Corticosterone concentrations in free-living spotted salamanders (Ambystoma maculatum)

Rebecca Newcomb Homan,* J. Michael Reed, and L. Michael Romero

Department of Biology, Tufts University, Medford, MA 02155, USA

Accepted 12 October 2002

Abstract

A non-lethal technique for drawing repeated blood samples from spotted salamanders (Ambystoma maculatum) was used to examine sex, seasonal, and capture technique differences in the physiological stress response under natural, undisturbed conditions. Baseline and stress-induced (30 min of handling and restraint) corticosterone (CORT) concentrations were measured at night during the spring migrations into and out of the breeding pond, as well as during the fall migration to over-wintering sites. Females had significantly higher CORT concentrations than males during the spring migration toward the breeding pond, but this difference was not present when animals emerged from the pond post-breeding or during the fall migration. CORT concentrations did not vary seasonally, but during the acute stress response, CORT concentrations nearly doubled during the spring inbound migration, exhibited a nearly significant increase during the spring outbound migration, and did not change in the fall. Allowing animals to sit overnight in the buckets in which they were captured elicited CORT concentrations that tended to be higher (although not statistically significant) than when animals were sampled on the night of capture. The ability to sample blood from spotted salamanders using a non-lethal technique offers an opportunity to better understand both amphibian physiology and ecology.

Keywords: Amphibian; Stress; Wild animal; Conservation; Glucocorticoid; Season

1. Introduction

Alterations to natural habitat caused by human activity often lead to stress, which in turn can have a significant influence on the survival and reproduction of native animal populations (Wingfield and Romero, 2001). In vertebrates, a classic response to an acute stress is the release of glucocorticoids, a class of steroid hormones that promote physiological and behavioral changes, such as rapid glucose production and the temporary cessation of reproduction (Sapolsky et al., 2000). Although enabling an animal to better survive a short-term stressful event (Sapolsky et al., 2000; Wingfield and Romero, 2001), these same glucocorticoid-mediated effects can be damaging in the long term, under situations of chronic stress where they can cause, among other things, neuronal cell death and complete reproductive failure (Sapolsky, 1992; Wingfield and Romero, 2001). The glucocorticoid response to stress can be measured with minimal danger to the animal, allowing an assessment of the animals’ health. This assessment is just beginning to be used by conservation biologists to identify populations in need of protection and in evaluating risk management strategies (e.g., Creel et al., 1997; Hopkins et al., 1997; Wassser et al., 1997; Homan et al., in press). Whereas relatively high glucocorticoid concentrations could be useful in identifying populations at high risk due to chronic stress, it is important to have basic information on a species’ typical glucocorticoid concentrations and responses, so that chronic elevations can be identified in presumed high-risk populations (Romero et al., 2000).

Amphibians may be an ideal taxon on which to use such endocrinological techniques to identify threatened populations or species. Many different threats seem to be facing various amphibian species, including pollution (Hopkins et al., 1997), ultraviolet radiation (Blaustein et al., 1994), and climate-induced pathogen outbreaks (Kiesecker et al., 2001). However, the greatest threat to
Spotted salamanders are pond-breeding amphibians that are common in the deciduous forests of eastern North America. They spend greater than 11 months of the year in the terrestrial habitat surrounding their breeding pond. In eastern Massachusetts, individual salamanders begin emerging from their over-wintering sites during the first cool rainy nights of early spring (generally late February to late March—pers. observation), and it may take a few rainy nights for an individual to reach the pond (Downs, 1989). In the pond, courtship followed by internal fertilization occurs, and the females deposit their eggs (Downs, 1989). Although the inbound breeding migration for the population can last for nearly 6 weeks, individuals generally spend between a few days and 3 weeks in the pond (Shoop, 1968), so there is a window in which animals are simultaneously entering the pond to breed and leaving the pond post-breeding. The only other major migratory event occurs during the fall, when climatic conditions are again cool and rainy. Animals then move around the terrestrial upland in no standard direction (Windmiller, 1996). Duellman (1954) proposed that this fall movement enables animals that were in sub-optimal over-wintering habitats to locate better spots before the coming cold and snow. We hypothesize that there may be seasonal differences in glucocorticoid levels, as there are in some amphibians (e.g., Dupont et al., 1979; Licht et al., 1983; Zerani and Gobbetti, 1993) and other vertebrates (Romero and Remage-Healey, 2000; Romero et al., 1997; Wingfield et al., 1994) and so we define three migratory seasons for our study: spring inbound to the breeding pond, spring outbound, and fall.

