Painted turtles (*Chrysemys picta*) may not flee earlier when chronically stressed

By

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Abstract

Glucocorticoids are released in response to stressful environmental stimuli, and can result in beneficial short-term effects. However, when elevated levels of glucocorticoids are long lasting, as is the case in chronic stress, glucocorticoids can have negative effects such as reproductive shutdown, decreased immunity and growth, and a reduced ability to cope with additional stressors. In recent years, the stress response is more commonly being used as a bio-monitor for potentially threatened populations, and chronic stress may be useful in predicting the survival of stressed populations. In the interest of conservation, this study aims to answer how chronically stressed individuals react to acute stressors in nature. I hypothesized that chronically stressed individuals have a reduced ability to cope with an additional acute stressor due to elevated levels of CORT. I predicted that individuals with chronically elevated levels of CORT (i.e. 2 CORT and 1 CORT) could tolerate an approaching threat to a greater degree than those without such elevated levels of CORT (i.e. SHAM and control). Painted turtles (*Chrysemys picta*) were implanted with slow-release silastic implants containing either corticosterone (i.e. simulating chronic stress) or sham, and the responsiveness to acute stressors was measured by approaching basking individuals and measuring their flight initiation distances. Results indicate a significant effect of treatment, between 1 CORT and 2 CORT treated individuals in particular.

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Introduction

Stress can be defined as the need for an individual to focus energy on coping with a short-term threat to survival at the cost of long-term investments such as reproduction and growth (Busch and Hayward, 2009). Glucocorticoids are released in response to stressful environmental stimuli, and the primary glucocorticoid found in reptiles is corticosterone (CORT) (Homan et al., 2003; Thaker et al., 2009; Norris, 2007). When an individual perceives a threat, the hypothalamic-pituitary-adrenal (HPA) axis is activated. Activation of the hypothalamus causes an increase in the secretion of corticotropin-releasing hormone (CRH), which binds to receptors in the anterior pituitary causing it to release adrenocorticotropic hormone (ACTH) into the bloodstream (Norris, 2007). ACTH affects the interrenal tissue of the adrenal gland allowing for on-demand synthesis of CORT, which in turn regulates transcription of target genes in tissues expressing CORT receptors to mediate its effects (Norris, 2007). At the same time, but at a much faster rate, the adrenergic system is activated which results in increases in circulating levels of catecholamine hormones, adrenaline and noradrenaline, from the chromaffin cells (Norris, 2007; Sloman et al., 2002). However, for the purpose of this study the focus is on the glucocorticoid aspect of the stress response.

A variety of factors affect the levels of circulating glucocorticoids, and may include such things as taxonomic group, the environment, and the life-history stage (Busch and Hayward, 2009). For example, non-breeding Texas horned lizards, *Phrynosoma cornutum*, show average plasma CORT levels at around 5ng/ml (Wack et al., 2007) whereas non-breeding fruit bats, *Pteropus hypomelanus*, show 1270 ng/ml on average (Widmaier and Kuntz, 1993). Furthermore, glucocorticoid levels vary within a species on the basis of season, sex, age, social status, and breeding stage (Romero, 2002). For example, most reptiles tend to show peaked levels of glucocorticoids during the breeding season. Sloman et al. (2002) have also shown higher levels of cortisol in subordinate fish compared to dominant fish.

The stress response can be divided into two different levels based on the duration for which high glucocorticoids are maintained. The first level of stress is acute stress where the effects typically show a marked increase in plasma CORT levels that lasts for minutes or hours (Sloman et al., 2002). When experienced for such a short period of time, high levels of glucocorticoids usually promote behaviours and physiology that help the individual cope with unexpected challenges (Busch and Hayward, 2009). For example, they can increase cardiac tone, increase production and utilization of glucose, regulate immune system and fluid volume, and suppress behaviours that would otherwise distract the individual from survival, such as reproduction (Busch and Hayward, 2009). However, when elevated levels of glucocorticoids last for days or weeks, as is the case in chronic stress, glucocorticoids can have negative effects such as reproductive shutdown, decreased immunity and growth, and neuronal cell death (Homan et al., 2003), as resources are directed away from these processes.

