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The effect of environmental provisioning on stress levels in captive green anole (Anolis carolinensis)

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Abstract

*In response to an increased awareness concerning the welfare of captive animals, several studies have investigated the effect of provisions on stress levels in model species, such as small mammals, birds and fish. In contrast, reptiles have received less attention. Although many reptilian species are becoming increasingly popular in the pet trade and are frequently used as model species in various branches of biology and a number of studies have explored how they react to stress in different contexts (eg social, predatory), little is known about how they react to stress induced by housing conditions or experimental treatments. In this study, we quantified the effect of provision of perches and leaves as refuges (provisioned) on the behaviour, morphology and physiology of the green anole (*Anolis carolinensis*). Our results showed that increased or decreased structural complexity of the cage had no effect on body mass, tail-base width, heterophil to lymphocyte ratios (H/L ratios), brightness, body colour, behaviour and faecal corticosterone metabolite (FCM) levels for both males and females in the experimental treatments (provisioned or deprived situation). 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, environmental provisioning, green anole, reptiles, stress*

Introduction

In response to an increased awareness concerning the welfare of captive animals, numerous studies have investigated stress (defined as the adverse effect that external or internal stimuli may have on the physical or mental well-being of the animal; Carstens & Moberg 2000) and how to avoid it in model species, such as small mammals (Pfister 1979; Weinberg & Wong 1986), birds (Asher *et al* 2009) and fish (Näslund *et al* 2013). It has been shown that stress, as a result of captivity, can affect the endocrinology, physiology and behaviour of animals. This may lead to a general decrease in welfare (Morgan & Tromborg 2007) and could also confound the results of scientific experiments or observations on captive study animals (Garner 2005). Several factors may affect stress levels in captivity, for example: cage size, sound, temperature, social structure, etc (Morgan & Tromborg 2007).

One measure that is widely thought to alleviate stress in artificial housing conditions is dubbed 'environmental provisioning' (EP): modifying the captive environment by, for example: adding structures, that could lead to an improvement in the biological functioning of the animals (Newberry & Shackleton 1997). The effect of EP on welfare is relatively well studied and proven to be effective in mammals (Cooper *et al* 1996; Townsend 1997) and birds (Newberry & Shackleton 1997; Dawkins *et al* 2003). Although a number of studies have explored how reptiles react to stress in different contexts (eg social; Greenberg *et al* 1984, Greenberg & Crews 1990; Greenberg 2003 and predatory; Hennig *et al* 1976; Hennig 1977), little is known about what effect EP has on welfare and, subsequently, stress in reptiles. It also remains difficult to assess stress levels in reptiles and amphibians in an objective manner due to their large phylogenetic distance from humans and mammals (Langkilde & Shine 2006).

Table 1 provides an overview of, as far as we are aware, all studies investigating EP in reptiles. Remarkably, and in contrast to previously mentioned research on mammals and birds, there does not appear to be a general consensus about the effect of EP on stress levels in reptiles. While the majority of research does demonstrate a positive effect of EP on the animals' well-being, the studies by Case *et al* (2005) and Therrien *et al* (2007) give mixed results whereby certain variables do not indicate a positive effect of EP. Research that should be discussed separately, given the fact that it is a good example of a study with negative findings

Table 1 Research investigating environmental provisioning (EP) in reptiles.

Animal	Species	Type of EP	Techniques of measuring stress	Effect of EP	Reference
Turtles	*Terrapene carolina*	Cage enrichment	Faecal corticosterone metabolites and bodyweight	Inconclusive	Case *et al* (2005)
			Heterophil to lymphocyte (H/L) ratio	Decreased	
			Escape behaviour	Decreased	
			Resting	Increased	
	Chelonia mydas, *Caretta caretta*	Object provisioning	Swimming activity	Inconclusive	Therrien *et al* (2007)
			Interactions with objects	Increased	
			Inactivity	Decreased	
			Stereotypical behaviour	Decreased	
			Aggression and hiding	Inconclusive	
Lizards	*Anolis carolinensis*	Cage enrichment	Duration of tonic immobility	Decreased	Henning & Dunlap (1978)
	Tiliqua scincoides	Food enrichment	Bodyweight	Increased	Phillips *et al* (2011)
			Hiding behaviour	Decreased	
	Sceloporus undulatus	Cage enrichment	Behaviour, growth, corticosterone, survival	Inconclusive	Rosier & Langskilde (2011a)
	Iguana iguana	Cage enrichment	Resting	Increased	Kalliokoski *et al* (2012)
			Faecal corticosterone	Decreased	
Snakes	*Elaphe obsoleta*	Food/cage enrichment	Solving behavioural tasks	Increased	Almli & Burghardt (2006)

that was not particularly well received, is the work of Rosier and Langkilde (2011a). They investigated the effect of EP (in the form of adding a wooden platform) on Eastern fence lizards (*Sceloporus undulatus*) and found no effect on any behaviour, growth, corticosterone, or survival measure. This study came in for much criticism, leading the authors to write a letter to the editor defending their findings (Rosier & Langkilde 2011b). These few published studies that did not find a positive effect of EP might indicate a publication bias whereby studies with negative results are not published as frequently as those with positive findings.

