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Behavioural and hormonal responses to capture stress in the male red-sided garter snake, *Thamnophis sirtalis parietalis*

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We measured the behavioural and hormonal responses to capture stress in male red-sided garter snakes. Four hours of capture stress resulted in no suppression of mating behaviour relative to control individuals. In contrast, the same stress resulted in a significant increase in plasma levels of corticosterone and a significant decrease in plasma levels of testosterone. There was a significant negative correlation between plasma levels of corticosterone and testosterone in both control and capture-stress groups, suggesting that the increase in corticosterone directly drives the decrease in testosterone. While there was no relation between body size and initial plasma levels of the two steroids, longer individuals had a significantly greater increase in corticosterone following capture stress than did shorter individuals. Snakes display indeterminate growth, suggesting that older individuals have decreased sensitivity to negative feedback in the hypothalamic–pituitary–adrenal axis and thus hypersecrete glucocorticoids. These results suggest that male red-sided garter snakes have uncoupled their behavioural stress response from their hormonal stress response to maximize reproductive opportunities.

During the breeding season, the hormonal and behavioural responses of an animal to a stressor are dependent on many factors including the individual's reproductive state and the environment it inhabits (Greenberg & Wingfield 1987; Wingfield 1988, 1994; Moore et al. 1991; Wingfield et al. 1992; Dunlap & Wingfield 1995). The hormonal stress response is typically seen as an increase in plasma glucocorticoid hormones (corticosterone in reptiles), while the behavioural response can be manifested as suppression of reproductive behaviours (Greenberg & Wingfield 1987). This generalized reproductive suppression occurs primarily because of negative interactions between the hypothalamic–pituitary–adrenal (HPA) axis, responsible for glucocorticoid release, and the hypothalamic–pituitary–gonadal (HPG) axis, responsible for sex steroid release (Greenberg & Wingfield 1987; Rivier & Rivest 1991). Where reproductive opportunities are limited, some animals will suppress their stress response (Wingfield et al. 1992, 1995). Although suppressing sensitivity or activity of the HPA axis could increase mortality, in some environments the benefit of increased reproductive success outweighs this potential cost (Wingfield et al. 1998).

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Most reptiles studied to date respond to capture stress and short-term confinement with increases in plasma corticosterone levels (reviewed by Lance 1990; Tyrrell & Cree 1998) and decreases in plasma testosterone (Moore et al. 1991; Knapp & Moore 1995, 1997). No studies in reptiles have investigated both the hormonal and behavioural effects of stress on reproduction. However, an amphibian, the rough-skinned newt, *Taricha granulosa*, has served as a model for understanding the behavioural effects of stress and corticosterone on reproductive behaviour. Short-term stress as well as exogenous corticosterone have been demonstrated to suppress reproductive behaviour directly (Moore & Miller 1984). We used an estimated effect size for the suppressive effects of capture stress on mating behaviour from Moore & Miller (1984) to investigate whether the male red-sided garter snake shows a similar suppression of reproductive behaviour in response to capture stress.

Because of its unique life history in Manitoba, Canada, the red-sided garter snake is a good model for investigating adaptations of the stress response. It has a short 4-month activity period each year followed by 8 months of winter dormancy. Mating occurs immediately upon emergence in the spring, in large mating balls of up to 100 males courting a single female, while plasma sex steroid levels are decreasing, gonads are regressed and glucocorticoid levels are elevated (Crews et al. 1984; Krohmer et al. 1987; Whittier et al. 1987). We

Table 1. Ethogram of courtship behaviour for the male red-sided garter snake, *Thamnophis sirtalis parietalis**

Courtship score	Description
1.0	Male investigates female, increased tongue-flick rate
2.0	Male chin rubs female with rapid tongue-flicks
3.0	Male aligns body with female
4.0	Male actively tail searches and attempts cloacal apposition and copulation with female; possible caudocephalic waves
5.0	Male copulates with female

*Modified from Crews et al. (1984).
Behaviours 3.0 and greater only occur during breeding.

hypothesized that males would suppress their behavioural and hormonal stress response during the brief breeding season to avoid the potentially deleterious effects on reproduction. We base this hypothesis on previous observations suggesting that male red-sided garter snakes are behaviourally and hormonally resistant to natural stressors and capture (Krohmer et al. 1987; Whittier et al. 1987).

