

Effect of Capture, Phenotype, and Physiological Status on Blood Glucose and Plasma Corticosterone Levels in Free-Ranging Dice Snakes

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ABSTRACT

Theoretically, animals integrate intrinsic and extrinsic factors to respond appropriately to the wide range of stressors they encounter during their life span. We examined how stress response varies between sexes and among morphotypes in wild dice snakes (*Natrix tessellata*). We also considered reproductive and feeding status and antipredator behavior. We used two indicators of stress (glucose [GLUC] and corticosterone [CORT] levels) at eight sampling time intervals (immediately after capture, up to 17 h after) and a large sample size ($N = 113$ snakes). Concentrations of both markers increased sharply after capture (an equivalent of predation). This acute phase occurred earlier for GLUC (30 min) compared to CORT (60 min). Then the values plateaued to very high levels without decline over time, indicating prolonged saturation of the hypothalamus-pituitary-adrenal axis. In contrast to our expectations, we found no effect of sex, morphotype, or reproductive status. Yet the CORT stress response of those individuals displaying death-feigning (DF) antipredator behavior was attenuated compared to those that did not. Low stress hormones levels may facilitate the expression of DF (high levels supporting fleeing behavior). The presence of partially digested material in the stomach was associated with higher blood GLUC during the

plateau. Assaying blood GLUC requires very little blood but was as good as CORT at gauging acute stress response. The prolonged plateau suggests that captivity should be minimized during field studies.

Keywords: antipredator behavior, glucose, *Natrix tessellata*, reptile, stress.

Introduction

Animals experience many abiotic and physiological constraints while taxonomic and individual variations generate a vast array of phenotypes. The resulting number of combinations between stressors and individual characteristics is thus immense. Remarkably, in vertebrates, the mechanisms involved in the mobilization of physiological resources during challenging situations are largely uniform (Cockrem 2013). A relatively small number of hormones (e.g., catecholamines, glucocorticoids) released in the bloodstream exert cascading pleiotropic effects that enable rapid, powerful, albeit highly customized reactions of the whole organism. Ever since seminal articles of Selye (1936, 1946) and Axelrod and Reisine (1984), the central role of the hypothalamus-pituitary-adrenal (HPA) axis in the regulation of stress responses has been established in all lineages of vertebrates examined (Romero and Butler 2007).

Following a stressful event, catecholamines are released within a few seconds and trigger generalized physiological and behavioral alarms (Goldstein 2003). With a short delay (tens of seconds), glucocorticoids are released and stimulate a wide range of processes to help the organism to overcome most challenges (Kassahn et al. 2009). Yet stress response is energetically demanding; the rise of both catecholamines and glucocorticoids induces a strong elevation of blood glucose (GLUC; Pickering et al. 1982). These hormones target multiple other systems; they regulate their own and each other's secretion and those of other hormones (Axelrod and Reisine 1984). This pluripotency is pivotal for orchestrating stress response, notably to hinder or suppress less urgent processes such as immune (Lance and Elsey 1986; Charmandari et al. 2005) and reproductive (Moore and Jessop 2003) functions.

Stress physiology is a powerful and flexible system capable to optimize both the intensity and the duration of the response according to individual characteristics and to the nature of the stressor (Korte et al. 2005). For example, body condition influences corticosterone (CORT) levels and thus the adjustment of reproductive investment in female starlings (Love et al. 2005). More drastically, glucocorticoid upsurges are also implicated in organ

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maturation and behavioral shifts concomitant to highly challenging life-history stages like birth or sexual maturity (Wada 2008).

In captive and wild endothermic vertebrates, many studies used stress hormones to examine how individuals cope with environmental pressures (Rivier and Rivest 1991; Möstl and Palme 2002; Carlstead and Brown 2005; Martin 2009; Breuner et al. 2013; Crespi et al. 2013). Less information is available in wild ectothermic vertebrates, though the usefulness of stress physiology to study how they respond to various events has been amply supported (Barton and Iwama 1991; Homan et al. 2003; Moore and Jessop 2003; Palacios et al. 2012; Cockrem 2013; Anderson et al. 2017; Bradshaw 2017). In squamate reptiles (one of the most species-rich vertebrate groups, with >10,400 species; Uetz et al. 2020), studies revealed differences in stress responses between sexes or among reproductive groups (review in Moore and Jessop 2003). For example, age, body size, body condition, morphs, feeding, or reproductive status can influence basal and acute stress levels (Dayger et al. 2013; Gangloff et al. 2017).