Although the effects of chronic stress on metabolism, condition, and immunocompetence have been studied in a fair amount of detail, the effects of chronic stress on an individual's responsiveness to acute stress is not well understood (Sloman et al., 2002). It has been shown that high levels of glucocorticoids needed to cope with one stressor may compromise an animal's ability to cope with additional stressors (Busch and Hayward, 2009). The results of a study conducted by Barton et al. (1987) demonstrate that chronic treatment with cortisol in rainbow trout eliminated the ability of fish to demonstrate an elevation in CORT in response to handling (an acute stressor). Van de Nieuwegiessen et al. (2008) found that catfish housed at high densities demonstrated signs of chronic stress, which was reflected by an absence of a cortisol response to an acute net stressor. Sloman et al. (2002) found similar effects in chronically stressed subordinate fish in response to social stress. Alternatively, jundia fingerlings (*Rhamdia quelen*) exposed to chronic stress, which was elicited by daily soft handling in a restricted space, did in fact show an acute cortisol response to netting (Barcellos et al., 2006).

Flight initiation distance (FID) is defined as the distance between an approaching stimulus and the focal individual when this individual flees, and an approaching stimulus is commonly used to simulate a predatory approach (Thaker et al., 2008; Berger et al., 2007). Rapid plasma glucocorticoid elevations typical of an acute stress response have been demonstrated in the tree lizard (Thaker et al., 2008) and the marine iguana (Berger et al., 2007) following a simulated predator approach used in FID measurements. Thaker et al. (2008) further found a positive correlation between FID and corticosterone levels in response to acute stress.

A potential problem associated with these approaches is habituation where an individual may become less responsive to a particular stressor with repeated encounters. For example, high levels of human visitations due to tourism at Magellanic penguin (*Spheniscus magellanicus*) colonies did not respond to the human presence compared to those exposed to lower levels of visitation (Fowler, 1999). A study by Bailey et al. (2009) on cottonmouths (*Agkistrodon piscivorus*) shows that this species too might have an adaptive mechanism allowing them to not be alarmed by the presence of a potential predator.

The Committee on the Status of Endangered Wildlife in Canada (COSEWIC) has reported that reptiles are proportionately the most threatened group in Canada, which means that conservation is of important interest for these organisms. The painted turtle, however, is an exception since it is the most common turtle in eastern North America. This makes the painted turtle a good model system for reptile conservation where gaining more insight on this species could contribute to potential future conservation practices.

Recently, a new branch of conservation has emerged, conservation physiology, that examines the physiological responses of organisms to human alteration of the environment that might be of cause or contribution to population declines (Wikelski and Cooke, 2006). For example, the stress response is more commonly being used as a bio-monitor for potentially threatened populations, and elevated glucocorticoid concentrations may be useful to predict the survival of stressed populations (Homan et al., 2003). The purpose of this study, which is part of a broader scale study looking at chronic stress in relation to parasitism, is to indirectly test whether the response to an environmental disturbance is attenuated during a simulated chronic stress response through the use of CORT implants in *Chrysemys picta.*

Thus, if CORT levels can be manipulated such that elevated levels can be maintained for a long period of time, and habituation can be avoided, FID measurements, which have been shown to positively correlate with CORT levels during acute stress (Thaker et al., 2008), can be used to indirectly and behaviourally measure the responsiveness of chronically stressed individuals to an acute stressor. Therefore, this study seeks to address the hypothesis that chronically stressed individuals have elevated levels of CORT, which act to reduce their ability to cope with an additional acute stressor. I predict that individuals with chronically elevated levels of CORT (i.e. 2 CORT and 1 CORT treatments) can tolerate an approaching threat to a greater degree than those without such elevated levels of CORT (i.e. SHAM and control treatments).