METHODS

We subjected male red-sided garter snakes to a capture-stress protocol (e.g. Wingfield 1994) that involved capture and isolation in a small cloth bag to determine their behavioural response and the sensitivity of the HPA and HPG axes to stress. All field studies were conducted at the Narcisse Wildlife Management Area in the Interlake region of Manitoba, Canada, during May 1997. All animals were held in captivity for less than 24 h and then returned to the site of capture.

Behavioural Stress Response

To describe the effects of capture stress on mating behaviour, we randomly captured 72 males, numbered each male on the head with a paint pen and randomly assigned them to capture-stress or control groups ($N_1=N_2=36$). Capture-stress males were held singly in cloth bags (20 × 20 cm) for 4 h prior to behavioural trials, while controls were tested immediately upon capture. We conducted mating behaviour trials with three capture-stress and three control males simultaneously introduced to a single female in a nylon cloth arena measuring 1 × 1 × 1 m. Males were introduced in groups of six to mimic natural conditions, where mating balls rarely contain fewer than five males courting a single female (Joy & Crews 1985). The observer was blind to the treatment group of each male. Males were observed for a period of 2 h after introduction or until mating occurred. Using an ethogram of male garter snake mating behaviour (Table 1), we recorded the highest score achieved by each male during the trial. We compared the distribution of maximal mating scores between the capture-stress and control groups. To compare our results with previous studies (Moore & Miller 1984), in which animals were scored as displaying courtship behaviour or not, male red-sided garter snakes achieving a score of 3.0 or greater were considered 'courting males' as these behaviours are only seen in the context of male courtship. With an effect size of 0.80, based on a Fisher's exact test, and sample sizes of $N_1=N_2=36$, we would have a good chance of detecting effect sizes of the magnitude reported in Moore & Miller (1984).

Hormonal Stress Response

We examined the hormonal response to stress in male red-sided garter snakes by analysing plasma hormone levels in control and capture-stress individuals. We randomly captured 18 males from the den site and stressed them by isolating them individually in cloth bags (20 × 20 cm) for 4 h, after which we obtained a blood sample. We randomly captured another group of 18 individuals and bled them within 90 s (mean 53 s) of capture. We obtained all blood samples between 1600 and 1720 hours on 5 and 6 May to avoid diel and seasonal variation in plasma hormone levels.

We obtained blood samples from the caudal vein using heparinized 1-cm^3 syringes and 25-g needles. Blood samples were stored on ice until we returned to the field station where the samples were centrifuged and the plasma separated. The plasma was then stored at −4°C until return to Oregon State University, where it was stored at −60°C until assayed.

We obtained body size measurements of snout–vent length (SVL) and body mass for each individual. Body condition was defined as each individual's residual from the regression of body mass on SVL for the population. A positive residual would indicate an above-average mass for a given SVL, while a negative residual would indicate the opposite.

Radioimmunoassay

We measured plasma levels of testosterone and corticosterone by radioimmunoassay following the procedures of Moore (1986) with slight modification. Briefly, plasma samples were fractionated on celite microcolumns to separate the steroids from one another and from neutral lipids, which interfere with the assay. Samples were assayed in duplicate and corrected for individual recovery variation. All samples were run in a single assay with an intra-assay variation of 14% for corticosterone and 10% for testosterone calculated from an assay of standards ($N=16$) by I.T.M.

Statistics

All statistical analyses were performed using Jandel SigmaStat version 2.0 software package (Jandel Corporation). Level of significance was $P<0.05$.