In snakes, extended variations of body size, sexual dimorphism, and distinct morphs generate discrete groups of individuals at the population level (Shine 1991; King 1992; Madsen and Shine 2000). Different cohorts exploit different ecological niches and thus experience different types of stressors (Shine 1991; Forsman 1995; Vincent et al. 2004; Isaac and Gregory 2013). The impact of stressors on chronic and acute stress responses has been studied in captive snakes (Schuett et al. 2004; Bonnet et al. 2013; Dupoué et al. 2014; Cusaac et al. 2016; Claunch et al. 2017; Van Waeyenberge et al. 2018) and in several species in natural conditions (Moore et al. 2000; Fauvel et al. 2012; Palacios et al. 2012; Holding et al. 2014; Owen et al. 2014; Herr et al. 2017; Bonnet et al. 2020). However, possible differences of stress response among groups, within or among populations, have rarely been investigated. Large garter snakes (*Thamnophis sirtalis*) exhibit higher CORT levels after capture compared to smaller ones (Moore et al. 2000), while habitat influences basal and acute stress levels (Gangloff et al. 2017). Two ecotypes of another garter snake species (*Thamnophis elegans*) show different stress-induced CORT responses and different thermal stress patterns of GLUC concentrations (Palacios et al. 2012; Gangloff et al. 2016). In the Hog Island boa (*Boa constrictor imperator*), stressful events prompt higher CORT levels in males than in females (Holding et al. 2014). Thus, available information in snakes indicates that individuals may adjust their stress response with regard to their own status. Yet the small number of studies precludes testing the validity of this concept across the large diversity of snakes (Shine 2005).

In this study, we examined the basal and stress-induced plasma levels of CORT and GLUC in free-ranging dice snakes (*Natrix tessellata*), a highly polymorphic species (Ajtić et al. 2013). We used a study system where individuals undergo intense avian predatory pressure and where three contrasted morphs occur in large numbers within a single population. Very high population density enabled us to standardize field procedure. Our first aim was to describe stress response pattern(s) immediately after capture (i.e., stress triggered by “predation”) and over a prolonged period (hours in a calico bag; i.e., stress caused by custody) in order to characterize

both acute and plateau phases. All snakes were captured by hand; thus, we induced the same type of stress to individuals belonging to different groups. Therefore, we considered sex, three distinct color morphs, reproductive and feeding status, and the type of antipredator behavior displayed at capture to assess whether phenotype and physiological status influence stress response. We hypothesized that different groups should exhibit a specific stress response pattern; plasma levels of CORT and GLUC should be adjusted to individual status. For example, because of the high energetic demands associated with reproduction, especially vitellogenesis (Bonnet et al. 1994; Van Dyke and Beaupre 2011), higher CORT and GLUC levels were expected in gravid compared to nongravid females.

Material and Methods

Study Species and Study Site

The dice snake, *Natrix tessellata*, is an amphibious, piscivorous, and oviparous species (Natricidae) widely distributed from Western Europe to the Middle East, to North Africa across central Asia, to eastern China (Gruschwitz et al. 1999). Dice snakes exhibit a great diversity of phenotypes, both within and among populations (review in Mebert 2011). Individuals were collected on the shores of Golem Grad Island (18 ha, 850 m a.s.l., Prespa Lake, National Park Galičica, North Macedonia) between July 8 and July 23 in 2017, 2018, and 2019. Each year, we selected this period to sample gravid and nongravid females; vitellogenesis is achieved in July in the study site, and many females do not breed annually (Ajtić et al. 2013). This narrow time window also minimized possible effects caused by seasonal variations (e.g., emergence from hibernation in April vs. hot summer in August). In this population, three color morphs coexist (i.e., dotted, black, and gray), and females attain larger body size than males (Ajtić et al. 2013). Dice snakes experience intensive avian predation in Golem Grad, but they are also frequently eaten by otters (*Lutra lutra*) and nose-horned vipers (*Vipera ammodytes*; Ajtić et al. 2013).