Methods

Study Location and Species

The field site of this study is situated in Gatineau Park (Quebec, Canada), which is a 361-km^2 natural area that is protected by the National Capital Commission (NCC). Sampling efforts were specific to Renaud Lake (45^{o}36’10”N, 76^{o}01’24”W), which is a small lake situated just south of the town of Sainte-Cécile-de-Masham, and is connected to both Philippe Lake, and Taylor Lake. This field site is most suitable for the study due to the relatively small size of the lake, which would allow for better opportunities of recapturing individuals several times after the initial implantation and marking process. Furthermore, the lake appears to house a fairly abundant sized population of at least 123 painted turtles (calculated using the Lincoln Peterson Index).

The painted turtle (*Chrysemys picta*) was used as the model species for this study for several reasons. Firstly, this species is found in relatively large numbers in the area (i.e. Southern regions of Ontario and Quebec). Moreover, aerial basking is a common behaviour of this species, which is advantageous for finding individuals and measuring FID. Furthermore, the body size of these individuals is sufficient enough to allow for blood sampling without drastically affecting the blood volume of the individual.

Treatments

Individuals were implanted subcutaneously with slow-release silastic implants. The implants were made with 2 cm long Silastic® medical grade tubing (Dow Corning), and the ends of each implant was sealed using Silastic® medical adhesive silicone sealant. Each individual was randomly assigned a treatment type and implanted with two implants using a needle implanter; one implant was placed on the most anterior portion in each of the hind limbs. Prior to the implantation process the incision site was anaesthetized using lidocaine cream (emla® cream), and disinfected using an alcohol swab. After the implant was inserted the incision site was sealed with Krazy® glue, and then allowed to dry before the turtle was released to its capture site.

Four different treatments were used in the study (N = 42): (1) two implants filled with corticosterone (Sigma Chemical Co.; 2 CORT; n = 14), (2) two empty sham implants (SHAM; n=14), (3) one implant filled with corticosterone in the right hind limb and one empty sham implant in the left hind limb (1 CORT; n = 14), and (4) no implants (Control). The 1 CORT and 2 CORT implants were used to see a graded dose effect. Prior to implantation these implants were left in saline solution for 24 hours. Corticosterone silastic implants have been used before in side-blotched lizards (*Uta sansburiana*; Denardo and Sinervo, 1994; Denardo and Licht, 1993; Miles et al., 2007), red-eared slider turtles (*Trachemys script elegans*; Cash and Holberton, 1999), and Japanese quails (*Coturnix coturnix japonica*; Pike and Petrie, 2006). These implants are meant to simulate a prolonged physiological stress response and are known to keep plasma CORT levels elevated for up to three months (Denardo and Licht, 1993).

Turtle Capture and Processing

During the months of June, July, and early August adult individuals were initially captured using a dip net or opportunistically by hand. Blood samples (300 μL) were taken within 2-3 minutes of initial disturbance, and 30 minutes following the initial disturbance from the caudal vein using pre-heparinized syringes (0.5 cc with 28 gauge needle). This allowed for a measurement of the baseline CORT levels in blood plasma and the CORT levels in response to the approach (stress-induced), respectively, as outlined by Romero and Reed (2005). In between blood sampling individuals were kept in covered plastic bins to avoid the effect of additional stressors besides the capture itself.

After blood sampling individuals were implanted on the basis of the treatment types outlined above. A number unique to each individual was marked on the carapace with Tremclad® rust paint, and each treatment had a representative colour. Individuals were also notched on the carapace with a unique code so that an individual could still be identified during its moulting period. Other variables were also measured: carapace length (mm), carapace width (mm), carapace height (mm), plastron length (mm), weight (g), air temperature (^{o}C), water temperature (^{o}C), cloacal temperature (^{o}C), and sex. Furthermore, implanted individuals were recaptured and monitored for CORT levels in addition to these other variables over the course of the field season.