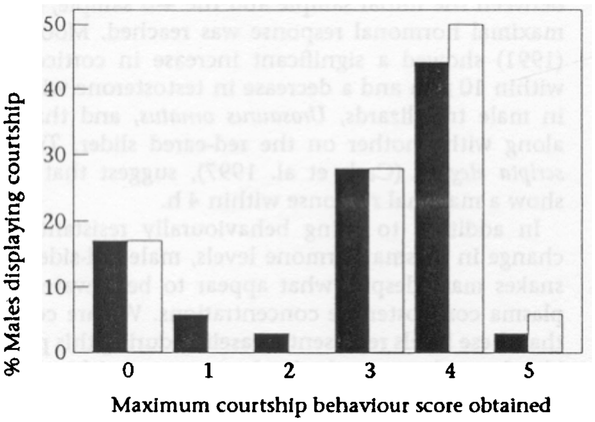

Figure 1. The distribution of maximal mating scores between control (□; N=36) and capture-stress (■; N=36) male red-sided garter snakes. Each individual was given the highest score it displayed during the mating trial.

RESULTS

Behavioural Stress Response

There was no difference between the distribution of maximal mating scores between the capture-stress and control groups (Fig. 1; chi-square analysis of contingency tables: χ^2_5=3.451, P=0.631, power=0.246). There was no difference in the percentage of male red-sided garter snakes receiving a score of 3.0 or better between capture-stress and control groups (Fig. 1; chi-square test: χ^2_1= 0.337, P=0.562, power=0.084). Power analysis (Cohen 1988) based on an effect size of 0.80, an alpha of 0.05, and our sample size (N_1=N_2=36) suggests we would have a greater than 99% chance of detecting a difference of similar magnitude to that reported by Moore & Miller (1984).

Hormonal Stress Response

Males responded to 4 h of capture stress with a significant increase in plasma corticosterone levels (Fig. 2a; Mann–Whitney U test: U=11, N_1=N_2=18, P<0.001) and a significant decrease in plasma testosterone levels (Fig. 2b; Mann–Whitney U test: U=20, N_1=N_2=18, P<0.001). Plasma levels of corticosterone increased 2.8 times during the 4 h of stress, while testosterone decreased 5.6 times. There was a significant overall negative correlation between testosterone and corticosterone levels (linear regression: R^2=0.678, N=36, P<0.001). In the control group, there was no relationship between the time it took to obtain the initial bleed and plasma testosterone or corticosterone levels (linear regression: testosterone: R^2=0.096, N=18, P=0.21, power=0.24; corticosterone: R^2=0.045, N=18, P=0.40, power=0.13).

There was also no correlation between SVL and initial levels of testosterone and corticosterone (Fig. 3; linear regression: corticosterone: R^2=0.0033, N=18, P=0.82, power=0.041; testosterone: R^2=0.0026, N=18, P=0.84, power=0.039). In addition, there was no correlation between SVL and testosterone levels in animals stressed for 4 h (linear regression: R^2=0.0015, N=18, P=0.88, power=0.035). However, longer males subjected to 4 h of capture stress had significantly higher levels of corticosterone than did shorter males (Fig. 3; linear regression: R^2=0.23, N=18, P=0.042). There was a significant positive relationship between SVL and body mass in the