Phenotypes and Antipredator Behaviors

All subjects ($N = 113$) were adults (table 1). Individuals were sexed, measured with a flexible ruler (snout-vent length [SVL] ± 0.5 cm), weighed (± 0.1 g), and palpated to detect food items in the stomach and/or developing eggs (table 1). Around Golem Grad, dice snakes are strictly piscivorous, and prey condition can be easily accessed using palpation; recently ingested fish are solid to the touch, while digested items are soft (Ajtić et al. 2013). Color pattern was recorded in most snakes ($N = 111$); 68 were dotted (39 F and 29 M), 22 were black (16 F and 6 M), and 21 were uniformly gray (14 F and 7 M).

During handling, the snakes exhibited different antipredator behaviors (e.g., hissing, shaking, vomiting, or defecating). All snakes shook vigorously at capture and hissed. By contrast, thanatosis, or death feigning (DF; Gregory and Gregory 2006; Rogers and Simpson 2014), characterized by motionless bradycardia, opened mouth, and protruding tongue, was not systematically expressed. Consequently, DF was the most discriminant

Table 1: Summary of blood samples taken in 113 wild dice snakes

Reproductive status, feeding status	Individuals	Resampled	Total
Male:			
Recent prey	7	9	16
Digested item	18	13	31
Empty	16	14	30
ND	1	1	2
Nonreproductive female:			
Recent prey	14	14	28
Digested item	18	15	33
Empty	21	16	37
ND	1	1	2
Gravid female:			
Recent prey	1	2	3
Digested item	3	3	6
Empty	13	14	27
Total	113	102	215

Note. Several individuals were sampled more than once (N (two times) = 84, N (three times) = 18, N (total) = 102 blood samples), while others were sampled only once. Overall, 215 blood samples were collected. Large follicles or ovulated eggs were easily assessed by palpation. “Recent prey” indicates that intact fish was (were) in the stomach of the snake (several were regurgitated); “digested item” indicates partly digested fish that are soft under palpation; “empty” indicates that nothing was detected in the stomach (palpation only enables the finding of small, less than 2-g items). ND = not determined.

antipredator behavior; its occurrence was recorded in 48% of the snakes sampled ($N = 54$). Body temperature was measured at capture with a laser thermometer in 52 individuals and did not influence our results (see the appendix, available online).

Blood Sampling

To obtain detailed GLUC and CORT variations caused by handling, we used eight time intervals (table 2). Some individuals were sampled immediately at capture, while others were restrained in calico bags (kept under shade) and sampled at different time intervals after capture. Because stress response is assumed to rise rapidly and then to reach a plateau when the physiological ca-

pacities of individuals are saturated, the first time intervals were short and the next ones more spaced (Gangloff et al. 2017). We also used a long-time interval to explore possible long-lasting response (17 h; table 2). Some individuals were sampled once, while others twice or three times (total number of blood samples, $N = 215$; table 1). This sampling design enabled us to explore the stress response of individuals over time and to compare independent groups at different time intervals while minimizing the number of punctures per individual (e.g., the maximal number of repeated punctures per individual was three).

To obtain basal levels, we minimized the time elapsed since the detection of a given snake until blood was actually retrieved (Herr et al. 2017). Snakes were detected from a short distance and expeditiously and carefully seized by hand. Swiftly was essential because snakes escaped among rocks very rapidly after encounter—a situation that somehow mimicked diurnal avian predation. We carried all the equipment to collect and process the blood with us in order to minimize the timing between detection, blood sampling, and blood storage. On average blood was retrieved at 219 ± 126 s (\pm SD, $102 < 95\%$ confidence interval [CI] < 163 , $N = 38$) after snake detection. We recorded sex and biometric data after blood sampling.

