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LABORATORY

The Effect of Long Term Captivity on Stress Levels in *Anolis carolinensis* Lizards

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ABSTRACT
The effect of long term captivity is a factor that is important for all research utilizing wild caught animals. Despite the fact that it can be considered to be one of the most fundamental potential sources of stress in captivity, it has received a low amount of interest in recent research on lizards. Given the wide variety in ecology and life history among lizards species, it would make sense to investigate the effect of long term captivity on wild caught lizards on a broader scale. In this study we investigated the effect of long term captivity (four months) on the physiology and behavior of male and female *Anolis carolinensis* lizards. Our results showed no negative effects of four months of captivity on physiological and behavioral measurements in male *A carolinensis* lizards. Similar results for females were found for all measurements except body mass and tail width. Here our results indicated a potential negative effect of four months of captivity on body mass and tail width in females.

KEYWORDS
Anolis carolinensis; reptiles; stress; long term captivity

Introduction

A factor that might play a role when conducting scientific research on wild caught animals is the stress these animals experience in captivity. Some excellent review studies have looked at potential sources of stress in captivity (Morgan & Tromborg, 2007) and the stress from laboratory routines (Balcombe, Barnard, & Sandusky, 2004). Nonetheless, it seems a matter of common sense that one of the greatest potential stressors in captivity would be restriction of movement and the inability to perform natural behaviors, and thus essentially captivity itself.

However, despite the fact that the effect of captivity can be considered as one of the most fundamental stressors for wild caught animals, it has received a very low amount of interest in scientific research on lizards. A literature search yielded only a handful of studies which looked at the effect of long term captivity on stress measurements in lizards. A short summary of the literature is given: 8 weeks of captivity significantly lowered metabolic capacity of *Amphibolurus nuchalis* (Garland, Else, Hulbert, & Tap, 1987) and 12 months of captivity was found to have the same effect in *Tupinambis nigropunctatus* (Bennett & John-Alder, 1984). In contrast, no effect of 8 weeks of captivity on metabolic capacity was found in *Sceloporus occidentalis* (Gleeson, 1979) and of 12 months in captivity on metabolic capacity in *Varanus gouldii* (Thompson, 1997). The study by Moore, Thompson, and Marler (1991) on *Urosaurus ornatus* showed an increase in plasma corticosterone and a drop in plasma testosterone after three weeks of captivity. Lastly, three months of

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captivity did not significantly affect progesterone or estradiol levels at any stage of the reproductive cycle in female *Sceloporus virgatus* (Weiss, Jennings, & Moore, 2002). The contrasting results of the aforementioned studies point to the necessity of further research on this topic. A possible reason for the low amount of research on this topic could be that studies that do not find an effect of long term captivity are published less frequently, which might indicate a publication bias. However, given the wide variety in ecology and life history among lizard species, it would make sense to investigate the effect of long term captivity on wild caught lizards on a broader scale.

In this study we quantified the physiological and behavioral impact of long term captivity (four months) on Green anole (*Anolis carolinensis*) lizards. This species is commonly kept as a companion animal (pet), especially in North America, and it has been described as an excellent reptilian model for laboratory studies of reproductive morphology and behavior in a review on the use of *A. carolinensis* in research by Lovern, Holmes, and Wade (2004). It has been used for scientific studies in a laboratory setting across a range of research fields, including behavioral biology, physiology, and morphology (e.g., Merchant, Fleury, Rutherford, & Paulissen, 2008; Montuelle, Daghfous, & Bels, 2008; Stellar & White, 2010; Waters et al., 2005). Furthermore, almost all researchers mention the use of wild caught individuals in their studies. Despite an enormous body of work available on many aspects of its behavior and ecology, to our knowledge no research exists looking at the effect of long term captivity on stress levels in *A. carolinensis*. We hypothesize that long term captivity of wild caught individuals will lead to an increase in stress and concordant changes in behavioral and physiological indices.