Apart from showing that there is no general consensus on the effect of EP in reptiles, Table 1 shows that the methods used for measuring stress vary widely among studies, possibly leading to disparate results on the effect of EP. While most studies focus on one technique of measuring stress, we use an integrative approach by combining different measurements to get a broader view of the response to a certain stressor, in this case variations in level of environmental provisioning. Using this integrative approach allows us to validate the different measurement techniques further.

Many reptilian species are becoming increasingly popular in the pet trade. They are also frequently used as model species in various branches of biology. The uncertainty regarding the effect of EP on stress and the variety in measurements used is therefore far from ideal. It is clear from the examples mentioned above that both the effect of environmental provisions on animal welfare in reptiles and which measurements should be used to assess welfare requires more research.

In this study, we quantified the impact of providing perches and leaves on the physiology and behaviour of the green anole (*Anolis carolinensis*). This animal 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 behaviour, physiology and morphology (eg Waters *et al* 2005; Merchant *et al* 2008; Montuelle *et al* 2008; Stellar & White 2010). We hypothesise that a greater level of environmental provisioning will lead to a decrease in stress and concordant changes in behavioural and physiological indices. Additionally, we seek to provide further validation for the different measuring techniques used.

Materials and methods

Study animals and housing

All procedures were carried out with the approval of the institute's ethical committee for animal experiments (Ethische Commissie Dierproeven, ECD, file nr 2013-70). Thirty-four adult *A. carolinensis* (21 males, 13 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 (30 × 40 × 70 cm; length × width × height); (see Appendix Figure 1A in the supplementary material to papers published in *Animal Welfare* on the UFAW website: https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material). Lizards were housed individually to permit the

collection of faecal samples. Light bulbs (45 W), placed at the top of the cages, 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). Relative humidity was kept constant at around 60% by monitoring using a hygrometer (TH50 hygrometer, Hama, Germany) and misting the terraria daily. 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 with a 2-cm diameter (the preferred diameter for *A. carolinensis*; Gilman & Irschick 2013) and two banana leaves under which lizards were able to hide. This experimental set-up is based upon standard practices used in literature. 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 three weeks after their arrival in the laboratory. This time interval will be referred to hereafter as the 'acclimatisation' period. At the end of this period, measurements were carried out as described below during which animals were randomly assigned to one of two groups. Individuals allocated to the 'deprived first' group were kept in cages containing no perches or leaves and thus were empty apart from the food and water trays' environmental provisions (deprived cages, see Appendix Figure 1B in the supplementary material to papers published in *Animal Welfare* on the UFAW website: https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material) for the next three weeks and their cages were subsequently altered to 'provisioned' cages (Appendix Figure 1C; https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material) containing three diagonal perches and four banana leaves. This set-up is a far closer approximation of the structure and complexity seen in the animals' natural habitat compared to the standard practice seen in scientific research. Individuals of the 'provisioned first' group received a reversed order of treatment. This cross-over design allowed testing for an effect of order of treatments.

Measurements

All measurements were carried out in the final 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 day 14–16 and behavioural observations were performed on day 19. Blood samples were collected on day 20 and the colour and morphological measurements calculated on day 21.

Morphometrics

We measured tail width (at the base of the tail) using digital calipers (SEM: ± 0.1 mm, Absolute Digimatic, Mitutoyo, USA) and body mass using an electronic balance (SEM: ± 0.01 g, Scout Pro, Ohaus, USA).

Heterophil to lymphocyte (H/L) ratio

Heterophils and lymphocytes are two types of white blood cells in reptiles. They both play a role in the 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 (max 60 µl) was obtained from the post-orbital sinus via insertion of a capillary tube (75 mm; 60 µl) between the eye and the eyelid (Maclean *et al* 1973). Animals were restrained by hand to facilitate drawing blood. The use of post-orbital sinus sampling is often difficult as it requires considerable skill to avoid damaging the animal, which is likely to have a considerable adverse welfare impact, and may not be suitable for some studies as the sample contains other fluids as well as blood. Our laboratory has extensive experience using this technique and no animals suffered any 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 (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, 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 experimentation he was not blinded for the behavioural analyses. The duration of the following behaviours was noted over 10-min observation periods (Table 2): 'sitting', 'hiding', 'basking', 'walking', 'climbing', 'foraging', 'licking', 'wiping', 'sprinting', 'foot pounding', 'tail trembling', 'standing up', 'pounding', 'jumping', 'biting'. 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.