Stress levels at various time intervals were obtained from snakes kept in shade in calico bags after capture (see “Results”). No snake showed any sign of injuries or distress after blood sampling (Bonnet et al. 2020) or after temporary captivity, and all individuals were released at the site of capture.

Blood was collected from the heart with 1.0-mL syringes fitted with small (27/30-G) heparinized needles. Heart puncture was preferred over puncture of the caudal plexus (often inappropriately named the caudal vein) to avoid hemolymph dilution, which can strongly affect the results (Bonnet et al. 2016), and also because tail puncture is painful, while heart puncture does not trigger any peculiar reaction (X. Bonnet, personal observations). The volume of each blood sample was approximately 150 μ L. Blood GLUC was immediately assayed with a drop (5 μ L) of blood using miniature glucometers (Bayer Contour Next/One, $N = 215$ samples). The blood was then centrifuged at 6,000 rpm (3 min); the plasma was collected in 1.5-mL cryotubes and stored in liquid nitrogen. Whole-blood GLUC concentrations assessed with portable glucometers were tested using GLUC concentrations on the same blood

Table 2: Summary of timing when blood samples ($N = 215$) were taken

Time group	NT	Second NT (\pm SD)	NP	Second NP (\pm SD)	Timing	~Timing
1	38	219 \pm 126	37	211 \pm 115	4 min	5 min
2	46	1,017 \pm 165	29	1,026 \pm 177	17 min	15 min
3	66	1,903 \pm 155	8	1,980 \pm 184	32 min	30 min
4	14	3,433 \pm 654	6	3,770 \pm 458	57 min	1 h
5	19	5,672 \pm 393	12	5,660 \pm 403	95 min	1.5 h
6	16	7,789 \pm 406	8	7,898 \pm 419	130 min	2 h
7	11	11,580 \pm 594	8	11,700 \pm 665	193 min	3 h
8	5	60,756 \pm 223	5	60,759 \pm 22.9	1,013 min	17 h

Note. “Time group” refers to the groups used as a categorical factor. “NT” provides the total sample size per group (including pseudoreplicates), “second NT” refers to the exact time since detection of the snake until the blood was collected (in seconds \pm SD). “NP” and “second NP” provide the values without pseudoreplicates ($N = 113$; note the very slight differences with NT). “Timing” and “~timing” provide convenient time intervals (notably, for comparison with published studies).

samples ($N = 115$ individual snakes from different species) measured with laboratory equipment (Pentra 500 Horriba spectrometer). The very high correlation ($r = 0.95$) obtained suggests that miniature glucometers provided reliable values, at least to monitor variations of circulating GLUC concentrations in snakes (X. Bonnet, unpublished data).

In order to shorten sampling, we rapidly processed the snakes in the field. The most efficient protocol was to catch a small number of individuals (one to three), take blood as fast as possible, and process samples before rapidly catching a second batch of snakes, and so forth. This offsetting method allowed us to rapidly collect blood samples while precisely controlling the time elapsed from capture to successive blood samplings. High population density enabled us to minimize snake searching. Procedures were approved by North Macedonia authorities (permit 03-246).

Radioimmunoassay

Plasma CORT concentration assays were determined using radioimmunoassay at the Centre d'Etudes Biologiques de Chizé laboratory (Bonnet et al. 2013). The steroids were extracted from 40 μL of the sampled tissue (plasma) using diethyl ether (mean extraction rate, $97.3\% \pm 5.2\%$); the sensitivity of the assay was of 1.9 pg/tube. Cross-reactions with other steroids were low ($<0.1\%$ for 11-deoxy-corticosterone, cortisol, testosterone, and androstenedione and 7% for compound S and progesterone). Intra- and interassays coefficients of variation remained lower than 4%. Intra- and interassay variations were, respectively, 7.07% and 9.99%. We did not assay CORT in all the blood samples; the total sample size for CORT was $N = 146$.