Flight Initiation Distance Data Collection

Fight initiation distances (FID) were measured in meters after the implantation period from the second week of August until the end of September to measure the responsiveness of the implanted and control individuals to an acute stressor. FID was measured as the distance it takes for an individual to flee during an approach, which was measured from the bow of the canoe to the position of the individual prior to fleeing. These measurements were made at least once per individual, however multiple measurements per individual were made when possible. FID measurements were made on 29 of the 42 individuals initially captured: 2 CORT (n = 9), 1 CORT (n =10), and SHAM (n = 10). FID measurements were also made on 21 individuals control individuals.

While in a canoe we approached basking individuals at a slow, but relatively constant speed on a typical sunny day to avoid any confounding effects due to weather and speed. The approach was practiced prior to the experiments to ensure the speed was kept constant. For reasons beyond one's control, maintaining a constant starting distance for the approach was difficult to do in the field; therefore we tried to keep the starting distance as far back as possible at approximately 20 m, which is greater than the largest observed fleeing distance. The initial body position of the turtle, which is the position of the turtle prior to the approach (i.e. facing forwards, sideways, etc.), were also noted to avoid any potential effects of body position on an individual's perception of the stressor. Water and air temperature were measured to control for the effect of temperature on a basking individual's willingness to flee. When possible, blood samples were obtained from individuals within 2-3 min and 30 min following the approach to allow for a direct measurement of the baseline CORT levels and the stress-induced CORT levels in response to the approach (n =18).

Statistical Analyses

All statistical analyses were carried out using S-PLUS® 8.0 software. The Restricted Maximum Likelihood (REML) variation of an ANCOVA was used for statistical analyses to look at the effect of treatment (1 CORT, 2 CORT, SHAM) while using repeated measures at the individual level (random effects) and controlling for all potential covariates (fixed effects) that could have an effect on an individual's CORT levels (i.e carapace length, temperature, sex). Square root transformations were used on the continuous variables to satisfy the assumptions of normality, linearity, and homoscedasity. A less complete ANCOVA including only water temperature, treatment and only the first FID measurement for each individual was used to include control individuals to see if the implantation process itself affected FID. Variables were transformed again using the square root function. To ensure that habituation did not occur among individuals for which repeated measures were made an ANOVA was run looking the square-root transformed FID against measurement time (i.e. first vs. last FID measurement), treatment, and sex. Multiple comparisons were made using Tukey HSD when significant effects were found.

Results

I chose to incorporate only water temperature in the REML model since both water and air temperatures were highly correlated ($p = 0$). Results from the REML model indicate that there is in fact a significant effect of treatment ($p = 0.0310$, df = 2, f ratio = 3.8098; Fig. 1) and sex ($p = 0.0127$) while there was no significant effect due to water temperature ($p = 0.4873$) and carapace length ($p = 0.6130$). Using a Tukey HSD multiple comparisons test a significant difference was found between the 1 CORT and 2 CORT treatments (Fig. 1). Individuals from the 1 CORT treatment fled first (mean = 8.023, SE = 2.132) while individuals from the 2 CORT treatment fled last (mean = 3.311, SE = 0.8819). CONTROL (mean = 6.108, SE = 1.116) and SHAM (mean = 4.122, SE = 1.110) treated individuals fled at intermediate distances. Results from the simplified one-way ANCOVA indicate that there is no significant effect of treatment when CONTROL individuals were incorporated ($p = 0.267$), and the mean FID is quite similar to SHAM individuals indicating that the implantation process itself likely did not have an effect on the results. Results from the ANOVA indicate that habituation did not occur among those individuals for which replicate measurements were made ($p = 0.481$).