In this paper, we used a non-lethal technique to sample blood from wild, free-living spotted salamanders in a habitat largely undisturbed by humans. We compared both unstressed (baseline) and stress induced (30 min of handling and restraint) concentrations of corticosterone (CORT), the primary glucocorticoid in amphibians (Jungreis et al., 1970). Sex and seasonal comparisons of CORT concentrations established a CORT profile for an apparently healthy population of spotted salamanders.

2. Methods

2.1. Animals

Spotted salamanders were captured during the springs of 1999, 2000, and 2001 and in the falls of 1999 and 2000, in the terrestrial habitat surrounding a semi-permanent breeding pond in Sudbury, Massachusetts (N42°22′, W71°25′). Animals were captured from April 9 to April 24 and from October 20 to November 2 in 1999, from March 16 to April 22 and from October 5 to November 10 in 2000, and from April 6 to May 12 in 2001. Differences in capture dates reflect differences in onset of climatic conditions that facilitate migration. Spring captures were made in two phases: (1) as salamanders were approaching the breeding pond from their terrestrial hibernacula; and (2) as salamanders were leaving the pond post-breeding. These samples overlapped somewhat in time, as discussed above. Fall captures included salamanders moving within the terrestrial habitat, presumably searching for better over-wintering locations before the first snowfall (Duellman, 1954). Animals were captured only once within each season, and given that we captured a small percentage of the total population for blood sampling, it is unlikely that animals were recaptured across years.

We used a system of drift fencing and pit fall traps to capture animals in all seasons (Dodd and Scott, 1994). This fencing is composed of black mesh (siltation) fencing buried approximately 0.2 m below ground and extending approximately 0.8 m above ground. Every 15 m, paired five-gallon (19 L) buckets were buried on either side of the fence so that the lip of the bucket was at ground level. Each bucket had a moist sponge on the bottom to help prevent animal desiccation. On rainy nights salamanders moving in the terrestrial habitat encounter the fences, and they walk along them until they eventually fall into a bucket. We captured animals in three ways to obtain blood samples. First, we walked the fences on rainy nights and captured salamanders moving along the fences, with the assumption that encountering the fence caused them no stress. Second, we collected salamanders that had already fallen into a bucket. These animals could have been trapped in the bucket for any time between a few minutes and 4 h. Finally, we obtained some blood samples from salamanders that had been in buckets all night. In this last case, animals were bled in the morning following a rainy night and could have been in the buckets anywhere between 5 and 13 h. All protocols were approved.
by the Tufts University Animal Care and Use Committee.

2.2. Bleeding technique

Animals were bled within 3 min of their being observed. Salamanders were subsequently held in small plastic containers for 30 min before having a second blood sample drawn to determine the CORT response to capture, bleeding, and handling. A few wet leaves added to the plastic containers provided moisture to prevent desiccation of the animal. Following the second bleed, salamanders were sexed by examining the relative swoleness of the cloaca (Downs, 1989) and returned to their original capture points.

Blood samples were collected by puncturing the ventral side of the tail along the midline and close to the cloaca with a 21 gauge needle. We scraped the sides of the tail vertebrae with the needle to break blood vessels on the outside of the bone. The upwelling blood was collected with heparinized microhematocrit capillary tubes (Fisher) and stored on ice for 2–18 h. Blood flow rarely exceeded our needs, but when necessary, pressure applied to the wound for less than 1 min stopped any bleeding. Samples were centrifuged at approximately 400 g for 10 min, and the plasma was removed and stored at −20 °C for future analyses.

2.3. Radioimmunoassay and statistical analysis

Plasma CORT levels were measured using radioimmunoassay, as previously described (Wingfield et al., 1992). Briefly, CORT was extracted with redistilled dichloromethane and resuspended in phosphate buffer. We used a small amount of tritiated corticosterone (New England Nuclear, Massachusetts) to determine the recovery of corticosterone through the assay. Samples were compared to a standard curve and adjusted by the percent recovery to determine actual concentrations of corticosterone. Two CORT antibodies that differed in sensitivity were used (B21-42 and B3-163 from Endocrine Sciences). Consequently, we standardized all assays using a pool of starling plasma (Sturnus vulgaris) as a constant. This starling pool had a 30% inter-assay variation between the two respective antibodies, thus requiring the standardization across assays. The intra-assay variation ranged from 2 to 16%. Samples below the detection limit for each assay were assigned the detection limit for statistical analysis. Twelve percent of spring inbound samples and 3% of spring outbound samples were below the detection limit of 1.70 ng/mL, whereas 12% of fall samples were below the detection limit of 3.01 ng/mL. The low spring samples were exclusively from males, the low fall samples were evenly divided among males and females.

2.4. Statistical analysis

We compared baseline and stress-induced CORT using repeated measures ANOVA with sex, season, and capture method as factors. Factors that had no main or interaction effects were removed in a stepwise fashion and new ANOVAs were run with subsequent independent variables removed. All comparisons of hormone concentrations were conducted using SYSTAT (6.0.1 student version).