Materials and methods

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) between August and December 2014. Twenty-nine adult *A. carolinensis* lizards (16 males, 52–60 mm; 13 females, 50–56 mm) were obtained from a licensed commercial supplier in Belgium. The animals had been caught in August in the field in Florida, USA, sent by air to Belgium, and arrived at our lab within two days. In the laboratory, lizards were placed into individual glass terraria (30 × 40 × 70 cm, width × length × height). 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 (6:00–20:00 hr), 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) 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 fecal pellets. Each cage contained a diagonally-placed wooden perch of ± 40 cm with a diameter of 2 cm (which is the preferred perch diameter for *A. carolinensis*, Gilman, Irschick, & Fox, 2013) and two banana leaves (± 20 × 10 cm) under which lizards could hide. Animals were provided with *ad libitum* water and were 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).

Experimental design

The lizards remained under the conditions described above for the entire experimental period. At one week, one month, and four months of captivity, measurements were carried out as described hereafter. The captive conditions are based on standard practices in the literature, only cage size was larger than what is normally used in research.

Measurements

Morphometrics

We measured snout-vent length (SVL) and tail width (measured at the base of the tail, tail width is considered to be an indicator of fat deposited and hence condition, Bauwens, 1985; Avery, 1974; Donoghue, Vidal, & Kronfeld, 1998; Vervust, Lailvaux, Grbac, & Van Damme, 2008) using digital calipers (0.1 mm, Absolute, Digimatic) and body mass using an electronic balance (0.01 g, Scout pro, Ohaus). Tail width measurements were corrected for SVL by using the residuals from a linear regression of tail width against SVL.

Heterophil to lymphocyte ratio

Heterophils and lymphocytes are two types of white blood cell that play a role in the immune system of reptiles. 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 (review in Davis & Maerz 2008), including reptiles (Borgmans, Palme, Sannen, Vervaecke, & Van Damme, 2018; Case, Lewbart, & Doerr, 2005; Chen, Niu, & Pu, 2007; Lance & Elsey, 1999; Morici, Elsey, & Lance, 1997; Saad & Elridi, 1988).

Blood samples (max 60 µl) were obtained from the post-orbital sinus by inserting a capillary tube (75 mm, 60 µl) between the eye and the eyelid (MacLean, Lee, & Wilson, 1973). Animals were held in hand to immobilize them to facilitate drawing blood. The use of post-orbital sinus sampling can cause some acute stress but is unlikely to have any long term effects as Balcombe et al. (2004) have shown that in rodents and lagomorphs the acute response lasted for up to 2 hr maximum and thus should have no effect on measurements taken on following days. Langkilde and Shine (2006) showed the same result in a reptilian species (*Eulamprus heatwolei*). Our lab has extensive experience using this technique 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 minand stained with Hemacolor®, Merck Millipore. The numbers of heterophils and lymphocytes visible in 10 fields (magnification: 40 × 10, field size: 0.2 × 0.2 mm, WILD Heerbrugg M20, Switzerland) were counted and used to calculate heterophil to lymphocyte (H/L) ratios.

Behavioral observations

The behavior of the lizards in their home cage was observed from a distance of 3 min a darkened room using continuous focal animal sampling with observation software (JWatcher v1.0, Blumstein, Evans, & Daniel, 2006). All observations were performed live by the same observer (Glenn Borgmans). The duration of the following behaviors was noted over 10-min observation periods (Table 1): "sitting," "hiding," "basking," "walking," and "climbing". In addition, the number of lateral head movements, dewlap extensions, push-ups, and head nods was recorded. All observations were carried out between 9:00 and 17:00 hr, when the lizards were fully active (personal observation). Individuals were observed in a random order within this active period.

Fecal corticosterone metabolites

The traditional technique of measuring plasma levels of corticosterone to assess physiological stress in vertebrates has been criticized because acute rises in corticosterone, associated with blood

Table 1. Ethogram behavioral observations.

Behavior	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, e.g., walls of the cages, wooden bar, leaves.
Walking	Time spent moving on horizontal structures.
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 times 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).

sampling, may mask more subtle variation due to mild, prolonged stress. Instead, fecal corticosterone metabolites (FCM) can be measured with minimal disturbance of the animal, and may reflect average stress over longer time periods (Möstl & Palme, 2002; Palme, Rettenbacher, Touma, El-Bahr, & Möstl, 2005). This alternative technique has recently been used in a large variety of vertebrates, including reptiles (Borgmans et al., 2018; Kalliokoski et al., 2012; Rittenhouse, Millspaugh, Washburn, & Hubbard, 2005); details on the use and validation of FCM can be found in the literature review by Keay, Singh, Gaunt, and Kaur (2006), Palme et al. (2005) and Palme (2019).