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
Wiping	Time spent wiping their snout on the substrate or structures in the cages
Sprinting	Time spent moving in bouts of extreme activity
Foot pounding	Time spent continuously pounding with one of their feet on the ground
Tail trembling	Time spent continuously trembling their tails
Standing up	Time spent standing up against the side of the cages
Pounding	Time spent continuously pounding with one of their feet against the side of the cage
Jumping	Time spent continuously jumping against the side of the cage
Biting	Time spent continuously trying to bite the side of the cage
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)

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 stress over longer time-periods (Möstl & Palme 2002; Palme *et al* 2005). This alternative technique has been recently used in a variety of vertebrates, including reptiles (Rittenhouse *et al* 2005; Kalliokoski *et al* 2012).

Cages were checked three times daily (0900, 1200 and 1500h) for three days and samples collected when available. Faecal pellets were collected from the lizards' home cages using tweezers. The pellets were stored in small plastic bags and 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 pellets weighed less than 10 mg (Sartorius CPA223S, 0.001 g, Sartorius, Germany), they were pooled with samples of the same individual within the same treatment. 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). Details of the EIA, including cross-reactions of the antibody have been previously described (Touma *et al* 2003). We performed a validation experiment (Touma & Palme 2005) to demonstrate the suitability of the chosen EIA for measuring FCM levels in *A. carolinensis*. Therefore, we artificially increased blood corticosterone levels in four male and four female individuals by applying an oil/hormone mixture as described by Meylan *et al* (2003). All voided faecal samples were collected three days before (baseline), during (six days) and after (five days) transdermal corticosterone application and FCM determined, as described above.

Colour

Body colour has been shown to change in response to different stressors in reptiles (eg Summers & Greenberg 1994; Vroonen *et al* 2012). Summers and Greenberg (1994) have investigated this in *A. carolinensis* and found that they become darker when stressed. However, this was in response to an acute stressor and not long-term stress.

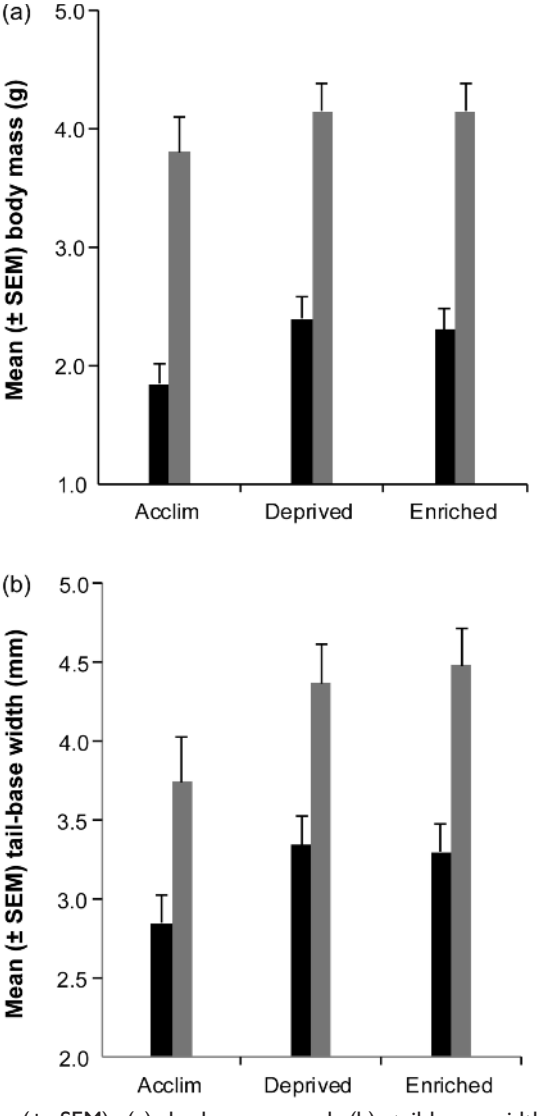
Reflectance of the skin was measured using spectrophotometry (range 250–1,000 nm, Avasoft spectrophotometer AvaSpec-2048-USB2-UA-50, Avantes, The Netherlands) with a deuterium-halogen light source and fitted with a fibre-optic probe (probe diameter: 1.3 mm). The probe, which was mounted within a metal holder to ensure reading at a constant distance from the surface, was always placed perpendicular to the skin of the animals. All measurements were expressed in relation to a white reference tile (WS2; Avantes). Four points on the body of each animal were sampled: one mid-dorsally on the head directly posterior to the eyes; one on the centre of the dewlap; and a central point on both flanks. These points were chosen to reflect the overall colouration of the animals' body. Reflectance spectra were calculated automatically from the subject's radiance by the software (AvaSpec75USB2).

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 effect between treatments and sex. The assumption of normality was tested with a Shapiro-Wilk test. H/L ratio and FCM level 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 previously explained) 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 combined to increase sample size. Occurrence of most of the behavioural variables (Table 1) was non-existent, 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 'climbing') and number of head movements were analysed using generalised linear model (GzLM).

