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

Animal Welfare 2019, 28: 455-464
ISSN 0962-7286
doi: 10.7120/09627286.28.4.455

The effect of cage size on stress levels 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

*Reptiles are often used as model species in scientific research and are popular in the pet trade, yet how they cope with captive conditions has not been well studied. Stress caused by captivity could affect the endocrinology, physiology and behaviour of animals, resulting in a general decrease in welfare and could confound the results of scientific experiments. One of the factors that could influence stress in a captive environment is the size of the cage. However, the effect of cage size on stress has rarely been investigated in reptiles. In this study, the effect of cage size on the behaviour, morphology and physiology of the green anole (*Anolis carolinensis*) was quantified. We were unable to find an effect of cage dimensions (range 0.05 to 0.2 m^3) on body mass, tail-base width, heterophil to lymphocyte ratios (H/L ratios), behaviour and faecal corticosterone metabolite (FCM) levels.*

Keywords: *animal welfare, cage size, captivity, green anole, reptiles, stress*

Introduction

Reptiles are popular in the pet trade and as model species in laboratory studies (eg Waters *et al* 2005; Lailvaux & Irschick 2007; Merchant 2008; Montuelle 2008; Stellar & White 2010). In the period between 1975 and 2014, 152 million reptiles were traded worldwide, compared to 79.8 million invertebrates, 24.1 million birds, 13 million mammals and 12.8 million fish (Harfoot *et al* 2018). In many animals, captivity is known to induce stress, especially when aspects of the captive housing conditions depart from the natural habitat (as they almost inevitably do: Morgan & Tromborg 2007). This stress is reflected in the endocrinology, physiology and behaviour of the subjects and may lead to a general decrease in welfare (Morgan & Tromberg 2007). It may also confound the results of scientific experiments or observations (Garner 2005).

Given the variation in natural history among species, the stress of limited locomotion due to enclosure size is expected to have varying degrees of impact (Clubb & Mason 2007). Generally, it is assumed that a small cage size would have a negative effect on animals given the disparity between the size of the cage in captivity and their natural home range. An argument that is often used in this context, is that a small cage size would not allow an animal to perform its full range of natural behaviours.

The effect of differences in enclosure size has received considerable attention in mammals (Hite *et al* 1977; Horton *et al* 1991; Pearce & Patterson 1993; Saito *et al* 1996; Kaufman *et al* 2004; Whitaker *et al* 2007), birds (Adams & Jackson 1970; Sefton 1976; Nicol 1987; Buchwalder & Huber-Eicher 2004; Jalal *et al* 2006) and fish (Kilambi *et al* 1977; Teng & Chua 1978; McGinty 1991; Rowland *et al* 2006), and while many aspects of the effect of captivity on reptiles (for a review, see Burghardt 2013; Michaels & Campbell-Palmer 2014) have been investigated, the consequences of changes in enclosure size on reptiles is rarely studied. Research on loggerhead turtles (*Caretta caretta*: Gregory *et al* 1996) and tuataras (*Sphenodon punctatus*: Tyrrell & Cree 1998) showed that also in reptiles, enforced confinement can lead to an acute stress response and the study by Wheler and Fa (1995) is a rare example investigating the effect of enclosure size in reptiles. They found that in Round Island geckos (*Phelsuma guentheri*) enclosure size does not appear to influence activity cycles, but large enclosures may encourage greater use of available space.

A problem that arises when conducting stress research in reptiles is that previous studies used a wide variety of ways to gauge captive stress, including: behavioural observations; resting and stereotypic behaviour (Therrien *et al* 2007); social behaviour (Phillips *et al* 2011); heterophil to lympho-

cyte ratio (Case *et al* 2005); level of faecal corticosterone metabolites (Case *et al* 2005; Kalliokoski *et al* 2012); survival (Rosier & Langkilde 2011a); body mass (Case *et al* 2005); to duration of tonic immobility (Hennig & Dunlap 1978). Because most studies have focused on one or two of these indicators, comparing results among studies, and different methodologies is difficult. Therefore, in the current study, we use an approach combining multiple variables.

In this study, we quantify the impact of cage size on the physiology and behaviour of the green anole (*Anolis carolinensis*), a small lizard of the Polychrotidae family. It is most commonly found in the south-eastern United States and on some of the Caribbean islands. The males are territorial and have a home range of 32–69 m^2 (32–44 m^2: Gordon 1956; 65.9 m^2: Schoener & Schoener 1982; 69 m^2: Jenssen & Nunez 1998), while females have a home range of 8 m^2 (Jenssen & Nunez 1998). 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 studies, physiology, and morphology (eg Waters *et al* 2005; Merchant *et al* 2008; Montuelle *et al* 2008; Stellar & White 2010). Despite its popularity in the pet trade and in research, guidelines on housing conditions for *A. carolinensis* vary considerably (see Table S1 in the supplementary material to papers published in *Animal Welfare*: https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material), the arbitrariness probably reflecting a lack of objective information.