Statistical Analysis

Several individuals were sampled more than once (table 1). Pseudoreplicates ($N = 102$ blood samples) were removed from all analyses, except from a single one presented in the appendix. Thus, results were free from pseudoreplication (i.e., each individual was included only once).

CORT concentrations were not normally distributed, even after log transformation. Therefore, we used a Box-Cox transformation that provided satisfactory outcomes with a normally distributed frequency histogram (Shapiro-Wilk $W = 0.993$, $P = 0.708$). Box-Cox-transformed GLUC data were also normally distributed (Shapiro-Wilk $W = 0.993$, $P = 0.397$). Similarly, it was necessary to use Box-Cox-transformed body size (SVL); the resulting distributions did not deviate from normality ($0.697 < P < 0.990$). Body mass (BM) values were normally distributed in each sex ($0.219 < P < 0.787$). In all the plots of the expected normal values versus transformed or untransformed data, the patterns were linear without outliers.

In several analyses, in addition to sex (F vs. M), the reproductive status of females was considered (gravid females vs. nonreproductive females). We obtained 54 categories of snakes: combining three reproductive stages, three color morphs, three feeding stages, and two antipredator behaviors (DF, or lack of). With 113 individuals (fewer when data were lacking), this high number of

categories precluded using a single global analysis. Therefore, we examined all possible combinations (e.g., color morph vs. reproductive status). This approach enabled us to examine interactions and to detect those variables that (never) contributed to the stress responses. We used Tukey post hoc tests because they are more conservative (minor type I error).

Our sample includes gravid and nonreproductive females (fig. 1). The presence of eggs in the oviducts is associated with a greater BM but not necessarily with higher body reserves; by mixing contrasted physiological statuses, body condition can generate spurious results, and thus, it was not used here (Bonnet 2011). Similarly, the body condition of snakes with prey in the stomach remains imprecise. SVL and BM were not recorded in a few individuals, while color morph and feeding status were missing in few others, generating minor fluctuations of sample size for several analyses. Statistics were performed with Statistica 13.5.0.17 (2018, TIBCO Software).

Results

Morphological Traits and Physiological Status

Females were larger than males (generalized linear model [GLM] with reproductive status and color morph as the factors and SVL as the dependent variable: $F_{2,99} = 126.484$, $P < 0.001$), without difference between gravid and nonreproductive females (post hoc Tukey honestly significant different [HSD] test: $P > 0.244$; fig. 1), and without effect of color morph ($F_{2,99} = 0.098$, $P = 0.906$; interaction with reproductive status: $F_{4,99} = 2.379$, $P = 0.057$). BM scaled by size was influenced by reproductive status (GLM with reproductive status and color morph as the factors, BM as the dependent variable, and SVL as the covariate: $F_{2,99} = 54.989$, $P < 0.001$), without effect of color morph ($F_{2,99} = 0.368$, $P = 0.693$; interaction with reproductive status: $F_{4,99} = 1.149$, $P = 0.338$). Gravid females were relatively heavier, males were relatively lighter, and nonreproductive females were in an intermediate state (appendix).

The proportion of individuals with recently ingested fish or digested material in the stomach or without any food in the stomach was influenced by reproductive status (table 1; $\chi^2 = 9.786$, $P = 0.044$). Most gravid females had an empty stomach (76% of $N = 17$), and only one contained an intact fish. Many nonreproductive females had recently ingested or half-digested fish in the stomach (26% and 34%, respectively, of $N = 53$). A modest proportion of males (17% of $N = 41$) had recently ingested fish in the stomach, and many had digested material in the stomach (44%). Overall, the proportion of fed snakes was high and similar in nonreproductive females and males (60%) but low in gravid females (24%).