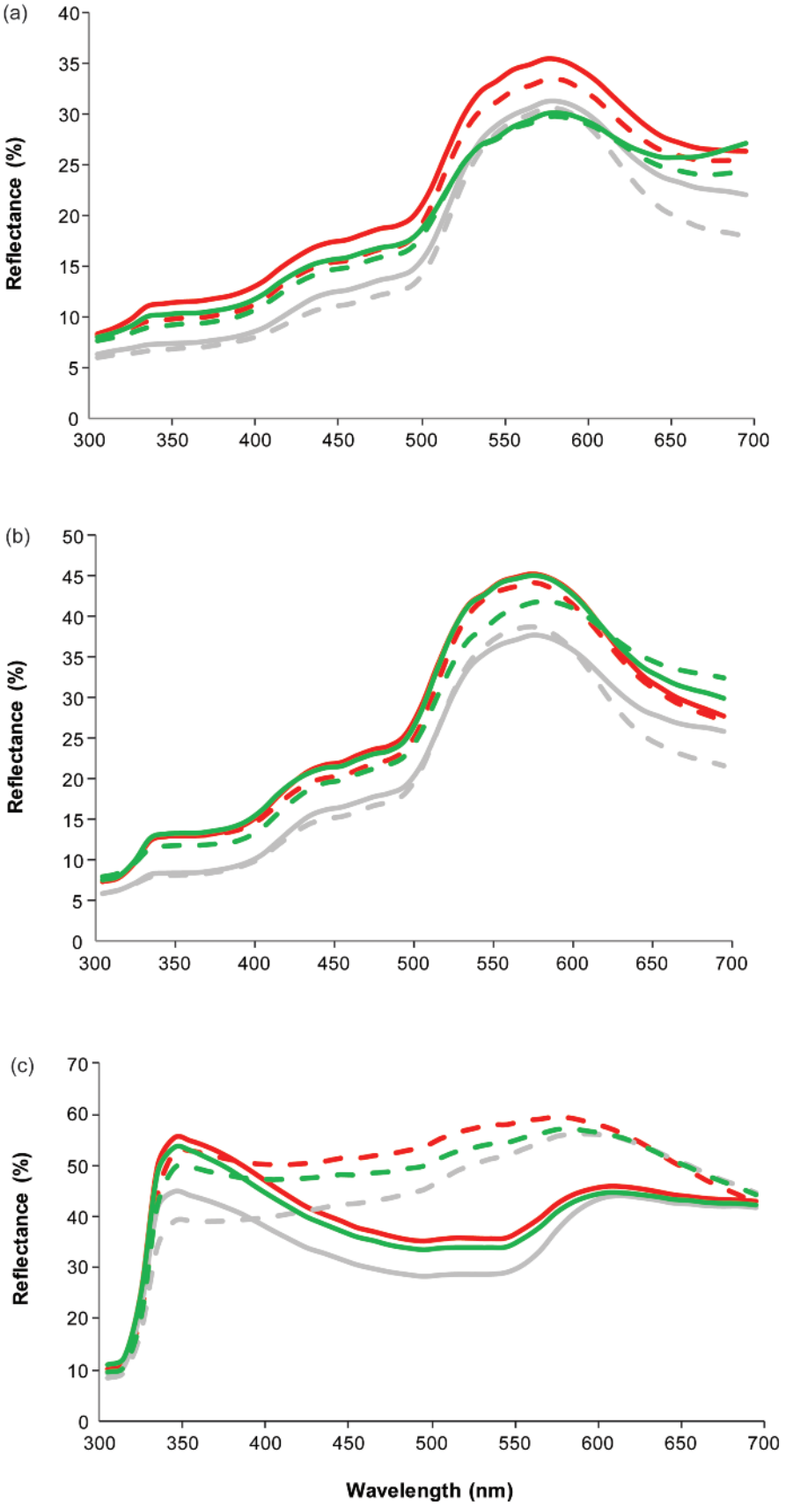
Spectral data were analysed in a different manner to the variables mentioned above. Each of our reflectance spectra originally comprised 690 data-points (0.59 nm reflectance intervals from 300 to 700 nm). Reflectance data were grouped into 10 nm bins resulting in mean values for 40 bins ranging from 300 to 700 nm. Principal component analysis (PCA) was used to reduce the number of variables for the reflectance data. Additionally, six commonly used reflectance indices were calculated from the reflectance spectra and these were then correlated with the principal component scores received from the principal component analysis of the three points (head, average values for the flanks and dewlap) used in the analysis (Griggio *et al* 2009): mean brightness (the mean percentage of reflectance from 300 to 700 nm), UV chroma ($R_{300\text{-}400}/R_{300\text{-}700}$), blue chroma ($R_{400\text{-}475}/R_{300\text{-}700}$), green chroma ($R_{475\text{-}550}/R_{300\text{-}700}$), yellow chroma ($R_{550\text{-}625}/R_{300\text{-}700}$) and red chroma ($R_{625\text{-}700}/R_{300\text{-}700}$) (Endler 1990; Montgomerie 2006). All above-mentioned indices are calculated as the proportion of the average percentage of reflectance of the specified ranges divided by the average percentage of reflectance from 300–700 nm. The correlation between these indices and the principal component scores was done to simplify the interpretation of possible differences in body colour. In analyses of spectral data, the first PC inevitably represents brightness variation and subsequent PCs represent colour variation (Cuthill *et al* 1999). A PCA was run for the three points on the body (head, average values for the flanks and dewlap). Components with eigenvalues > 1.5 and which contributed more than 10% to variance were retained and one-way repeated measures ANOVAs were carried out on their component scores to test for statistical differences between treatments. This resulted in three principal components being retained (PC1, eigenvalue = 42.8, 35.7% of variation; PC2, eigenvalue = 22.3, 18.6% of variation; PC3, eigenvalue = 15.7, 13.1% of variation). Whenever a repeated measures ANOVA found a statistical difference, a *post hoc* analysis with Bonferroni adjustment was carried out to investigate pair-wise differences.