When looking into online care-sheets (specifically on *A. carolinensis*), 26 were found (Table S1; https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material), out of this non-exhaustive list of 26, 15 suggest a 10-gallon tank (0.038 m^3) as the minimal cage size for one individual. Two recommended smaller dimensions (0.019 m^3), five recommended larger dimensions (range: 0.057–0.22 m^3) and four did not suggest any particular cage size. Home cage dimensions used in scientific studies on *A. carolinensis* varied from 0.019 to 0.11 m^3 (eg Plavicki *et al* 2004; Waters *et al* 2005; Irschick *et al* 2006; Merchant *et al* 2008; Montuelle *et al* 2008; Stellar & White 2010). None of the care sheets or research studies provide any scientific references indicating that the cage size used would be appropriate for housing *A. carolinensis* lizards. Even the review study on the use of *A. carolinensis* as a model for laboratory studies by Lovern *et al* (2004) does not provide references for the suggested cage size. Government guidelines on the housing of pet or laboratory animals from ten countries (Australia, Austria, Canada, Germany, India, New Zealand, Norway, Switzerland, UK, USA) contained no recommendations on reptile housing dimensions. This variety in suggested sizes and lack of legislation on housing indicates that fundamental research looking solely at the effect of cage size on stress levels is needed.

The dimensions of cages recommended and actually used to house *A. carolinensis* in captivity are obviously only a fraction of their natural home range. We used an integrative approach by combining different behavioural and physiological measurements to obtain a broader view of the response to variation in cage sizes. We hypothesised that a small cage size would lead to a chronic increase in stress and concordant changes in behavioural and physiological indices, in addition we hypothesised that a decrease in stress would be found for a large cage size.

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-four adult *A. carolinensis* lizards (21 males, 13 females) were originally obtained from a licensed commercial supplier in Belgium. During the experiments, two males and one female died of natural causes, resulting in a sample size of 31 (19 males, 12 females). The animals had been caught in the field in Florida, USA, less than one week before being sent by air to Belgium. In the laboratory, lizards were placed into individual glass terraria (40 × 30 × 70 cm; length × width × height; 0.08 m^3). Full spectrum halogen reflector light bulbs with a 30° light arc (40 W) were provided and placed in the roof of the cages. The lamps were switched on during daytime (0600–2000h), providing a shallow thermogradient (air temperatures between 20 and 30°C) within the cages. The maximum temperature of 30°C falls within the range of mean preferred temperature (MPT) of *A. carolinensis* (Licht 1968) and corresponds to mean body temperatures found in the field (Lailvaux & Irschick 2007). At night, ambient temperature was never below 20°C. Relative humidity was monitored with a hygrometer (TH50 hygrometer, Hama, Germany) and kept constant at around 60% by misting the terraria when necessary. The walls of adjacent cages were lined with white paper to preclude visual contact between individual lizards. The bottom of the cages was covered with white paper towels to facilitate the detection and collection of faecal pellets. Each cage contained a diagonally placed wooden perch of 40 × 2 cm (length × diameter) (2 cm being the preferred perch diameter for *A. carolinensis*: Gilman & Irshick 2013) and two banana leaves (on average 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*). Also, 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 three weeks after their arrival in 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, submitted). At the end of this period, measurements were carried out as described below with animals randomly assigned to one of two treatments. As this experiment was part of a larger set

of experiments, the 'acclimatisation' period was not directly followed by the two experimental treatments. There was a gap of approximately 12 weeks between the 'acclimatisation' period and the experimental treatments. This 12-week period consisted of two other experiments, one investigating the effect of handling frequency (Borgmans *et al* submitted) and one investigating the effect of environmental provisioning (Borgmans *et al* 2018). Individuals allocated to the 'small cage first' group (eight males, seven females) were kept in glass cages of 30 × 30 × 50 cm (0.05 m^3) for three weeks and subsequently moved to the 'large' glass cages (50 × 40 × 100 cm; 0.2 m^3) for a further three weeks. Individuals of the 'large cage first' condition (eleven males, five females) received a reversed order of treatment. This crossover design allowed us to control for an effect of order of treatments. The level of enrichment (length of the diagonal perches and size of the leaves) in the experimental cages was scaled to match the cage dimensions (80 cm perches and 40 × 20 cm leaves for the large cages; 20 cm perches and 10 × 5 cm leaves for the small cages). All terraria were cleaned between treatments to prevent an effect of previous occupancy.

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 considered the first day of a three-week period, then faecal samples were collected on days 14–16 and behavioural observations carried out on day 19. Blood samples were collected on day 20 and morphological measurements were taken on day 21.

Morphometrics

Snout-vent length (SVL) and tail width (at the base of the tail, which is considered to be an indicator of fat deposition and hence condition: Bauwens 1985) were measured using digital calipers (smallest increment = 0.1 mm, Absolute, Digimatic, Mitotoyo, USA) and body mass using 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 two types of white blood cells that play a role in the reptile immune system. Heterophils (neutrophils in mammals and amphibians) are part of the innate immune system, while lymphocytes are part of the acquired immune system. High ratios of heterophils to lymphocytes in blood samples are considered an indication of high glucocorticoid and stress values in all vertebrate taxa (for a review, see Davis & Maerz 2008), including reptiles (Saad & Elridi 1988; Morici *et al* 1997; Lance & Elsey 1999; Case *et al* 2005; Chen *et al* 2007).

Blood samples (max 60 µl) were obtained from the post-orbital sinus by inserting a capillary tube (length: 75 mm, maximum volume: 60 µl) between the eye and the eyelid (MacLean *et al* 1973). Animals were held by hand to immobilise them to facilitate drawing blood. 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 Hemacolor® (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.