Effect of Time since Capture on Stress Response

GLUC increased rapidly after capture and reached a plateau approximately 30 min later (fig. 2A; ANOVA with the eight time intervals as the factor and GLUC as the dependent variable: $F_{7,105} = 18.977$, $P < 0.001$). Post hoc tests revealed significant changes during the rise phase, first from 5 min to 15 min

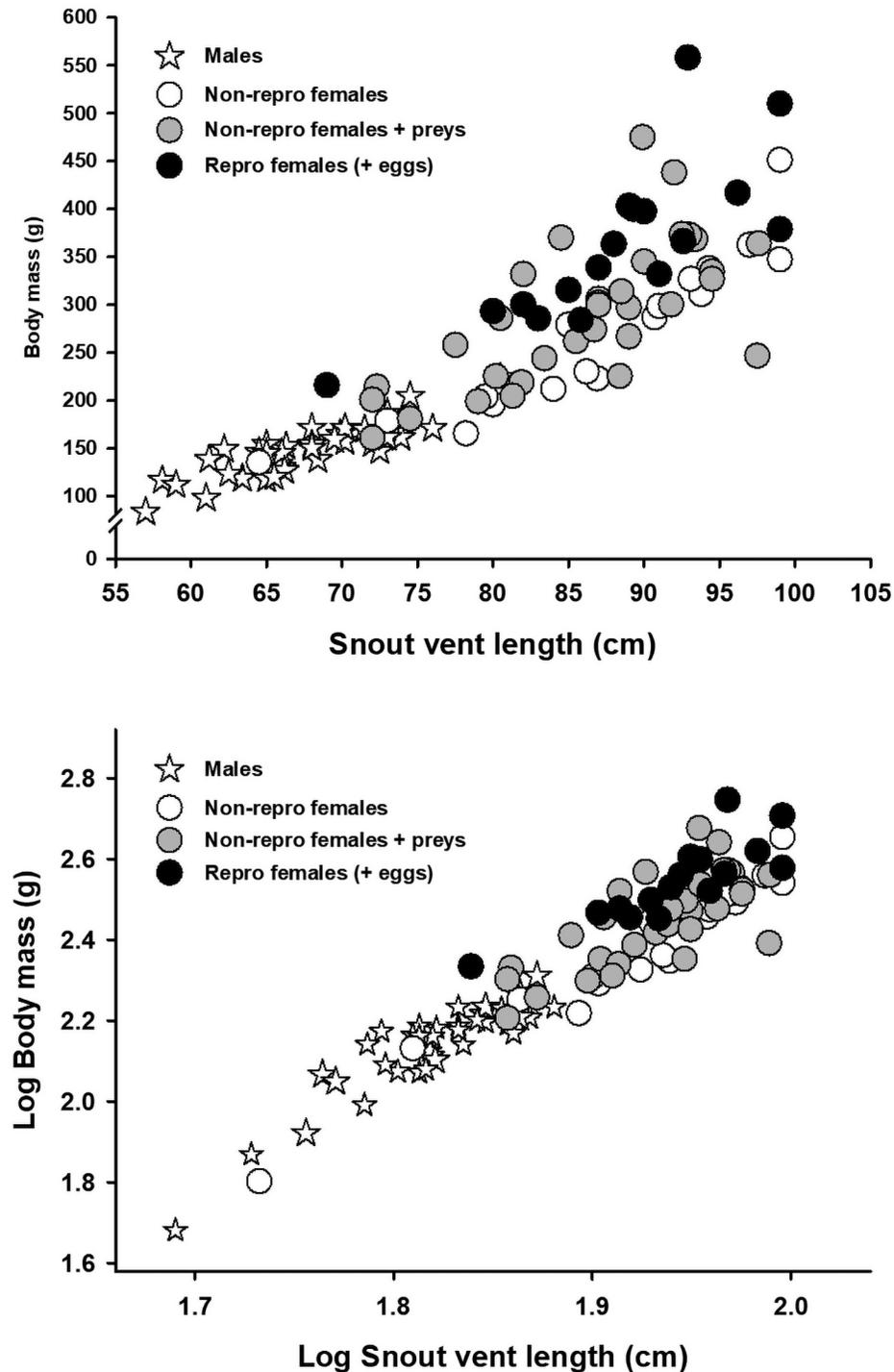


Figure 1. Relationship between body mass and body size in different cohorts of dice snakes (*top*, untransformed values; *bottom*, log-transformed values). Males were smaller and relatively lighter than females. Nonreproductive and gravid females did not differ in size, but the presence of a clutch in the abdomen or of fish in the stomach influenced body mass. Nonreproductive females with an empty stomach were relatively lighter.