Figure 1



Mean (± SEM) (a) body mass and (b) tail-base width for *Anolis carolinensis* lizards in the acclimatisation situation, the deprived and the enriched situation for females (black) and males (grey).

Figure 2



Average reflection of the skin of the (a) head, (b) flanks and (c) dewlap of *Anolis carolinensis* lizards in the acclimatisation situation (grey lines), the deprived situation (red lines) and the enriched situations (green lines). Values for males (full lines) and females (dashed lines) are shown separately.

Results

Morphometrics

Lizards in both the deprived and the provisioned cages exhibited a rise in body mass compared with the initial 'acclimatisation' period (Figure 1[a], treatment effect: $F_{1.566, 50.113} = 15.9$; $P < 0.001$). The rise was similar between sexes (sex × treatment effect: $F_{1.566, 50.113} = 0.762$; $P = 0.442$). However, body masses of lizards in the 'deprived' and 'provisioned' situations were not significantly different (*post hoc* test: $P > 0.9$). Males did have an overall higher body mass ($F_{1,31} = 29.167$; $P < 0.001$).

Tail width (corrected for SVL) exhibited a similar effect of treatment (Figure 1[b], $F_{2,64} = 35.04$; $P < 0.001$), with high values in the deprived and provisioned cages compared with the 'acclimatisation' period. The change was similar for males and females (treatment × sex effect: $F_{2,64} = 1.65$; $P = 0.2$). The difference between the deprived and provisioned situations was not significant ($P > 0.9$). Males had an overall higher tail width ($F_{1,31} = 25.85$; $P < 0.001$).