Behavioural observations

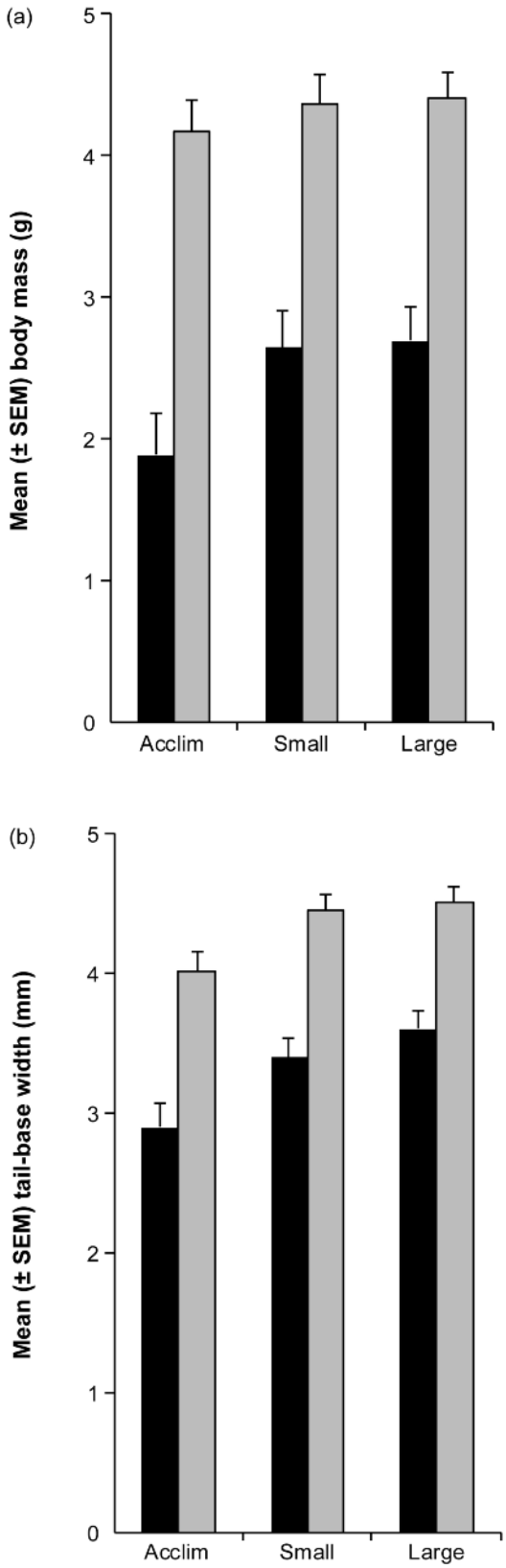
The behaviour of the lizards in their home cage was observed, from a distance of 3 m in a darkened room, using continuous focal animal sampling with observation software (JWatcher v1.0: Blumstein *et al* 2006). Observation via camera was not possible since none were available. All observations were performed live by the same observer (GB). The duration of the following behaviours was noted over 10-min observation periods (see Table S2, in the supplementary material to papers published in *Animal Welfare*: https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material): 'sitting', 'hiding', 'basking', 'walking', 'climbing', 'foraging' and 'licking'. In addition, the number of lateral head movements, dewlap extensions, push-ups, head nods and yawning 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.

Faecal corticosterone metabolites (FCM)

The traditional technique of measuring plasma levels of corticosterone to assess physiological stress in vertebrates has been criticised because acute rises in corticosterone, associated with blood sampling, may mask more subtle variation due to mild, prolonged stress. Instead, faecal corticosterone metabolites (FCM) can be measured with minimal disturbance to the animal and may reflect average levels of stress over longer time-periods (Möstl & Palme 2002; Palme *et al* 2005). This alternative technique has recently been used in a large variety of vertebrates, including reptiles (Rittenhouse *et al* 2005; Kalliokoski *et al* 2012), details on the use and validation of FCM can be found in the literature review by Palme *et al* (2005), Keay *et al* (2006) and Palme (2019)

Cages were checked three times daily (0900, 1200 and 1500h) for three days and all faecal pellets found were collected using tweezers. The pellets were stored in small plastic bags and frozen at –21°C immediately following collection. Tweezers were cleaned with 90% ethanol between consecutive collections to avoid contamination and faecal data weighted per mg of faeces. When pellets weighed less than 10 mg (Sartorius CPA223S, 0.001 g,

Figure 1



Showing (a) body mass (n = 19 for males; n = 12 for females) and (b) tail width (n = 19 for males; n = 12 for females) for *Anolis carolinensis* lizards in the 'acclimatisation' period, the small and the large cage treatments. Indicated are means and standard errors for females (black bars) and males (grey bars)

Sartorius, Germany), they were pooled with samples of the same individual of the same day within the same treatment. A minimum 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 2,500 g) 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 of a previously carried out validation experiment, which were reported in Borgmans *et al* (2018), showed that this EIA was most suitable for measuring FCM levels in *A. carolinensis*. Details of the EIA, including cross-reactions of the antibody can be found in Touma *et al* (2003).

Colour

Spectrophotometry was initially also included as a measure for stress as 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 are not convinced that body colour is valid as a tool for measuring chronic stress in *A. carolinensis* lizards. Therefore, information on the body colour measurements will not be included in this manuscript. The full methods and results of the body colour measurements (including Table S3 showing the component matrix of the colour PCA analysis and Figure S1 showing the average reflection of the skin of the head, the flanks and the dewlap for males and females in the different treatments) can be found in the supplementary material to papers published in *Animal Welfare*: https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material.