Cages were checked three times a day (9:00, 12:00, and 15:00) for three days and all fecal pellets found were collected 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 avoid contamination. The fecal data were weighted per milligram of feces. When pellets weighed less than 10 mg (Sartorius CPA223S, 0.001 g) 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 (personal observation). To extract FCM, 0.5 ml of a 60% methanol solution (60:40, methanol:water) was added to each sample (Palme, Touma, Arias, Dominchin, & Lepschy, 2013). Samples were then mixed for 2 min using a vortex and centrifuged (at 5000 rpm) for 5 min. An aliquot of 0.1 ml from each mixture was stored at −21°C until analysis. Extracts were analyzed 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, Sachser, Möstl, and Palme (2003).

Statistical analyses

Statistical analyses were carried out with SPSS (IBM SPSS statistics v.22). All measured variables were analyzed for effects of duration of captivity, sex, and for an interaction effect between duration and sex. The assumption of normality was tested with a Shapiro-Wilk test. H/L ratio was log10-transformed and FCM levels were square root 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 duration of captivity as a within-subject factor were used to test for an effect of the duration of captivity for body mass, tail width, H/L ratio, and FCM level. Some of the behaviors (Table 1) did not occur during the observations. The only behaviors to be observed were walking, climbing, sitting, hiding, basking, and head movements. The combination of all the "passive" sitting behaviors (sitting, hiding, and basking) can be considered the inverse of the more "active" state behaviors (walking and climbing) and would yield similar results when analyzed. Therefore, only total time spent moving (combination of "walking" and "climbing") and number of head movements were analyzed using a generalized linear model (GzLM). A value of 0.5 was added to the data of the behavioral observations and the data was subsequently square root transformed to control for zero inflated data.

Results

Morphometrics

A strong difference in change of average body mass over time was found between sexes, where males had an overall higher body mass than females (Figure 1A, duration*sex effect: $F_{2,54}$ = 10.52, $P < 0.001$; sex effect: $F_{1,27}$ = 37.24, $P < 0.001$). Male body mass was found to increase significantly over time ($F_{2,30}$ = 3.77, $P < 0.05$). A 4.3% increase in body mass was found after one month of captivity and the increase after four months was found to be 13.2%. Female body mass decreased significantly ($F_{2,24}$ = 8.8, $P < 0.01$). A 5.1% decrease in body mass was found after one month of captivity and the decrease after four months was found to be 13.3%.

Tail width (corrected for SVL) changed significantly over time (Figure 1B, $F_{2,54}$ = 14.58, $P < 0.001$). The change in tail width differed between sexes (duration*sex effect: $F_{2,54}$ = 3.54, $P < 0.05$) and males were found to have an overall higher tail width (sex effect: $F_{1,27}$ = 20.64, $P < 0.001$). Based on these results, sexes were analyzed separately. Male tail width did not change significantly over time ($F_{2,30}$ = 2.42, P = 0.11), while female tail width did ($F_{2,24}$ = 17.76, $P < 0.001$). An 8.4% decrease in tail base width was found after one month of captivity and the decrease after four months was found to be 11.1%. Figure 2 shows the change in SVL over time. Female SVL increased by 1.4 mm after four months of captivity, which equates to a 2.7% increase.

Physiology

H/L ratios decreased significantly over time (Figure 2A, $F_{2,54}$ = 28.1, $P < 0.001$). The change over time was similar for both sexes (duration*sex effect: $F_{2,54}$ = 0.04, P = 0.96), but females did have overall higher H/L ratios (sex effect: $F_{1,27}$ = 10.9, $P < 0.01$). Post hoc analyses showed that H/L ratios at one week of captivity were significantly higher than at one month ($P < 0.001$) and four months of

