Physiology and Biochemistry 10*: 67-73. https://doi.org/10.1007/BF00004655

Foo J and Lam T 1993 Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in the tilapia, *Oreochromis mossambicus*. *Aquaculture 115*: 145-158. https://doi.org/10.1016/0044-8486(93)90365-6

Garner JP 2005 Stereotypies and other abnormal repetitive behaviors: potential impact on validity, reliability, and replicability of scientific outcomes. *ILAR Journal 46*: 106-117. https://doi.org/10.1093/ilar.46.2.106

Gilman CA and Irschick DJ 2013 Foils of flexion: the effects of perch compliance on lizard locomotion and perch choice in the wild. *Functional Ecology 27*: 374-381. https://doi.org/10.1111/1365-2435.12063

Girling JE and Cree A 1995 Plasma corticosterone levels are not significantly related to reproductive stage in female common geckons (*Hoplodactylus maculatus*). *General and Comparative Endocrinology 100*: 273-281. https://doi.org/10.1006/gcen.1995.1158

Gregory LF and Schmid JR 2001 Stress responses and sexing of wild Kemp's ridley sea turtles (*Lepidochelys kempii*) in the northeastern Gulf of Mexico. *General and Comparative Endocrinology 124*: 66-74. https://doi.org/10.1006/gcen.2001.7683

Griggio M, Serra L, Licheri D, Campomori C and Pilastro A 2009 Moult speed affects structural feather ornaments in the blue tit. *Journal of Evolutionary Biology 22*: 782-792. https://doi.org/10.1111/j.1420-9101.2009.01700.x

Hennig CW and Dunlap WP 1978 Tonic immobility in *Anolis carolinensis*: Effects of time and conditions of captivity. *Behavioral Biology 23*: 75-86. https://doi.org/10.1016/S0091-6773(78)91180-X

Hoffmayer ER and Parsons GR 2001 The physiological response to capture and handling stress in the Atlantic sharpnose shark, *Rhizoprionodon terraenovae*. *Fish Physiology and Biochemistry 25*: 277-285

Kalliokoski O, Timm JA, Ibsen IB, Hau J, Frederiksen A-MB and Bertelsen MF 2012 Fecal glucocorticoid response to environmental stressors in green iguanas (*Iguana iguana*). *General and Comparative Endocrinology 177*: 93-97. https://doi.org/10.1016/j.ygcen.2012.02.017

Kreger MD and Mench JA 1993 Physiological and behavioral effects of handling and restraint in the ball python (*Python regius*) and the blue-tongued skink (*Tiliqua scincoides*). *Applied Animal Behaviour Science 38*: 323-336. https://doi.org/10.1016/0168-1591(93)90030-S

Lailvaux S and Irschick D 2007 Effects of temperature and sex on jump performance and biomechanics in the lizard *Anolis carolinensis*. *Functional Ecology 21*: 534-543. https://doi.org/10.1111/j.1365-2435.2007.01263.x

Lance VA and Elsey RM 1999 Plasma catecholamines and plasma corticosterone following restraint stress in juvenile alligators. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 283*: 559-565. https://doi.org/10.1002/(SICI)1097-010X(19990501)283:6<559::AID-JEZ7>3.0.CO;2-4

Lance VA and Lauren D 1984 Circadian variation in plasma corticosterone in the American alligator, *Alligator mississippiensis*, and the effects of ACTH injections. *General and Comparative Endocrinology 54*: 1-7. https://doi.org/10.1016/0016-6480(84)90192-8

Langkilde T and Shine R 2006 How much stress do researchers inflict on their study animals? A case study using a scincid lizard, *Eulamprus heatwolei*. *Journal of Experimental Biology 209*: 1035-1043. https://doi.org/10.1242/jeb.02112

Licht P 1968 Response of the thermal preferendum and heat resistance to thermal acclimation under different photoperiods in the lizard *Anolis carolinensis*. *American Midland Naturalist 79*: 149-158. https://doi.org/10.2307/2423160

MacLean GS, Lee AK and Wilson KJ 1973 A simple method of obtaining blood from lizards. *Copeia 1973*: 338-339. https://doi.org/10.2307/1442973

Mathies T, Felix TA and Lance VA 2001 Effects of trapping and subsequent short-term confinement stress on plasma corticosterone in the brown treesnake (*Boiga irregularis*) on Guam. *General and Comparative Endocrinology 124*: 106-114. https://doi.org/10.1006/gcen.2001.7694

Merchant M, Fleury L, Rutherford R and Paulissen M 2008 Effects of bacterial lipopolysaccharide on thermoregulation in green anole lizards (*Anolis carolinensis*). *Veterinary Immunology and Immunopathology 125*: 176-181. https://doi.org/10.1016/j.vetimm.2008.04.014

Montgomerie R 2006 Analyzing colors. In: Hill GE and McGraw KJ (eds) *Bird Coloration* pp 90-147 Harvard University Press: Cambridge, MA, USA

Montuelle SJ, Daghfous G and Bels VL 2008 Effect of locomotor approach on feeding kinematics in the green anole (*Anolis carolinensis*). *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 309*: 563-567. https://doi.org/ 10.1002/jez.484