Statistical analysis

Statistical analyses were carried out with SPSS (IBM SPSS statistics v22). All measured variables were analysed for effects of treatment, sex and for an interaction between treatments and sex. The 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 with situation as a within-subject factor and order (in which order animals received different treatments as explained in *Experimental design*) as a between-subject factor were used to test for differences between the treatments for body mass, tail width, H/L ratio and FCM level. No significant effect of order or the interaction between order and situation 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. Some of the behavioural variables (Table S2: 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, sitting, hiding, basking and head

movements. The combination of all the 'passive' sitting behaviours (sitting, hiding and basking) can be considered the inverse of the more 'active' state behaviours (walking and climbing). These 'passive' behaviours would yield similar results as the 'active' behaviours when analysed. Therefore, only total time spent moving (combination of walking and 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 in both the 'small cage' and 'large cage' treatments had a significantly higher body mass compared to the 'acclimatisation' period (Figure 1[a], treatment effect: $F_{1.313,38.08} = 13.95$; $P < 0.001$). This difference was slightly more pronounced in females (sex × treatment-effect: $F_{1.313,38.08} = 4.40$; $P < 0.05$), and males had an overall higher body mass (sex-effect: $F_{1,29} = 37.59$; $P < 0.001$). However, cage size ('small cage' versus 'large cage' treatments) did not affect body mass change ($P = 1.0$).

Tail width (corrected for SVL) exhibited a similar effect of treatment (Figure 1[b], $F_{2,58} = 13.46$; $P < 0.001$), with high values in the small and large cage situations compared to the 'acclimatisation' period. The difference was similar for males and females (treatment × sex effect: $F_{2,58} = 1.27$; $P = 0.29$) and there was no difference in tail width between males and females (sex-effect: $F_{1,29} = 0.001$; $P = 0.98$). The difference in tail width between animals in the 'small cage' and 'large cage' treatments was not significant ($P = 0.12$).

Physiological traits

The H/L ratios were high in the initial 'acclimatisation' period and equally low in the other two treatments (Figure 2[a], treatment effect: $F_{2,50} = 20.89$; $P < 0.001$). The same pattern was observed in males and females (sex-effect: $F_{1,25} = 0.84$; $P = 0.37$, sex × treatment effect: $F_{2,50} = 0.60$; $P = 0.55$). Lizards in the 'small cage' and 'large cage' treatments exhibited highly similar ratios ($P = 1.0$).

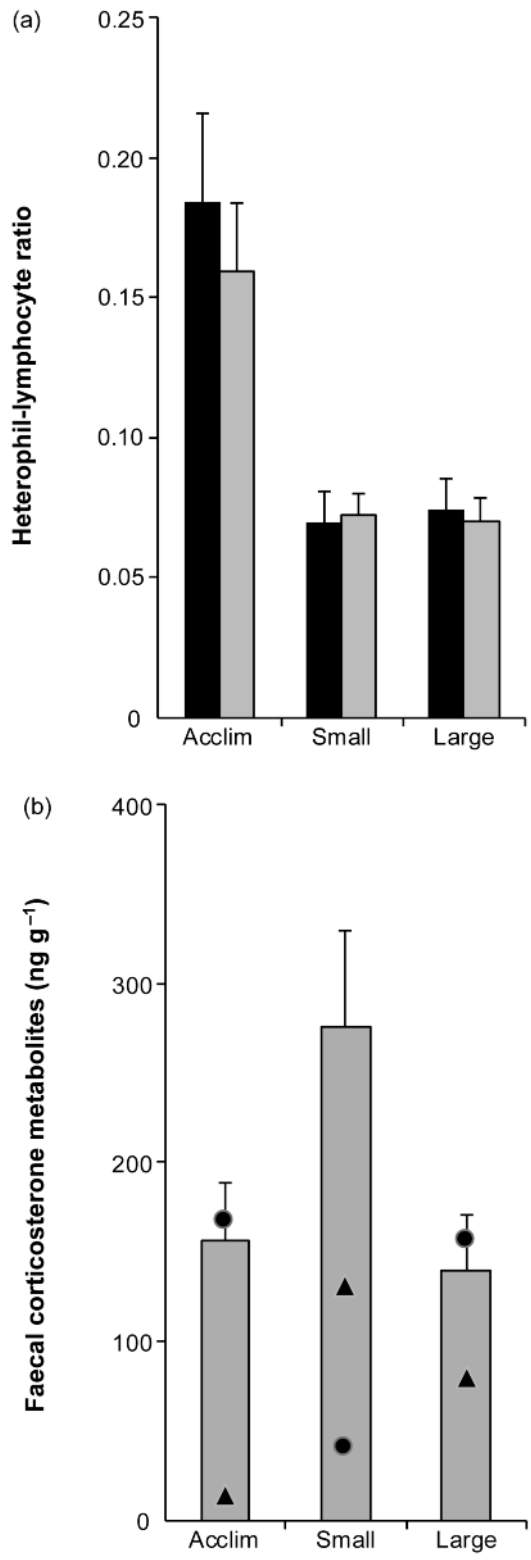
Male FCM levels did not differ among treatments (Figure 3[b], $F_{2,12} = 1,65$; $P = 0.23$).

Behavioural traits

The total time spent moving (walking and climbing) did not differ among treatments (GzLM, treatment-effect: Wald $\chi^2_2 = 0.15$; $P = 0.93$; Figure 3[a] nor between sexes (treatment × sex interaction effect: Wald $\chi^2_2 = 4.01$; $P = 0.13$, sex-effect: Wald $\chi^2_2 = 0.07$; $P = 0.79$).