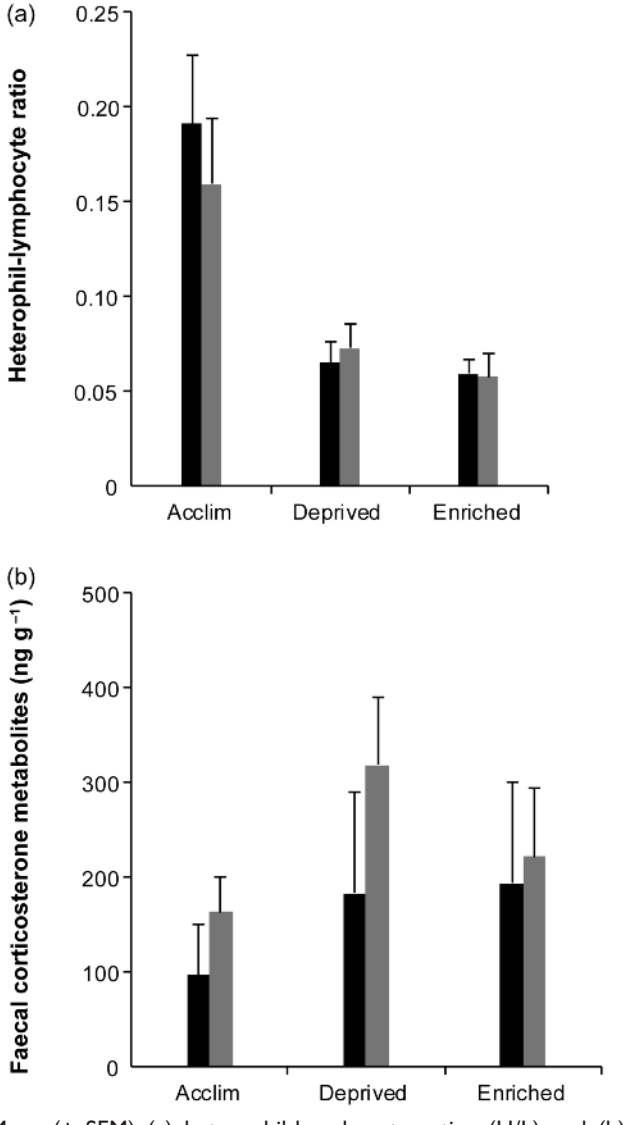
Colour

In all three body parts measured, the first principal component correlated positively with reflectance at all wavelengths (Table 3, see Appendix in supplementary material to papers published in *Animal Welfare* on the UFAW website: https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material) and the scores were positively correlated with the mean reflectance colour chroma (Pearson correlation: heads 0.696, flanks 0.630 and dewlap 0.376) and can therefore be considered a 'brightness' axis. The brightness of the head, flank and dewlap colours exhibited significant variation among treatments (Figure 2; $F_{2,64} = 87.606$; $P < 0.001$), but not between sexes (sex-effect: $F_{1,32} = 2.39$; $P = 0.132$; sex × treatment-effect: $F_{2,64} = 0.06$; $P = 0.942$). Brightness was lower in the 'acclimatisation' period than in the deprived (*post hoc*; $P < 0.001$) and the provisioned situation ($P < 0.001$). There was no difference between the deprived and the provisioned situation ($P = 0.14$).

The second principal component correlated positively with reflectances at almost all wavelengths for the dewlap, indicating that the second component represents colour variation in the dewlap. There were no significant differences between the situations for the colouration of the dewlaps (Figure 2[c], $F_{2,64} = 1.58$; $P = 0.213$). We did find a significant difference for the colouration of the dewlap between both sexes ($F_{1,32} = 79.907$; $P < 0.001$) which serves as adjunct evidence that the PCA method is valid for measuring differences in colouration.

The third principal component correlated positively with reflectances at almost all wavelengths for the head and negatively with reflectances for the flanks, indicating that the third component represents colour variation in the heads and flanks. Also, there were no significant differences between the situations for the colouration of the heads and flanks (Figure 2[a] and [b]; $F_{2,64} = 1.17$; $P = 0.315$).

Figure 3



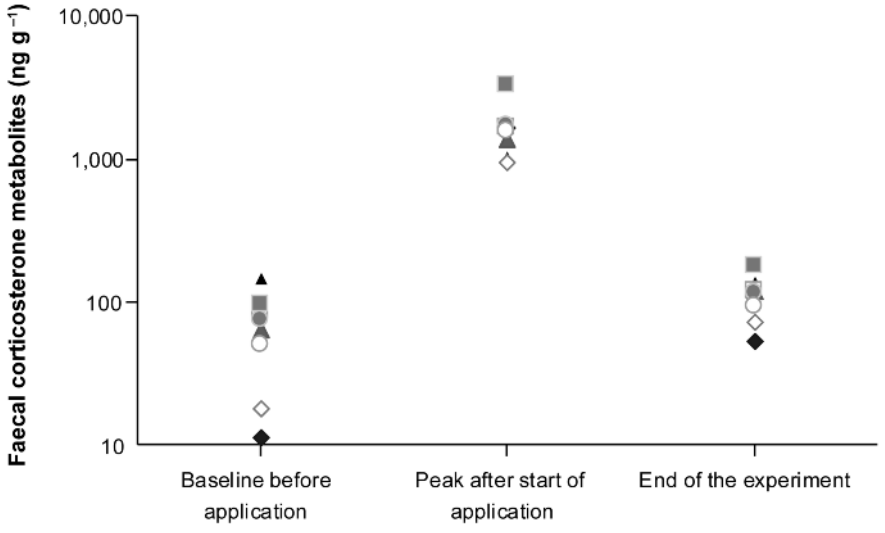
Mean (± SEM) (a) heterophil-lymphocyte ratios (H/L) and (b) faecal corticosterone metabolite levels (FCM) for n = 9 male and n = 4 female *Anolis carolinensis* lizards during the acclimatisation phase, and in the deprived and enriched conditions. Females shown with black bars and males with grey.

Physiology

The H/L ratios were high in the initial 'acclimatisation' phase and equally low in the other two situations (Figure 3[a], treatment effect: $F_{2,56} = 45.12$; $P < 0.001$). The same pattern was observed in males and females (sex × treatment effect: $F_{2,56} = 0.75$; $P = 0.48$, sex effect: $F_{1,28} = 0.55$; $P = 0.46$). Lizards in the deprived and provisioned cages exhibited highly similar rations ($P = 0.60$).

The results of the validation experiment showed no differences between sexes ($P > 0.34$). Baseline FCM levels varied between 12 and 147 ng g^{-1} faeces. After transdermal corticosterone application, levels increased reaching maximum concentrations in the second half of the application period. Peak concentrations were 7 to 144 (median: 27) times

Figure 4



Concentrations (males [full symbols; n = 4], females [open symbols; n = 4]) of faecal corticosterone metabolites (FCM) before (median of all baseline levels) and after (peak levels) corticosterone application, and at the end of the experiment. Please note the logarithmic y-axis. Different symbols represent different individuals. Not all individual peak values can be seen due to overlap.

higher than baseline levels. After stopping the application, levels decreased again, although baseline was not reached in all individuals within five days (Figure 4).