Discussion

It was hypothesized that chronically stressed individuals have a reduced ability to cope with an additional acute stressor. It was predicted that individuals with chronically elevated levels of CORT could tolerate an approaching threat to a greater degree than those without such elevated levels of CORT. Therefore, one would expect that 2 CORT treated individuals would have a shorter mean FID in comparison to SHAM and CONTROL treated individuals, while 1 CORT treated individuals would demonstrate an intermediate mean FID. Results showed that there was a significant effect of treatment, particularly between 1 CORT and 2 CORT treated individuals. This is not quite as expected since it was predicted that mean FID in increasing order would be 2 CORT, 1 CORT, and CONTROL/SHAM meaning that a significant effect, if any, should have occurred between 2 CORT and SHAM/CONTROL individuals to be consistent with the prediction.

I am unsure of the exact reason as to why a significant treatment effect was found between 2 CORT and 1 CORT treatments. One potential explanation relates to locomotor ability. It has also been shown that chronic stress can lead to reduce levels of activity and reduce home ranges in free-ranging male side-blotched lizards implanted with CORT implants (Miles et al., 2007; DeNardo and Sinervo, 1994). It seems as though there could be an added advantage to individuals with the 1 CORT treatment that increases locomotory activity for fight-or-flight, and that the 2 CORT treatment is detrimental to the individuals in some way reducing locomotory activity. Potentially, levels of corticosterone high enough to promote chronic stress were achieved only in the 2 CORT treatment.

Another plausible explanation for the observed treatment effect is related to the fact that chronic stress has been shown to decrease an individual's performance at memory-related skills (Busch and Hayward, 2009). Thaker et al. (2009) used male eastern fence lizards (*Sceloporus undulates*) to look at the importance of acute CORT elevation in aversive learning of a novel stressor. They found that by inhibiting CORT elevation during a simulated novel encounter, using an FID approach, immediate escape responses were impaired. In addition, their results showed that FID increased with repeated encounters for control individuals whereas FID stayed the same when CORT elevations were inhibited, indicating that there was limited learning and recall during repeated encounters. In the current study, it is possible that if chronic stress was only achieved by 2 CORT treated individuals, escape responses could have been impaired since the acute response would be attenuated, whereas the acute response in the 1 CORT treated individuals would proceed normally allowing for escape.

Although the treatment effect was between 1 CORT and 2 CORT treated individuals the significant treatment effect itself indicates that there is evidence supportive of the idea that chronic stress may affect this population's ability to respond to a human disturbance (i.e. an acute stressor) in their natural environment. This is in line with results from previous studies showing a decreased responsiveness to additional acute stressors during chronic stress in trout (Sloman et al., 2002), and catfish (Nieuwegiessen et al., 2008). However, there is a question of whether or not these implants provoke more or less dramatic responses than naturally occurring stress would do. It is known that CORT implants are not a true mimic of stress, but they still provide a valuable tool to examine a key part of the stress response, the rise in circulating glucocorticoids, as was explored in this study (DeNardo and Sinervo, 1994).

It is important to note that there are several limitations to this study. Firstly, this study was limited to a single field season, which ultimately limited the time available for data collection. This affected the total sample size we were able to collect, and as a result, the sample size for each treatment was rather small (N = 42, n =14). Also, CORT samples were collected to look at the dose effect of the implants and to look at the direct correlation between FID and CORT levels, which could contribute to the interpretation of these results, but these have yet to be analyzed. Furthermore, even though effort was put towards experimentally and statistically controlling for potential covariates to the stress response there are inevitably factors for which are beyond our control.

There are several implications to this study. As previously mentioned, the stress response is more commonly being used as a bio-monitor for potentially threatened populations, and elevated glucocorticoid concentrations could be useful in predicting the survival of stressed populations (Homan et al., 2003). Reptiles are proportionally the most threatened group in Canada (COSEWIC). Thus, this study uses a commonly distributed reptile, the painted turtle, as a model to integrate into the new discipline of conservation physiology (Wikelski & Cooke, 2006) where knowledge of the effects of chronic stress on this species that may result from anthropogenic alterations to the environment can give some insight into potential future conservation practices for reptiles in general. This study was carried out in the natural environment, which provides a more realistic setting with which to indirectly measure stress responsiveness. There has been little work, if any, done on stress physiology of the painted turtle; therefore this study sheds some light onto the stress response in turtles, and reptiles alike. In addition, there is continued uncertainty as to the effects of chronic stress on the responsiveness to acute stress, and few studies have looked at this from a behavioural perspective.