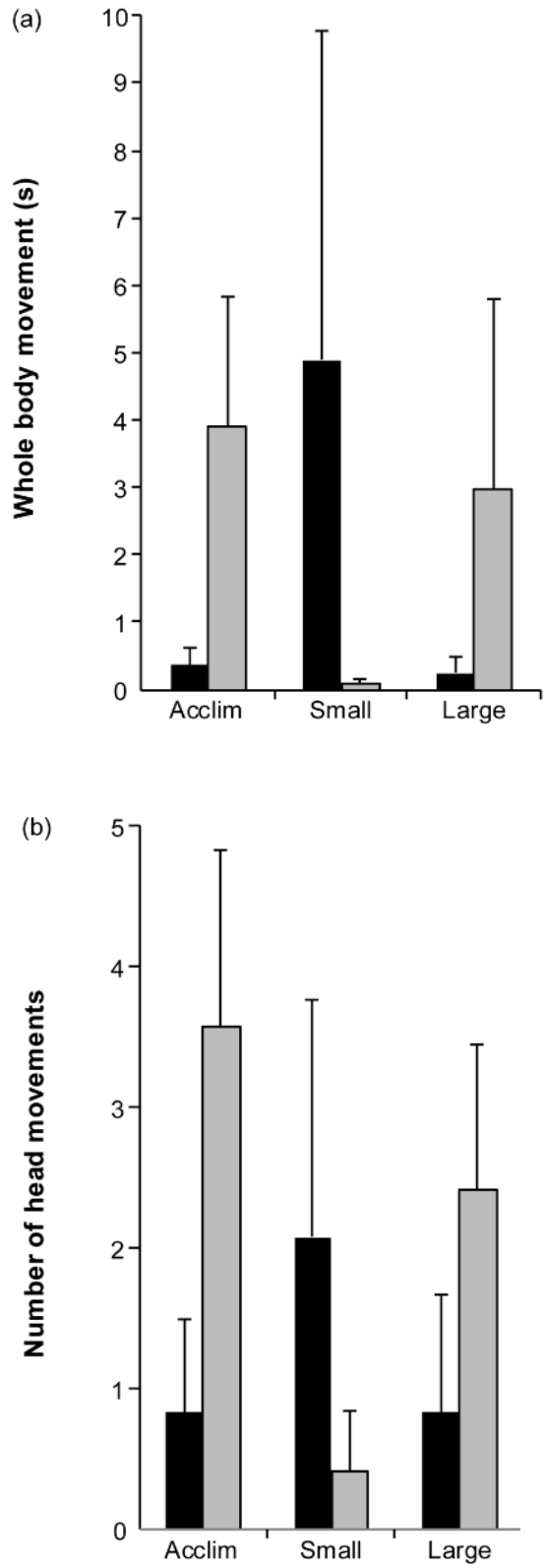
Head movements followed a similar pattern. There was no significant difference in number of head movements among treatments (GzLM, treatment-effect: Wald $\chi^2_2 = 0.857$; $P = 0.65$; Figure 3[b]) nor between sexes (treatment × sex interaction effect: Wald $\chi^2_2 = 4.84$; $P = 0.09$, sex-effect: Wald $\chi^2_2 = 1.1$; $P = 0.29$).

Figure 2



Showing (a) heterophil-lymphocyte ratios (H/L, n = 17 for males; n = 10 for females) and (b) faecal corticosterone metabolite levels (FCM, n = 7 for males; n = 2 for females) in *Anolis carolinensis* lizards during the 'acclimatisation' period, and in the small and large cage treatments. Indicated are means and standard errors for females (black bars) and males (grey bars). The two females in the FCM graph (b) are represented by the black circles and triangles.

Figure 3



Showing (a) the time spent moving (walking or climbing, n = 19 for males; n = 12 for females) and (b) the number of head movements (n = 19 for males; n = 12 for females) exhibited by *Anolis carolinensis* lizards during the 'acclimatisation' period, and in the small and large cage treatments. Indicated are means and standard errors for females (black bars) and males (grey bars).

Discussion

Our results show no effect of cage size on a series of variables that have been used as proxies of stress level. Individuals of *A. carolinensis* that were kept for three weeks in a 0.05 m^3 cage exhibited comparable changes in general condition (as indicated by body mass and tail-base width), leukocyte profiles (H/L ratios), levels of faecal corticosterone metabolites and behaviour as did individuals residing in 0.2 m^3 cages. A question that arises is why we found these negative results.

First of all, a possible explanation is that differences in cage size of the magnitude used in our study were within a range that did not have an effect on stress levels in *A. carolinensis*. It could be that animals did not experience the limited cage size, as such, and that it therefore did not lead to a clear increase in stress levels. This could, in part, be explained by the fact that *A. carolinensis* are a generalist species that are known to easily adapt to changing circumstances, also in captivity. Lovern *et al* (2004) discuss this in greater detail in their review on the use of *A. carolinensis* in laboratory studies. It is possible that species with a more specialised ecology experience a larger effect of changing environmental factors, such as a change in cage size. This could be investigated by undergoing research assessing the effect of changes in cage size in more specialised species. Furthermore, it is difficult to compare our results on the effect of cage size to previous studies on reptiles. As mentioned in the *Introduction*, some research has shown an effect of enforced confinement in, for example,: tuatara and loggerhead turtles (Gregory *et al* 1996; Tyrrell & Cree 1998) and Wheler and Fa (1995) provide a rare example of a study that looked at the effect of cage size on reptiles. To our knowledge, no other research has been carried out investigating solely the effect of cage size on stress levels in lizards. The range of cage sizes used in our experiments is comparable to the array of dimensions typically found in husbandry recommendations or housing descriptions of scientific reports, making our results very relevant in this respect. However, an option for future research could be to investigate the effect of cage sizes smaller and larger than those described in the current study.