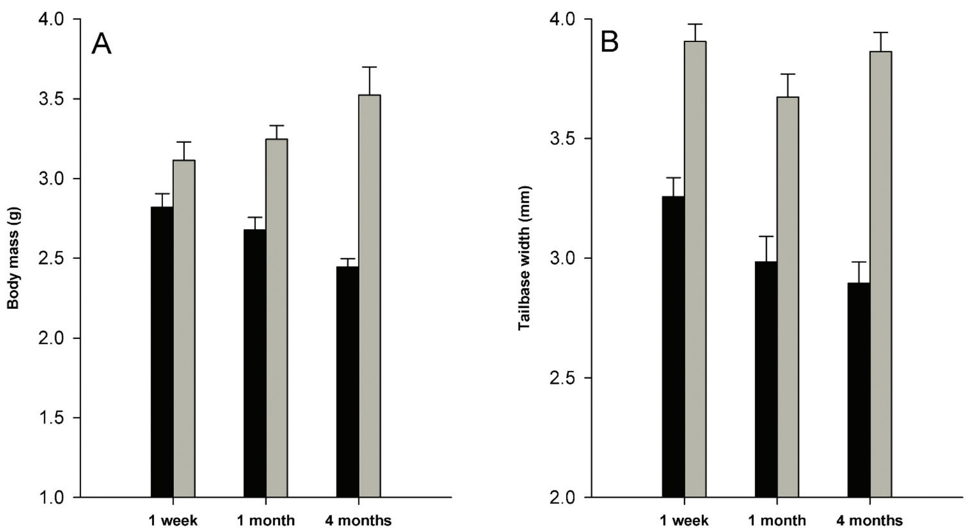

Figure 1. Body mass (A, n = 16 for males and n = 13 for females) and tail width (B, n = 16 for males and n = 13 for females) for *Anolis carolinensis* lizards after one week, one month, and four months of captivity. Indicated are means and standard errors for females (black bars) and males (gray bars).

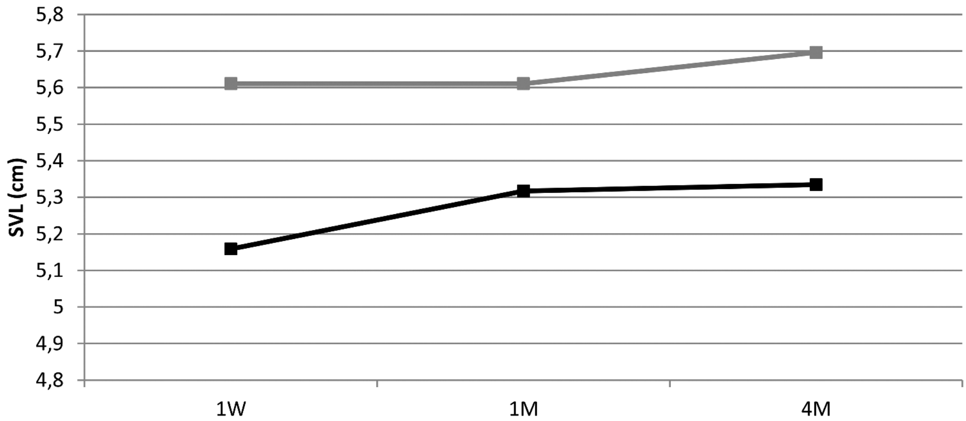

Figure 2. Average growth curves (n = 16 for males and n = 13 for females) for male (gray lines) and female (black lines) *Anolis carolinensis* lizards across time points. Time points represent, in chronological order, one week (1 W), one month (1 M), and four months (4 M) of captivity.