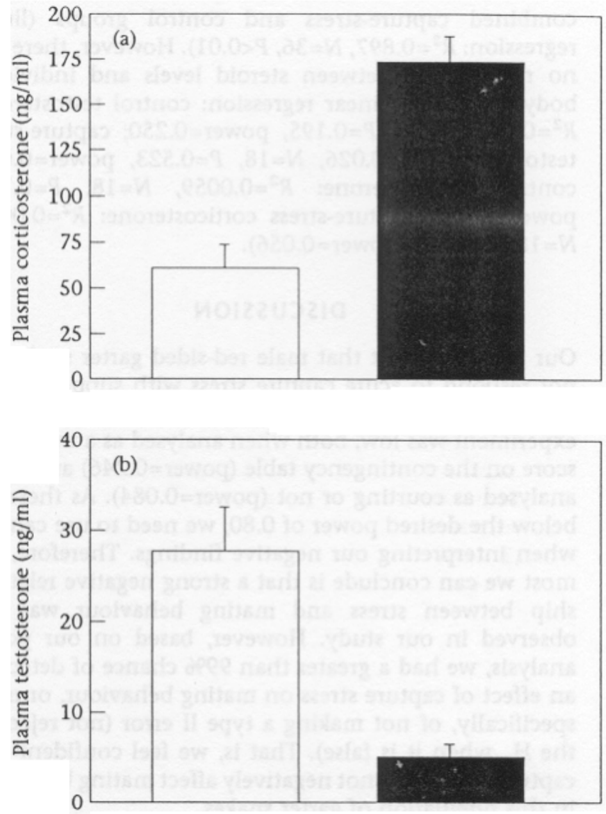

Figure 2. Mean+SE plasma levels of (a) corticosterone and (b) testosterone for control (□; N=18) and capture-stress (■; N=18) male red-sided garter snakes.

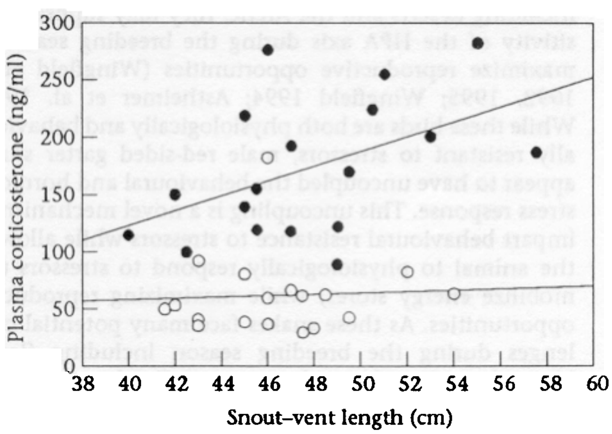

Figure 3. Regression of plasma corticosterone levels on snout–vent length for control (○) and capture-stress (●) male red-sided garter snakes.

combined capture-stress and control groups (linear regression: R^2=0.897, N=36, P<0.01). However, there was no relationship between steroid levels and individual body condition (linear regression: control testosterone: R^2=0.103, N=18, P=0.195, power=0.250; capture-stress testosterone: R^2=0.026, N=18, P=0.523, power=0.092; control corticosterone: R^2=0.0059, N=18, P=0.763, power=0.048; capture-stress corticosterone: R^2=0.0090, N=18, P=0.708, power=0.056).

DISCUSSION

Our results suggest that male red-sided garter snakes do not respond to acute capture stress with suppression of mating behaviour. However, the statistical power of this experiment was low, both when analysed as a maximum score on the contingency table (power=0.246) and when analysed as courting or not (power=0.084). As these are below the desired power of 0.80, we need to use caution when interpreting our negative findings. Therefore, the most we can conclude is that a strong negative relationship between stress and mating behaviour was not observed in our study. However, based on our power analysis, we had a greater than 99% chance of detecting an effect of capture stress on mating behaviour, or more specifically, of not making a type II error (not rejecting the H_o when it is false). That is, we feel confident that capture stress does not negatively affect mating behaviour in this population of garter snakes.

Suppression of the stress response during the mating season has been described in other classes of animals, but a different mechanism appears to be responsible. Studies in birds have demonstrated that in severe habitats, including deserts and the Arctic, they may suppress sensitivity of the HPA axis during the breeding season to maximize reproductive opportunities (Wingfield et al. 1992, 1995; Wingfield 1994; Astheimer et al. 1995). While these birds are both physiologically and behaviourally resistant to stressors, male red-sided garter snakes appear to have uncoupled the behavioural and hormonal stress response. This uncoupling is a novel mechanism to impart behavioural resistance to stressors while allowing the animal to physiologically respond to stressors (e.g. mobilize energy stores) while maximizing reproductive opportunities. As these snakes face many potential challenges during the breeding season including floods, blizzards and predation by crows (I. T. Moore, personal observation) the ability to respond physiologically without suppressing mating behaviour would be adaptive.