($P < 0.025$ in all pair comparisons) and then from 15 min to 30 min ($P < 0.025$ in all pair comparisons except with group 8). Post hoc tests also revealed a lack of significant variation during the plateau ($0.663 < P < 1.00$ in all pair comparisons).

The last blood sample was taken ~17 h after capture in five snakes kept overnight in calico bags and not previously sampled

(fig. 2). GLUC slightly decreased overnight, but not significantly compared to the plateau or to groups 2 or 3 (all post hoc tests: $P > 0.317$), and it remained significantly more elevated than the basal level (post hoc: $P = 0.003$).

The CORT profile largely mirrored the GLUC profile, with a rapid increase during the first hour after capture followed by

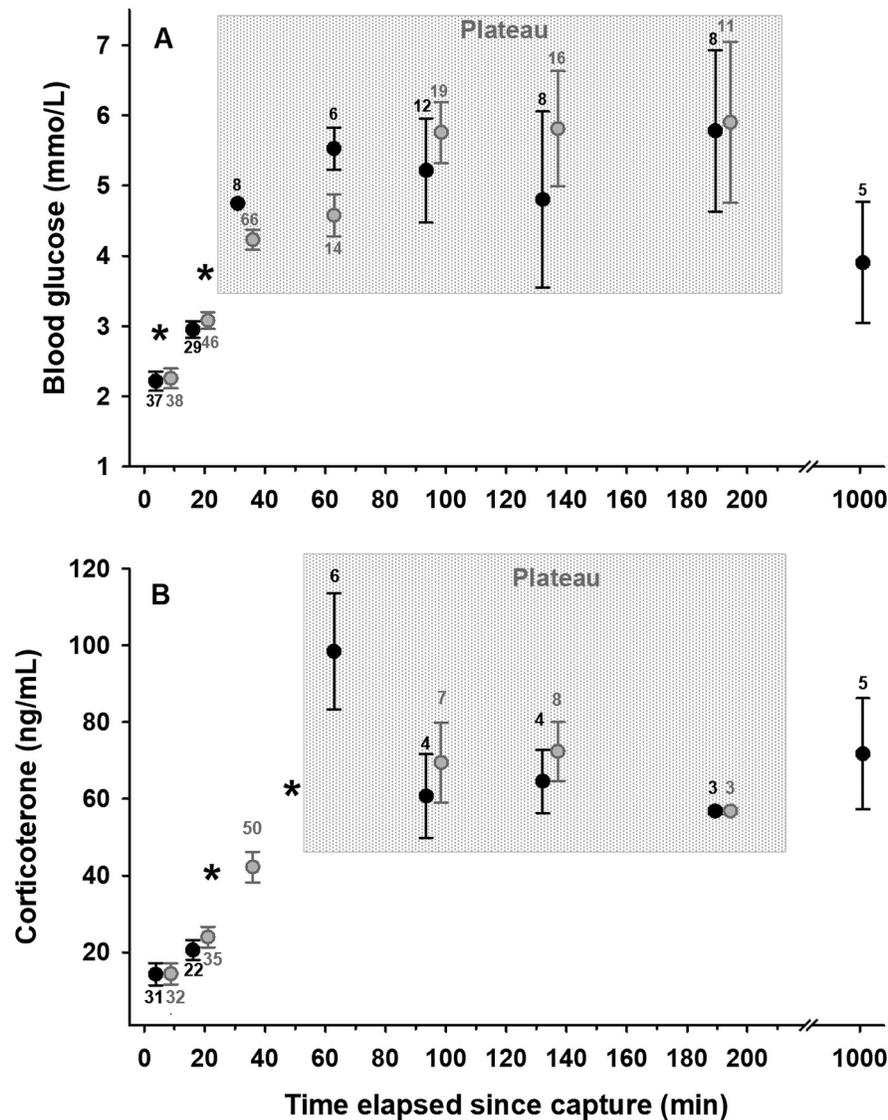


Figure 2. Shortly after capture (~3.7 min) a first group of snakes was sampled, and then at different time intervals, other groups of snakes were progressively sampled. Mean values of glucose (GLUC; A) and corticosterone (CORT; B) are presented \pm SE and with sample size. Black symbols indicate independent mean values collected in 113 individuals ($N = 75$ for CORT). Gray symbols (slightly offset) show mean values including pseudoreplicates ($N = 102$ for GLUC, $N = 71$ for CORT). Statistics were performed without pseudoreplicates (except one test); asterisks indicate significant differences. During the plateau (gray area) mean values did not differ from each other. The last mean value was taken in five snakes sampled more than 17 h after capture.