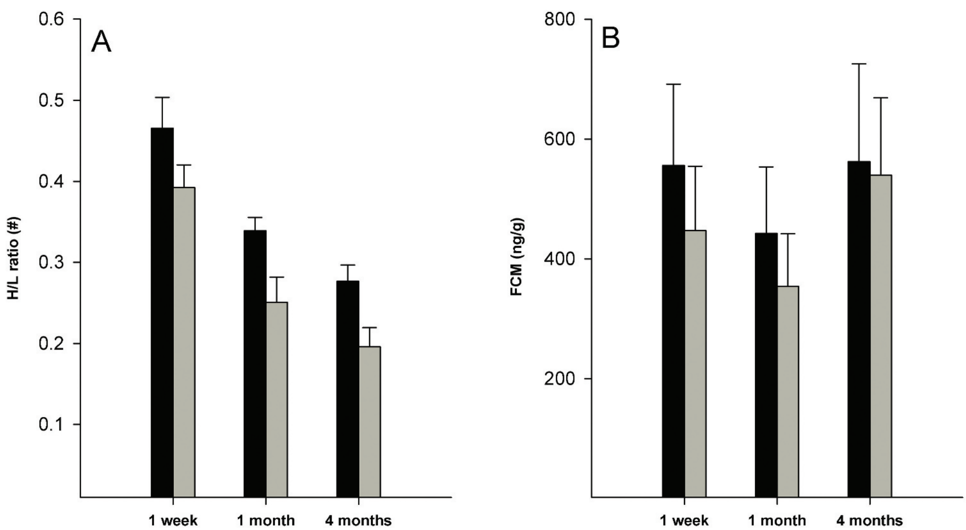

Figure 3. Heterophil–lymphocyte ratios (H/L; A, n = 16 for males and n = 13 for females) and fecal corticosterone metabolite levels (FCM; B, n = 8 for males and n = 5 for females) in *Anolis carolinensis* lizards after one week, one month, and four months of captivity. Indicated are means and standard errors for females (black bars) and males (gray bars).

captivity ($P < 0.001$). There was a trend that H/L ratios also differed between the latter two ($P = 0.06$).

FCM levels did not differ among treatments (Figure 2B, $F_{2,22} = 0.85$, $P = 0.44$), or between sexes (sex effect: $F_{1,11} = 0.64$, $P = 0.44$, treatment*sex effect: $F_{2,22} = 0.17$, $P = 0.85$).

Behavior

The total time spent moving (walking and climbing) did not differ among treatments (Figure 3A, GzLM, treatment-effect: Wald $\chi^2_2 = 4.03$, $P = 0.13$; treatment*sex interaction effect: Wald $\chi^2_2 = 3.54$, $P = 0.17$). Females did on average spend more time moving (sex effect: Wald $\chi^2_1 = 14.4$, $P < 0.001$).

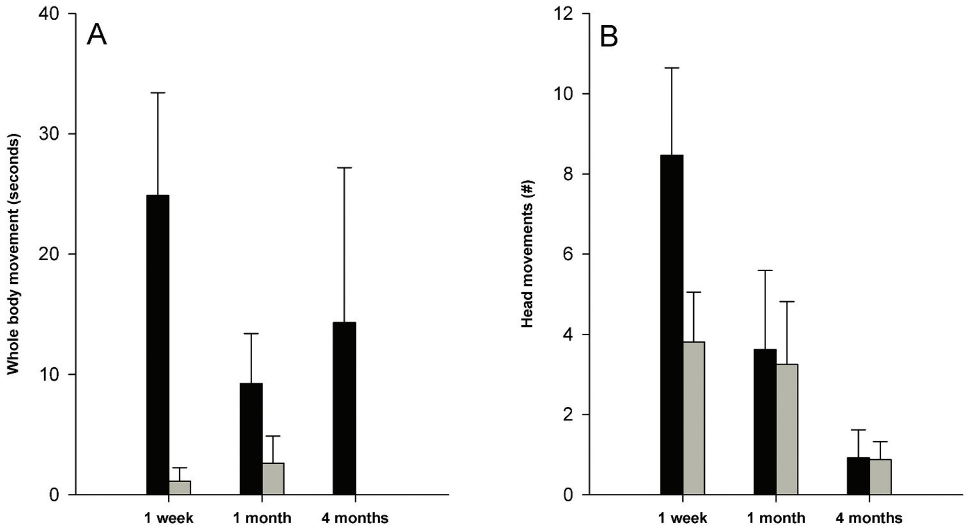

Figure 4. The time spent moving (walking or climbing, A, n = 16 for males and n = 13 for females) and the number of head movements (B, n = 16 for males and n = 13 for females) exhibited by *Anolis carolinensis* lizards after one week, one month, and four months of captivity. Indicated are means and standard errors for females (black bars) and males (gray bars).

Number of head movements decreased significantly over time (Figure 3B, GzLM, treatment-effect: Wald $\chi^2_2 = 14.06$, $P < 0.01$). The number of head movements performed after one week of captivity was significantly higher than after one month ($P = 0.058$) or four months ($P < 0.001$), but did not differ between the latter two ($P = 0.26$). No difference between sexes was found (treatment*sex interaction effect: Wald $\chi^2_2 = 3.38$, $P = 0.18$; sex effect: Wald $\chi^2_1 = 2.19$, $P = 0.14$).