3. Results

3.1. Sex and seasonal differences

Female spotted salamanders had significantly higher CORT concentrations than did males during the nocturnal spring inbound migration (F = 4.339, df = 1, 22, p = 0.049, Fig. 1a). There were no sex differences, however, during the spring outbound migration or during the fall migration (F = 0.227, df = 1, 8, p = 0.646 and F = 0.046, df = 1, 43, p = 0.832, respectively, Figs. 1b and c). Spotted salamanders demonstrated a significant CORT response to capture, bleeding, and handling during the spring inbound migration (although the sample sizes were small, F = 3.946, df = 1, 8, p = 0.082, Fig. 1b) and no response during the fall (F = 2.017, df = 1, 43, p = 0.1627, Fig. 1c). In no case was there a significant interaction between sex and CORT response (p > 0.05).

When sexes were analyzed separately, neither females (F = 0.089, df = 2, 27, p = 0.915) nor males (F = 0.769, df = 2, 46, p = 0.470) expressed differences in CORT concentrations among the three seasons. Because there was no sex difference within two seasons (spring outbound and fall), we combined sexes and retested for seasonal differences, but again there was no significant difference in CORT concentrations (F = 0.305, df = 2, 76, p = 0.738).

3.2. Capture technique differences

We tested whether capture technique affected corticosterone concentrations. We looked first at whether being caught at night walking along the fence and at night in a bucket elicited different corticosterone baselines or responses. Because of sex differences measured in the spring inbound migration, fence captures and bucket captures were analyzed separately by sex for this season. Sexes were combined for spring outbound and fall migrations. For all seasons, there was no significant difference in CORT concentrations between night bucket captures and night fence captures (p > 0.05 for all comparisons).
We also compared CORT levels from blood samples taken at night and in the morning during both the spring outbound and fall migrations. Because there was no significant difference between bucket or fence captures at night, these were combined and compared to bucket captures the morning following a rain event. Although there is a trend in the spring toward higher CORT levels in animals bled in the morning than at night, the difference is not statistically significant ($F = 2.762, \text{df} = 1,13, p = 0.120$, Fig. 2a). There was a significant response to handling in the both morning and night spring captures ($F = 7.361, \text{df} = 1,13, p = 0.018$, Fig. 2a). There was no significant difference between night and morning bleeds during the fall ($F = 0.101, \text{df} = 1,52, p = 0.752$, Fig. 2b) and no significant stress response to handling ($F = 0.153, \text{df} = 1,52, p = 0.698$, Fig. 2b).

4. Discussion

In general, the CORT concentrations that we measured were similar to those measured in other amphibian species. Studies of wild amphibians moving toward the breeding pond have found baseline CORT concentrations ranging from approximately 5 ng/mL in male marbled salamanders, *Ambystoma opacum* (Houck et al., 1996) and male green frogs, *Rana esculenta* (Paolucci et al., 1990) to nearly 60 ng/mL in male toads, *Bufo bufo* (Giacoma et al., 1994), whereas our measurements averaged approximately 7 ng/mL in migrating male spotted salamanders. Although some studies report increases in CORT in less than 3 min, they are the minority of studies in the literature. Even then, these studies usually report values far lower than values obtained after 30 min of stress (Sapolsky et al., 2000; Wingfield and Romero, 2001). Consequently, even though we do not know whether CORT levels change in less than 3 min in spotted salamanders, we are confident that the values reported here are at, or very near, pre-stress baseline levels. The CORT concentrations we measured after 30 min in captivity were similar to those measured after 24 h of captivity in green frogs (Paolucci et al., 1990).

Sex differences in baseline CORT concentrations and the subsequent response to stress of spotted salamanders do exist, but not year-round. As females migrate to the
breeding pond in the spring, they have significantly higher baseline and stress-induced CORT concentrations than males. Sex differences disappeared, however, when salamanders were leaving the breeding pond, with females and males converging at intermediate CORT concentrations relative to those observed during the spring inbound migration. Furthermore, sex differences were absent in the fall when animals were moving in an attempt to find more suitable over-wintering habitat. Wilson and Wingfield (1994) found a similar pattern in the side-blotched lizard (*Uta stansburiana*), where females had higher baseline CORT concentrations during their reproductive period but not post-breeding. They also found that during the peak of reproduction, females tended to have a reduced dry body mass, leading Wilson and Wingfield (1994) to hypothesize that the greater glucocorticoid concentrations in females may be a result of the females’ need to catabolize different energy reserves to maintain the eggs they are carrying. Their hypothesis is consistent with our data and data from other studies (McDonald and Taitt, 1982; Tyrrell and Cree, 1998), and it may explain the seasonally dependent sex differences we observed.