In conclusion, it is becoming increasing more obvious that chronic stress can be detrimental to individuals, and ultimately entire populations. Future efforts should be directed towards documenting CORT responses to flight to look at their correlation. It could be interesting to conduct a similar study in a laboratory setting to see how indicative such a setting is in comparison to the natural environment. If results were similar this would allow for more comprehensive studies with larger samples sizes to be done more efficiently. Future studies should also look at the idea of locomotor activity during chronic stress, which could involve implanting individuals and tracking their activity and movement patterns through the use of transmitters. Furthermore, future studies could look at aversive learning in response to chronic stress by seeing how individuals respond to regular and repeated exposures to predatory encounters.

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Figures

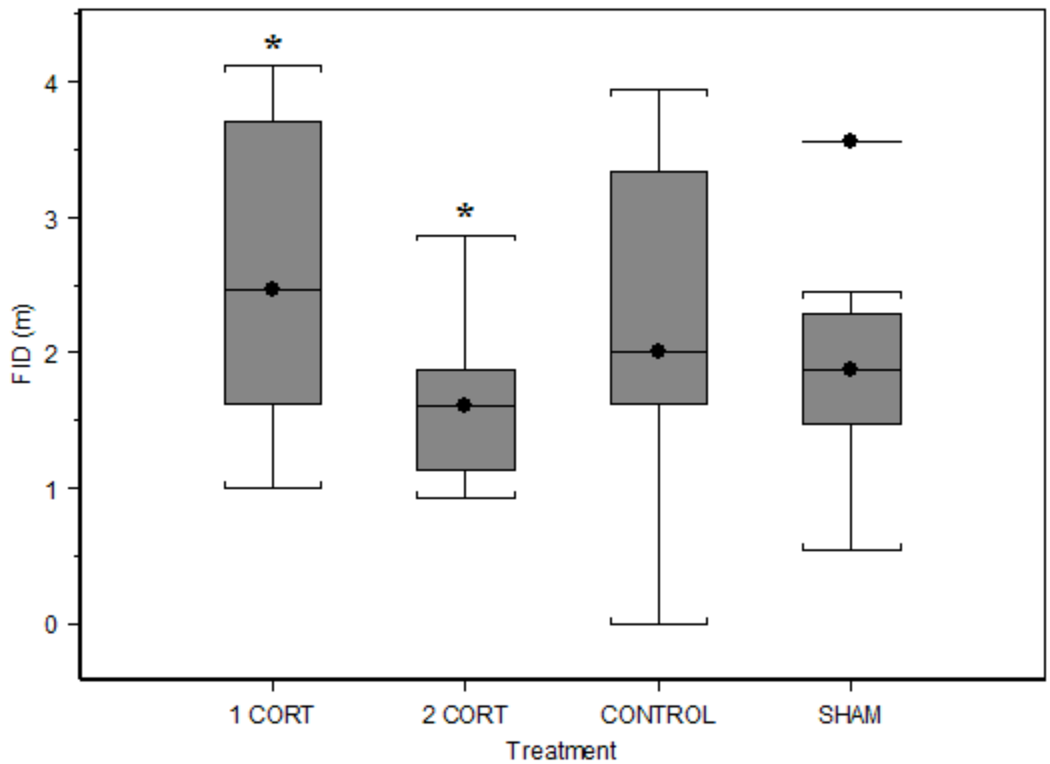

Figure 1: Mean FID (m) on a square root scale with the upper and lower limits corresponding to the 25th and 75th percentiles for each respective treatment type. The "*" indicates between which treatments a significant effect was observed ($p = 0.031$).