An aspect of cage size that is also worth noting is the shape of the cages. When animals are provided with a cage that has a shape that does not conform to their ecological needs, it could negatively impact their stress levels. For example, when *A. carolinensis* (a well-known arboreal species) are provided with a cage that is more long than it is high, it could be expected for this to have a negative effect on their stress levels since they are limited in carrying out aspects of their natural behaviour. Table 1 (https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material) shows that only 58% (15 out of 26) of consulted care-sheets mentioned that cages should be taller than long or wide for *A. carolinensis* lizards. This is an important point to consider when evaluating cage sizes, ie not only volume needs to be taken into account but also the shape of the cage. However, this point might

become irrelevant when lizards are housed in very large cages (eg a few cubic meters) which cannot be compared to the range of normal commercial cage sizes.

Another possible reason could be that the experimental period used was not long enough to elicit a clear response in the animals. Due to strict timing of the experiments we were limited to a three-week period. However, multiple studies have used a comparable experimental period to ours to investigate behavioural and physiological response in a similar set-up as in our study (Hennig & Dunlap 1978: 13-day period, tonic immobility; Moore *et al* 1991: three-week period, plasma CORT level; Case *et al* 2005: 1-month period, H/L ratio and behaviour; Phillips *et al* 2011: 2-week period, body mass and behaviour; Kalliokoski *et al* 2012: 8-day period, FCM and behaviour) showing that the length of our experimental period should be adequate. Despite this, it seems reasonable that, in general, a longer period would be beneficial when testing effects of chronic stressors. This could be studied by conducting similar experiments with a longer experimental period. Further research is needed to investigate these possibilities.

As mentioned in the *Introduction*, when we compare the home range of *A. carolinensis* to what is suggested/used in both care-sheets and literature, we see that the recommended cage sizes are but a fraction of the natural home range size. An extra factor here is that males use more space during certain periods of the year, as was shown by Jenssen *et al* (1998) where they found that breeding males had a home range of 174 m^2. This could potentially increase the effects of small cage sizes. However, it is unlikely that this had an effect in our study as the onset of the breeding season in this species is a period of decreased temperature and day length (Licht 1971) and the temperature and light cycle in our set-up was kept constant throughout the entire experimental period, prohibiting the onset of the breeding season. Female home range size is much smaller than that of the males, which could indicate that females would experience less effect of the differences in cage size. However, our results did not show any differences between males and females in their reaction to the differences in cage sizes.

Lastly, it could be that differences in cage size did have an effect on stress levels, but that we were not able to measure it. It could have been that our instruments were not precise enough to measure differences. This seems unlikely seeing as the instruments and techniques we used are all validated in previous research. It could also have been that we did not measure the appropriate variables. However, this also seems unlikely since the variables we used have all been linked to stress responses in previous research and we did find differences between the 'acclimatisation' period and the 'small cage' and 'large cage' treatments. Body mass and tail width were lower and H/L ratio was higher in the 'acclimatisation' period, indicating a higher stress level.

No differences in FCM levels and behaviour were found between the 'acclimatisation' and experimental situations. Although FCM has been presumed by many to be one of the most accurate measurements to which the other variables could be compared, the results did not show a similar outcome to those for the other variables. No difference between the 'acclimatisation' and the experimental treatments was observed. This difference might be explained by the fact that due to technical limitations (samples under a weight of 10 mg are too small to be accurately analysed via EIA) sample size was greatly reduced. This small sample size could have resulted in inaccurate results for the FCM measurements. A possible solution for the low weights could be to pool samples from an individual per treatment. However, given that FCM analyses are relatively recent in reptiles, it is not known yet whether pooling of samples has an effect on results. An experiment that could test this would be to divide different samples and analyse them both individually and pooled. Another solution could be to collect samples over a longer period of time. However, when the period in which samples are being collected gets longer, it is possible that the effect of a stressor differs between a sample that is collected in the beginning of the experimental period and one that is collected at the end. Although further studies are necessary, the use of FCM might be difficult for reptilian species that produce scant faeces.

The behavioural observations also did not show similar results to those found for the other variables. No differences were found between the treatments for the observed behaviours, making it impossible to draw any conclusions on the effect of the stressor on behaviour. We acknowledge that the behavioural observations could have been improved by the use of video monitoring instead of live observations. However, due to practical limitations, we were not able to use video monitoring. The darkened observation room did not allow for the use of camera observations. The 10-min observation periods might also not have been long enough to obtain a true representation of their behaviour in captivity. However, due to strict timing, all behavioural observations had to be carried out on the same day and given the 8-h period (0900–1700h) when animals were considered fully active, 10-min observations were the longest that could practically be performed. A longer observational period could address this issue for future research.

Our results showed that animals had higher stress levels for most of the measured variables in the 'acclimatisation' period. This finding has been discussed in detail in Borgmans *et al* (2018) as that study utilised the same 'acclimatisation' period as the current study. Even when we take into account that the high levels of stress found in the 'acclimatisation' period are most likely an artefact remaining from the scenarios animals found themselves in prior to our experiments, the fact still remains that we found no differences between the experimental situations.

Animal welfare implications

The major findings of this study are that differences in cage sizes, with regard to the specific dimensions used in this study, do 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. 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. Our results do suggest that decisions on maintaining reptiles in captivity based on the perception of their needs might not be ideal as a guide and that species-specific research into different aspects of captivity is required.