Despite sex differences during the spring inbound migration, we found no overall seasonal differences for either sex considered separately or together. This finding contrasts with many other studies, including those of the edible frog, *R. esculenta* (Dupont et al., 1979), the bullfrog, *Rana catesbeiana* (Licht et al., 1983), the crested newt *Triturus carnifex* (Zerani and Gobbetti, 1993) and the desert tortoise, *Gopherus agassizii* (Lance et al., 2001), all of which found a significant peak in CORT concentrations during the reproductive season. One other exception to the studies mentioned above is the seasonal variation seen in the male tuatara (Tyrrell and Cree, 1998). The tuatara shows multiple peaks in CORT throughout the year. However, these peaks do not seem to correlate with reproductive activity. Instead, the CORT concentrations peak when the weather is most suitable for nocturnal movements (Tyrrell and Cree, 1998). This pattern may be true for spotted salamanders as well, but is difficult to study, as these amphibians are only easily accessible on rainy nights in the spring or fall, conditions under which they emerge from underground burrows. It is possible then, that CORT concentrations are relatively uniform on occasions when these animals are likely to travel above ground. Without finding an efficient way to locate burrows and rapidly extract salamanders, we may not be able to study true seasonal variation in wild caught *Ambystoma*.

Although the overall CORT profiles did not vary seasonally, the presence of an acute stress response did differ between seasons. There was a significant stress response during the spring inbound migration, a nearly significant response to capture, bleeding, and handling during the spring outbound migration (the lack of statistical significance may be due to small sample sizes) and no stress response in the fall. One possible explanation for the variation relates to the different purposes for each migration. Spring migrations are necessary for any salamander intending to breed (inbound), or having completed breeding (outbound), as eggs and larvae are obligately aquatic and adults are terrestrial. In the fall, however, the animals are moving from burrow to burrow throughout the terrestrial habitat. For example, salamanders in the fall might make a judgement about the quality of their current habitat, with salamanders in sufficiently sub-optimal habitat choosing to move. Therefore, the animals we see during this season may already be experiencing higher-than-normal baseline CORT concentrations. If this were true, we might not expect a significant increase in CORT in response to the acute stress that we cause with capture, bleeding, and handling, because our “baseline” measurement may in

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**Fig. 2.** Baseline corticosterone concentrations and elevated responses to capture and handling of spotted salamanders relative to the time of capture. All data are reported as means ± SE. (a) Night of migration (n = 10) or the morning, having spent the night in a bucket (n = 5) as animals were leaving the breeding pond in the spring. (b) Night of migration (n = 45) or in the morning following capture in a bucket (n = 9) in the fall.
fact be a “response” measurement. This possibility also offers an additional explanation for the lack of seasonal variation in overall glucocorticoid levels discussed above. Animals remaining in their burrows in the fall may have baseline CORT concentrations that are much lower than those seen in the spring, but these animals are not available for us to measure, so we may get a biased result.

For practical purposes, we wanted to know whether variations in terrestrial amphibian capture techniques would significantly affect circulating CORT concentrations. For night sampling, salamanders that have already fallen into buckets (known as pit fall traps) had CORT concentrations no different from those of individuals sampled walking along the drift fence, making it easier to obtain suitable sample sizes in a relatively short period of time. With some ecological studies of amphibian populations, animals are often handled the morning following capture, after the animals spend the night in a pit fall trap (Dodd and Scott, 1994). Although we did not find a statistically significant increase in CORT concentrations in the morning during the spring outbound or fall migrations, caution seems warranted, because in the spring a strong trend exists toward higher CORT concentrations in the morning than at night. It is currently unknown whether the trend toward higher CORT concentrations in the morning than at night reflects a result of a circadian rhythm (Dupont et al., 1979) or the result of the stress of being in a bucket for between 5 and 13 h.

Overall, it is clear that CORT concentrations can be non-lethally measured in spotted salamander adults. We were able to establish a profile of CORT concentrations for both sexes and across seasons in an apparently healthy population. We feel that this ability affords the opportunity both for greater understanding of Ambystoma physiology and for conservation related comparisons with other populations.

Acknowledgments

We thank J. Fahey, D. Homan, A. Kennedy, J. O’Brien, M. Pokras, J. Regosin, L. Remage-Healey, A. Robbins, D. Rodriguez, E. Schlüter, and B. Windmiller for help in breeding salamanders. This project was funded by a National Science Foundation grant IBN-9975502 to LMR, by the Massachusetts Environmental Trust, by mitigation monies made available to the Massachusetts Natural Heritage and Endangered Species Program to Bryan Windmiller, and from contributions from Hyla Ecological Services.

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