Although male red-sided garter snakes do not respond to capture stress by suppressing mating behaviour, they do show a significant increase in plasma corticosterone and a significant decrease in plasma testosterone. This is similar to the hormonal stress response shown by most other reptiles (reviewed by Lance 1990; Tyrrell & Cree 1998). In addition, we found a strong negative correlation between plasma levels of corticosterone and testosterone, which suggests a direct negative interaction between the HPA and HPG axes. However, we do not know how quickly the increase in corticosterone or the decrease in testosterone occurred, as samples were not obtained between the initial sample and the 4-h sample, or if the maximal hormonal response was reached. Moore et al. (1991) showed a significant increase in corticosterone within 10 min and a decrease in testosterone within 4 h in male tree lizards, *Urosaurus ornatus*, and that study along with another on the red-eared slider, *Trachemys scripta elegans* (Cash et al. 1997), suggest that reptiles show a maximal response within 4 h.

In addition to being behaviourally resistant to the change in plasma hormone levels, male red-sided garter snakes mate despite what appear to be elevated initial plasma corticosterone concentrations. We are confident that these levels represent a baseline during this period as blood samples were obtained on average within 1 min of capture and there was no correlation between initial corticosterone and testosterone levels and the time to bleed. In addition, the hormone levels we report correspond well with levels previously reported in this species (Krohmer et al. 1987; Whittier et al. 1987). The initial elevated levels of corticosterone probably serve to mobilize energy stores during the energetically demanding period of courtship. During this 3–4-week period, males do not feed and lose almost 1% of their body mass each day (I. T. Moore, unpublished data). The ability of male red-sided garter snakes to mate with elevated corticosterone levels may represent an adaptation to the harsh environment, lack of food and limited breeding opportunities.

We found initially elevated levels of testosterone that decreased rapidly following the onset of capture stress. These high initial levels are in contrast to Crews et al. (1984) but are in accord with Krohmer et al. (1987) and Weil (1985). Our samples were obtained during the first week of spring emergence, similar to when Krohmer et al. (1987) found that testosterone levels were elevated before declining over the following 3 weeks. We hypothesize that the decrease in plasma testosterone following capture stress is representative of a decreased rate of production of the steroid from the testes.

In contrast to our findings on male red-sided garter snakes, Whittier et al. (1987) reported that during the spring emergence, females caught immediately after mating responded to 6 h of capture stress with a significant decrease in corticosterone and a spike in oestrogen. The activity of the HPG axis in response to mating could suppress the HPA axis resulting in no hormonal stress response. Our protocol needs to be performed with females to determine whether the difference is a result of techniques or sexual dimorphism.

While we saw no relation between SVL of individuals and their initial plasma corticosterone levels, we did see a significant trend for longer animals to show a greater increase in corticosterone in response to capture stress than shorter ones. No such trend in testosterone was evident. This was not a simple effect of body condition as there was no relationship between individual body condition and plasma levels of corticosterone or testosterone. While there is a lot of individual variation (Waye & Gregory 1998), snakes display indeterminate growth, thus longer individuals are generally older than shorter individuals (Fitch 1965). There is precedence, in laboratory rats, for older individuals to hypersecrete glucocorticoids in response to stress due to decreased sensitivity to negative feedback of the HPA axis (e.g. Sapolsky et al. 1986). We believe this is the first study to suggest hypersecretion of glucocorticoids in aged individuals of a free-living animal.

To our knowledge no previous studies have demonstrated an uncoupling of the physiological stress response from the behavioural stress response. We hypothesize that this is an adaptation to the unique life history traits displayed by red-sided garter snakes. The possession of a hormonal stress response that could mobilize energy stores, yet not suppress mating behaviour, would enable these animals to mate during challenging periods. Future studies will attempt to determine whether this uncoupling of the behavioural stress response from the hormonal stress response is adapted to this specific environment or is a conserved trait of garter snakes.

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