There was no effect of treatment on FCM levels (Figure 3[b]; $F_{2,22} = 1.07$; $P = 0.359$). Males and females exhibited similar among-situation variation (treatment × sex effect: $F_{2,22} = 0.211$; $P = 0.812$) and FCM levels (sex-effect: $F_{1,11} = 1.906$; $P = 0.195$).

Behaviour

The total time spent moving (walking and climbing) did not vary between treatments (GzLM, interaction-effect: Wald $\chi^2_2 = 0.299$; $P = 0.861$; Figure 5[a]) nor between sexes (treatment × sex interaction effect: Wald $\chi^2_2 = 4.343$; $P = 0.114$, sex-effect: Wald $\chi^2_2 = 0.138$; $P = 0.71$).

Head movements also did not vary between treatments (GzLM, interaction-effect: Wald $\chi^2_2 = 0.854$; $P = 0.653$; Figure 5[b]) nor between sexes (treatment × sex interaction effect: Wald $\chi^2_2 = 2.29$; $P = 0.318$, sex-effect: Wald $\chi^2_2 = 0.025$; $P = 0.875$).

Discussion

Contrary to our expectations, we found no effect of added or decreased EP on *A. carolinensis* body mass, tail width, H/L ratios, behaviour, brightness and colouration of the heads, flanks, dewlaps and FCM levels for both males and females.

Our findings have a number of possible explanations of which the most likely ones are discussed below. First of all, a possible explanation is that EP in the form of added or decreased complexity does not have an effect on stress levels in *A. carolinensis*. These results are highly comparable to what was found by Rosier and Langkilde (2011a). They found no effect of EP (in the form of adding a wooden platform) on survival, behaviour, physiological stress (corticosterone), growth and body condition for Eastern fence lizards (*S. undulatus*). The fact that we found little evidence of an effect of EP does not mean that other forms or combinations of EP do not play a role in improving a captive situation. Different forms of EP (for example, providing a thermal gradient, appropriate humidity or providing live prey) probably play an important role in the welfare of captive lizards, as also stated by Rosier and Langkilde (2011a). These results are not in line with what is generally found in the literature on the effect of EP on reptiles (Table 1). However, this again could be due to the fact that studies finding negative results are under-represented in published literature due to a publication bias.

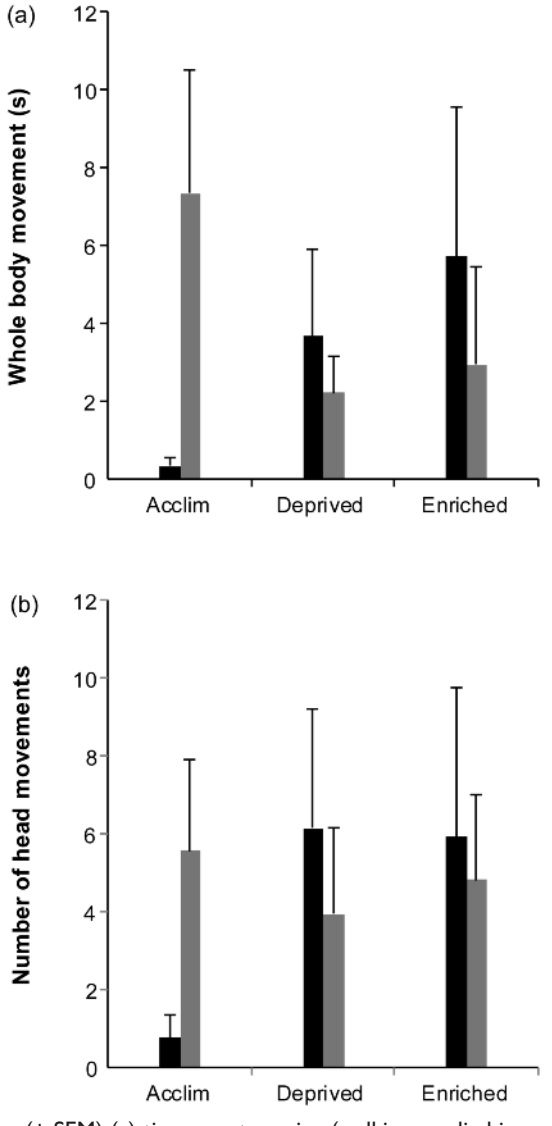
A second possible reason why we were not able to measure an effect might be that we did not use an appropriate form of EP. *A. carolinensis* are excellent climbers and although no actual climbing or hiding structures were present in the deprived situation, they could still climb on the sides of the cages and sit in the top corners, which could have provided them with sufficient possibilities to hide. Another aspect of this could be that the provision provided is not perceived as such by the animals due to the fact that the standard branches and banana leaves are not botanical species that *A. carolinensis* is familiar with. This could be tested in future research by investigating the effect of different forms of EP. Another possible reason could be that the experimental period used was not long enough to elicit a response in the animals. This could be studied by carrying out similar experiments with a longer experimental period. Further research is needed to investigate these possibilities.