Discussion

Our results showed a difference in effect of long term captivity on stress levels between male and female *A. carolinensis* lizards. Male lizards did not seem to experience any negative effects of long term captivity on physiological and behavioral measurements. Half of the measurements did not show significant differences over time. The measurements that did change were body mass, H/L ratio, and number of head movements. The low body mass and high H/L ratio in the first week of captivity and the subsequent changes of these measurements can be attributed to an acclimatization effect due to the residual stress from capture and transport by the commercial supplier; an effect which has been observed in this species in previous research (Borgmans et al., 2018, p. 2019). The change in number of head movements can be attributed to a similar acclimatization effect. It can be expected that animals are more alert (perform more lateral head movements, which is a proxy for alertness) when placed in a novel environment and that this effect will decline over time. Overall, the results for the males do not indicate a negative effect of long term captivity, which falls in line with what was found in previous research (Gleeson, 1979; Thompson, 1997; Weiss et al., 2002). These studies found no effect of 8 weeks of captivity on metabolic capacity in *Sceloporus occidentalis* (Gleeson, 1979) and of 12 months in captivity on metabolic capacity in *Varanus gouldii* (Thompson, 1997), and 3 months of captivity did not significantly affect progesterone or estradiol levels at any stage of the reproductive cycle in female *Sceloporus virgatus* (Weiss et al., 2002). Important to note here is that these results apply only to the specific circumstances in which our animals were housed and in no way should they be interpreted as a carte blanche to keep male *A. carolinensis* lizards in any circumstance.

Results for the females showed no significant differences over time for whole body movements and FCM levels. Similar to males, females' results also showed a decrease in H/L ratio and number of head movements, for which the most likely reasons are the same as for males and are discussed above. However, in contrast to the results we found for the males, females showed a decrease in both body mass and tail width over time. This could potentially indicate a negative effect of long term captivity on females. However, these morphometric results are contradictory to the results of the H/L ratio, where a decrease over time was observed. We are not sure as to what caused this difference between sexes. Previous research on Brown-Headed Cowbirds (*Molothrus ater obscurus*) also found sexual dimorphism in the effect of captivity (Day, Guerra, Schlinger, & Rothstein, 2008). Their study showed a decrease in female hippocampal size as an effect of captivity, which was strongly correlated to the species' natural behavior, where females solely look for new nest sites. Animals were kept at temperatures which maintained them in their reproductive states (Lovern et al., 2004), and some ovipositioning was observed anecdotally. It seems unlikely that the ovipositioning of a very small number of females would have caused differences to the degree observed for the body mass and tail width results. This potential negative effect of long term captivity on females deserves more attention in future research as it could potentially have an effect on studies investigating correlations between body mass and/or tail width and other factors.

When we looked at the difference in behavior between sexes, we found that females moved between 3 and 22 times more than males. We do not yet know what caused this higher activity in females. The fact that animals were kept at temperatures which maintained them in their reproductive states might be a possible reason why we observed the difference in behavior. Perhaps females were attempting to find environments better suited for ovipositioning. Another possible explanation for the difference in activity might be found in the recent paper by Kamath and Losos (2017), which is causing shifts in the traditional views of mating systems in Anolis lizards. Whereas it was long assumed that *A. carolinensis* lizards have a polygynous mating system with females mating only with the male whose territory overlapped with theirs, current views are pointing more toward a polygynandrous mating system where females can mate with multiple males. The dissertation by Gordon (1956) on the biology and biodemography of *Anolis carolinensis* has already stated that females might wander more than males and thus our results might indicate that females are more active in trying to find suitable mates. More research is needed to investigate these finding as they could influence results of future studies looking at the effect of specific factors on differences in behavior between sexes.

Conclusion

Our results show no negative effects of four months of captivity on physiological and behavioral measurements in male *A. carolinensis* lizards. Similar results for females were found for all measurements except body mass and tail width. Here our results indicate a potential negative effect of four months of captivity on body mass and tail width in females.

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