Conclusion

Our results suggest there to be no effect of differences in cage size across the range (with regard to the specific dimensions) we have investigated on stress levels in *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

Adams AW and Jackson ME 1970 Effect of cage size and bird density on performance of six commercial strains of layers. *Poultry Science 49(6)*: 1712-1719. https://doi.org/10.3382/ps.0491712

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*)*. Universitaire Instelling Antwerpen, Belgium. [Title translation: Demographical characteristics and population dynamics in a population of 459 viviparous *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 Routine handling does not lead to chronic stress in captive *Anolis carolinensis*. *Animal Welfare,* submitted

Buchwalder T and Huber-Eicher B 2004 Effect of increased floor space on aggressive behaviour in male turkeys (*Meleagris gallopavo*). *Applied Animal Behaviour Science 89(3)*: 207-214. https://doi.org/10.1016/j.applanim.2004.07.001

Burghardt GM 2013 Environmental enrichment and cognitive complexity in reptiles and amphibians: concepts, review, and implications for captive populations. *Applied Animal Behaviour Science 147*: 286-298. https://doi.org/10.1016/j.applanim.2013.04.013

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

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(13)*: 1380-1386. https://doi.org/10.1111/j.1365-2109.2007.01813.x

Clubb R and Mason GJ 2007 Natural behavioural biology as a risk factor in carnivore welfare: how analysing species differences could help zoos improve enclosures. *Applied Animal Behaviour Science 102(3)*: 303-328. https://doi.org/10.1016/j.applanim.2006.05.033

Cuthill I, Bennett A, Partridge J and Maier E 1999 Plumage reflectance and the objective assessment of avian sexual dichromatism. *The American Naturalist 153(2)*: 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(1)*: 197-201. https://doi.org/10.1670/06-277R2.1

Garner JP 2005 Stereotypies and other abnormal repetitive behaviors: potential impact on validity, reliability, and replicability of scientific outcomes. *Ilar Journal 46(2)*: 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(2)*: 374-381. https://doi.org/10.1111/1365-2435.12063

Gordon RE 1956 *The biology and biodemography of* Anolis carolinensis *Voigt*. PhD Dissertation, Tulane University, New Orleans, LA, USA

Gregory LF, Gross TS, Bolten AB, Bjorndal KA and Guillette JLJ 1996 Plasma corticosterone concentrations associated with acute captivity stress in wild loggerhead sea turtles (*Caretta caretta*). *General and Comparative Endocrinology 104(3)*: 312-320. https://doi.org/10.1006/gcen.1996.0176

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(4)*: 782-792. https://doi.org/10.1111/j.1420-9101.2009.01700.x

Harfoot M, Glaser SA, Tittensor DP, Britten GL, McLardy C, Malsch K and Burgess ND 2018 Unveiling the patterns and trends in 40 years of global trade in CITES-listed wildlife. *Biological Conservation 223*: 47-57. https://doi.org/10.1016/j.biocon.2018.04.017

Hennig CW and Dunlap WP 1978 Tonic immobility in *Anolis carolinensis*: Effects of time and conditions of captivity. *Behavioral Biology 23(1)*: 75-86. https://doi.org/10.1016/S0091-6773(78)91180-X

Hite M, Hanson H, Bohidar N, Conti P and Mattis P 1977 Effect of cage size on patterns of activity and health of beagle dogs. *Laboratory Animal Science 27(1)*: 60-64

Horton G, Malinowski K, Burgher C and Palatini D 1991 The effect of space allowance and sex on blood catecholamines and cortisol, feed consumption and average daily gain in growing lambs. *Applied Animal Behaviour Science 32(2-3)*: 197-204. https://doi.org/10.1016/S0168-1591(05)80043-7

Irschick DJ, Ramos M, Buckley C, Elstrott J, Carlisle E, Lailvaux SP and Vanhooydonck B 2006 Are morphology–performance relationships invariant across different seasons? A test with the green anole lizard (*Anolis carolinensis*). *Oikos 114(1)*: 49-59. https://doi.org/10.1111/j.2006.0030-1299.14698.x

Jalal MA, Scheideler SE and Marx D 2006 Effect of bird cage space and dietary metabolizable energy level on production parameters in laying hens. *Poultry Science 85(2)*: 306-311. https://doi.org/10.1093/ps/85.2.306

Jenssen TA and Nunez SC 1998 Spatial and breeding relationships of the lizard, *Anolis carolinensis*: Evidence of intrasexual selection. *Behaviour 135(7)*: 981-1003. https://doi.org/10.1163/156853998792640341

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(1)*: 93-97. https://doi.org/10.1016/j.ygcen.2012.02.017

Kaufman BM, Pouliot AL, Tiefenbacher S and Novak MA 2004 Short and long-term effects of a substantial change in cage size on individually housed, adult male rhesus monkeys (*Macaca mulatta*). *Applied Animal Behaviour Science 88(3)*: 319-330. https://doi.org/10.1016/j.applanim.2004.03.012

Keay JM, Singh J, Gaunt MC and Kaur T 2006 Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. *Journal of Zoo and Wildlife Medicine 37*: 234-244. https://doi.org/10.1638/05-050.1

Kilambi RV, Adams JC, Brown AV and Wickizer WA 1977 Effects of stocking density and cage size on growth, feed conversion, and production of rainbow trout and channel catfish. *The Progressive Fish Culturist 39(2)*: 62-66. https://doi.org/10.1577/1548-8659(1977)39[62:EOSDAC]2.0.CO;2

Lailvaux S and Irschick D 2007 Effects of temperature and sex on jump performance and biomechanics in the lizard *Anolis carolinensis*. *Functional Ecology 21(3)*: 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(6)*: 559-565. https://doi.org/10.1002/(SICI)1097-010X(19990501)283:6<559::AID-JEZ7>3.0.CO;2-4