Last, it could be the case that EP did have an effect on the well-being of the animals, but that we were unable to

measure this effect. A possible reason for this might be that it is not known which indicators should be used to measure stress accurately in these animals. Table 1 shows the variety of measurements used in stress research and, as stated in the *Introduction*, an additional goal of this study was to further validate the use of morphometrics, H/L ratio, colouration via spectrophotometry and behavioural analyses as measurements to gauge stress accurately. However, our mixed results did not provide further support for the use of the above-mentioned indicators as measurements of stress in an experimental set-up. Possibly, other, long-term, measurements, such as reproduction or survival, could be used as an ultimate measure of stress. Mention must be made of the use of FCM as a measure of adrenocortical activity. Our validation experiment clearly demonstrated its validity. FCM values increased dramatically after applying a method (transdermal application) which increases plasma steroid levels (Meylan *et al* 2003; Baeckens *et al* 2017). However, although FCM measurement is very promising as an easy-to-use, non-invasive technique, it has some restrictions. Samples under a weight of 10 mg are too small to be accurately analysed via EIA. This caused a reduction in sample size in our study. 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 yet known 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, FCM might be difficult for reptilian species that produce scant faeces.

However, it seems unlikely that our measurements were not able to detect differences in stress given the fact that, while we found few changes between our experimental situations, we did notice strong differences in values of variables measured between the experimental situations and the 'acclimatisation' period. Body mass and tail width were lower and H/L ratio was higher in the 'acclimatisation' period, indicating more stress in this period. Brightness was found to be lower in the 'acclimatisation' period for the head, flanks and dewlap. This is in line with the findings of other studies investigating the effect of stress on body colour (Ackermann 1997; Denardo 2006), where they found that animals become darker when stressed. Summers and Greenberg (1994) investigated this in *A. carolinensis*, but observed changes in colour visually. The use of spectrophotometry allows us to look at the animals' brightness and our results suggest that brightness could possibly be used as a variable of measuring stress. No differences in colouration of the heads, flanks or dewlaps were found between the experimental situations. It is worth pointing out here that due to its instantaneous nature, changes in body colour might not be suitable for measuring long-term stress effects.

Figure 5



Mean (± SEM) (a) time spent moving (walking or climbing and (b) number of head movements exhibited by *Anolis carolinensis* lizards during the acclimatisation period, and in the deprived and enriched situations for females (black bars) and males (grey bars).

When animals were removed from their enclosures prior to the colour measurements, a change in body colour could often be perceived. Taylor and Hadley (1970) showed that the reaction of the body colour in response to melanophore stimulating hormone (MSH, which is responsible for the change in colour) is almost maximal in 7 min and the classic literature by Carlton (1903) states that body colour changes take, on average, 4 min and although this is significantly longer than the time it takes us to perform colour measurements (under 1 min on average), this rapid change in colour could have had an effect on the measurement of body colour and mask any possible effects from the experimental treatments.

Lastly, no differences in FCM levels and behaviour were found between the situations. Although FCM was presumed to be a 'golden standard' with which the other variables could be compared, the results did not show the same

variation as was seen for the other variables. As mentioned above, this difference might be explained by the fact that, due to limitations, sample size was greatly reduced. This small sample size could have resulted in inaccurate results for the FCM measurements. Another possible explanation is discussed in the study by Case *et al* (2005). They state that, although acute stress is often associated with an increase of plasma corticosterone, chronic stress could actually lead to a suppression of corticosterone production. This has already been shown in studies on rodents (Mar Sanchez *et al* 1998), domestic pigs (De Jong *et al* 2000) and alligators (Lance & Elsey 1999). It could have been the case that animals experienced high stress during transport and/or their stay at the commercial supplier (this was approximately one week) that an effect could still be measured in the 'acclimatisation' period. This would explain why the corticosterone results appear to be contradictory to what was found for the other stress indicators. The results from the behavioural observations also did not show the same variation as was found for the other variables. Furthermore, as mentioned before, total time spent moving and head movements were the only behaviours that could be observed. These behaviours are not related to stress and it is therefore not possible to link the behaviours to a response from the stressor.

These results show us that animals had higher stress values for most of the measured variables in the 'acclimatisation' period, which leads us to conclude that animals experienced 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 especially remarkable when taking into account that measurements in the 'acclimatisation' period were taken after three weeks of stay in our standard set-up. This was surprising as this period was originally intended to be a baseline against which our experimental situations could be compared. These unexpectedly high levels of stress in the 'acclimatisation' period made it more difficult to interpret the results of the experimental situations.

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 we found no differences between the experimental situations of providing the specified environmental provisions.

Animal welfare implications

The major findings of this study are that differences in environmental provisions, as used in this study, do not have an effect on stress in *A. carolinensis*. This, however, does not mean that other forms or combinations of EP do not play a role in improving a captive situation. Different forms of EP (for example, providing a thermal gradient, appropriate humidity or providing live prey) probably play an important role in the welfare of captive lizards. Our results do suggest that decisions on the provision of possible environmental enrichment for 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 providing environmental enrichment is required.

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