Moore IT, Lemaster MP and Mason RT 2000 Behavioural and hormonal responses to capture stress in the male red-sided garter snake, *Thamnophis sirtalis parietalis*. *Animal Behaviour 59*: 529-534. https://doi.org/10.1006/anbe.1999.1344

Moore MC, Thompson CW and Marler CA 1991 Reciprocal changes in corticosterone and testosterone levels following acute and chronic handling stress in the tree lizard, *Urosaurus ornatus*. *General and Comparative Endocrinology 81*: 217-226. https://doi.org/10.1016/0016-6480(91)90006-R

Morgan KN and Tromborg CT 2007 Sources of stress in captivity. *Applied Animal Behaviour Science 102*: 262-302. https://doi.org/10.1016/j.applanim.2006.05.032

Morici LA, Elsey RM and Lance VA 1997 Effects of long-term corticosterone implants on growth and immune function in juvenile alligators, *Alligator mississippiensis*. *The Journal of Experimental Zoology 279*: 156-162. https://doi.org/10.1002/(SICI)1097-010X(19971001)279:2<156::AID-JEZ6>3.0.CO;2-N

Möstl E and Palme R 2002 Hormones as indicators of stress. *Domestic Animal Endocrinology 23*: 67-74. https://doi.org/10.1016/S0739-7240(02)00146-7

Palme R 2019 Non-invasive measurement of glucocorticoids: advances and problems. *Physiology and Behaviour 199*: 229-243. https://doi.org/10.1016/j.physbeh.2018.11.021

Palme R, Rettenbacher S, Touma C, El-Bahr S and Möstl E 2005 Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. *Annals of the New York Academy of Sciences 1040*: 162-171. https://doi.org/10.1196/annals.1327.021

Palme R, Touma C, Arias N, Dominchin M and Lepschy M 2013 Steroid extraction: get the best out of faecal samples. *Wiener Tierarztliche Monatsschrift 100*: 238-246

Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML and Schreck CB 2009 Whole-body cortisol response of zebrafish to acute net handling stress. *Aquaculture 297*: 157-162. https://doi.org/10.1016/j.aquaculture.2009.08.035

Rittenhouse CD, Millspaugh JJ, Washburn BE and Hubbard MW 2005 Effects of radiotransmitters on fecal glucocorticoid metabolite levels of three-toed box turtles in captivity. *Wildlife Society Bulletin 33(2)*: 706-713. https://doi.org/10.2193/0091-7648(2005)33[706:EORFGM]2.0.CO;2

Romero LM and Wikelski M 2002 Exposure to tourism reduces stress-induced corticosterone levels in Galápagos marine iguanas. *Biological Conservation 108*: 371-374. http://dx.doi.org/10.1016/S0006-3207(02)00128-3

Saad AH and El Ridi R 1988 Endogenous corticosteroids mediate seasonal cyclic changes in immunity of lizards. *Immunobiology 177*: 390-403. https://doi.org/10.1016/S0171-2985(88)80007-X

Schuett GW, Taylor EN, van Kirk EA and Murdoch WJ 2004 Handling stress and plasma corticosterone levels in captive male western diamond-backed rattlesnakes (*Crotalus atrox*). *Herpetological Review 35*: 229-232

Stellar JE and White DJ 2010 Social influences on female choice in green anole lizards (*Anolis carolinensis*). *Behavioral Processes 83*: 282-286. https://doi.org/10.1016/j.beproc.2010.01.005

Summers CH and Greenberg N 1994 Somatic correlates of adrenergic activity during aggression in the lizard, *Anolis carolinensis. Hormones and Behavior 28*: 29-40. https://doi.org/10.1006/hbeh.1994.1003

Touma C, Sachser N, Möstl E and Palme R 2003 Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. *General and Comparative Endocrinology 130*: 267-278. https://doi.org/10.1016/S0016-6480(02)00620-2

Tyrrell C and Cree A 1998 Relationships between corticosterone concentration and season, time of day and confinement in a wild reptile (tuatara, *Sphenodon punctatus*). *General and Comparative Endocrinology 110*: 97-108. https://doi.org/10.1006/gcen.1997.7051

Vervust B, Lailvaux SP, Grbac I and Van Damme R 2008 Do morphological condition indices predict locomotor performance in the lizard *Podarcis sicula*? *Acta Oecologica 34*: 244-251. https://doi.org/10.1016/j.actao.2008.05.012

Walberg J 2001 White blood cell counting techniques in birds. *Seminars in Avian and Exotic Pet Medicine 10*: 72-76. https://doi.org/10.1053/saep.2001.22051

Waters RP, Emerson AJ, Watt MJ, Forster GL, Swallow JG and Summers CH 2005 Stress induces rapid changes in central catecholaminergic activity in *Anolis carolinensis*: restraint and forced physical activity. *Brain Research Bulletin 67(3)*: 210-218. https://doi.org/10.1016/j.brainresbull.2005.06.029

Woodley SK and Moore MC 2002 Plasma corticosterone response to an acute stressor varies according to reproductive condition in female tree lizards (*Urosaurus ornatus*). *General and Comparative Endocrinology 128(2)*: 143-148. https://doi.org/10.1016/S0016-6480(02)00068-0