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*. *The American Midland Naturalist 79(1)*: 149-158. https://doi.org/10.2307/2423160

Licht P 1971 Regulation of the annual testis cycle by photoperiod and temperature in the lizard *Anolis Carolinensis*. *Ecology 52(2)*: 240-252. https://doi.org/10.2307/1934582

Lovern MB, Holmes MM and Wade J 2004 The green anole (*Anolis carolinensis*): a reptilian model for laboratory studies of reproductive morphology and behavior. *Ilar Journal 45(1)*: 54-64. https://doi.org/10.1093/ilar.45.1.54

MacLean GS, Lee AK and Wilson KJ 1973 A simple method of obtaining blood from lizards. *Copeia 2*: 338-339. https://doi.org/10.2307/1442973

McGinty AS 1991 Tilapia production in cages: Effects of cage size and number of non-caged fish. *The Progressive Fish Culturist 53(4)*: 246-249. https://doi.org/10.1577/1548-8640(1991)053<0246:IPICEO>2.3.CO;2

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(1-2)*: 176-181. S0165-2427. https://doi.org/10.1016/j.vetimm.2008.04.014

Michaels C, Downie J and Campbell-Palmer R 2014 The importance of enrichment for advancing amphibian welfare and conservation goals. *Amphibian Reptile Conservation 8*: 7-23

Montgomerie R 2006 Analyzing colors. In: Hill GE and McGraw KJ (eds) *Bird Coloration, Volume I: Mechanisms and Measurements* 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(9)*: 563-567. https://doi.org/10.1002/jez.484

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(2)*: 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(2)*: 156-162. https://doi.org/10.1002/(SICI)1097-010X(19971001)279:2<156::AID-JEZ6>3.3.CO;2-Y

Möstl E and Palme R 2002 Hormones as indicators of stress. *Domestic Animal Endocrinology 23(1)*: 67-74. https://doi.org/10.1016/S0739-7240(02)00146-7

Nicol CJ 1987 Effect of cage height and area on the behaviour of hens housed in battery cages. *British Poultry Science 28(2)*: 327-335. https://doi.org/10.1080/00071668708416965

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(1)*: 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(9-10)*: 238-246

Pearce G and Paterson A 1993 The effect of space restriction and provision of toys during rearing on the behaviour, productivity and physiology of male pigs. *Applied Animal Behaviour Science 36(1)*: 11-28. https://doi.org/10.1016/0168-1591(93)90095-7

Phillips C, Jiang Z, Hatton A, Tribe A, Le Bouar M, Guerlin M and Murray P 2011 Environmental enrichment for captive Eastern blue-tongue lizards (*Tiliqua scincoides*). *Animal Welfare 20(3)*: 377-384

Plavicki J, Yang EJ and Wilczynski W 2004 Dominance status predicts response to nonsocial forced movement stress in the green anole lizard (*Anolis carolinensis*). *Physiology and Behaviour 80(4)*: 547-555. https://doi.org/10.1016/j.physbeh.2003.10.009

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:EORFG]2.0.CO;2

Rowland SJ, Mifsud C, Nixon M and Boyd P 2006 Effects of stocking density on the performance of the Australian freshwater silver perch (*Bidyanus bidyanus*) in cages. *Aquaculture 253(1)*: 301-308. https://doi.org/10.1016/j.aquaculture.2005.04.049

Saad AH and El Ridi R 1988 Endogenous corticosteroids mediate seasonal cyclic changes in immunity of lizards. *Immunobiology 177(4-5)*: 390-403. https://doi.org/10.1016/S0171-2985(88)80007-X

Saito T, Motomura N, Taniguchi K, Hokao R, Arkin A, Takahashi K and Sato N 1996 Effect of cage size on sexual behavior pattern in male rats. *Contemporary Topics in Laboratory Animal Science 35(5)*: 80-82

Schoener TW and Schoener A 1982 Intraspecific variation in home-range size in some *Anolis* lizards. *Ecology 63*: 809-823. https://doi.org/10.2307/1936801

Sefton AE 1976 The interactions of cage size, cage level, social density, fearfulness, and production of single comb white Leghorns. *Poultry Science 55(5)*: 1922-1926. https://doi.org/10.3382/ps.0551922

Stellar JE and White DJ 2010 Social influences on female choice in green anole lizards (*Anolis carolinensis*). *Behavioural Processes 83(3)*: 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(1)*: 29-40. https://doi.org/10.1006/hbeh.1994.1003

Teng SK and Chua TE 1978 Effect of stocking density on the growth of estuary grouper, *Epinephelus salmoides* Maxwell, cultured in floating net-cages. *Aquaculture 15(3)*: 273-287. https://doi.org/10.1016/0044-8486(78)90037-6

Therrien CL, Gaster L, Cunningham-Smith P and Manire CA 2007 Experimental evaluation of environmental enrichment of sea turtles. *Zoo Biology 26(5)*: 407-416. https://doi.org 10.1002/zoo.20145

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(3)*: 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(2)*: 97-108. https://doi.org/10.1006/gcen.1997.7051

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

Wheler CL and Fa JE 1995 Enclosure utilization and activity of Round Island geckos (*Phelsuma guentheri*). *Zoo Biology 14*: 361-369. https://doi.org/10.1002/zoo.1430140407

Whitaker J, Moy SS, Saville BR, Godfrey V, Nielsen J, Bellinger D and Bradfield J 2007 The effect of cage size on reproductive performance and behavior of C57BL/6 mice. *Lab Animal 36(10)*: 32. https://doi.org/10.1038/laban1107-32