a plateau 1 h after capture (fig. 2; ANOVA with seven time intervals as the factor and CORT as the dependent variable: $F_{6,68} = 18.692$, $P < 0.001$; pseudoreplicates not included). The initial rise of CORT from 5 min to 15 min was not significant (post hoc: $P = 0.214$), but the basal level was different compared to all other time intervals (all $P < 0.001$). CORT mean values did not differ within the plateau or compared to the last 17-h group (all $P > 0.727$). The 30-min group involved pseudoreplicates ($N = 50$, not included in this analysis), limiting time comparisons. Yet an inspection of figure 2B suggests that the 30-min CORT mean value was intermediate between basal and plateau mean values (see the appendix for additional tests). Using the simple time grouping

as above (i.e., all plateau intervals pooled) did not change the results.

CORT and GLUC were highly correlated ($r = 0.675$, $F_{1,73} = 61.241$, $P < 0.001$; fig. 3), and thus, both proxies of stress response provided relatively similar patterns. But this correlation explained only 46% of the variance.

Influence of Phenotype and Physiological Status

Almost none of the characteristics (e.g., color morph, reproductive status) significantly influenced GLUC or CORT (values scaled by time since capture), whatever the combination of factor(s) considered. The exceptions were, first, a

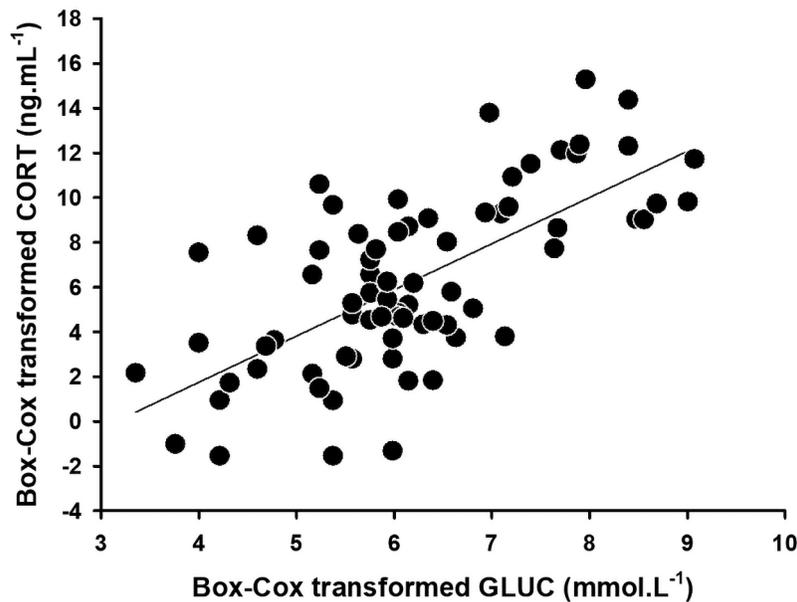


Figure 3. Relationship between two blood markers of stress in the dice snake: plasma corticosterone (CORT) and blood glucose (GLUC). Values were Box-Cox transformed before analyses. Both variables were correlated ($r = 0.675$, $F_{1,73} = 61.241$, $P < 0.001$).

significant interaction between sex and feeding status on GLUC ($F_{2,105} = 6.955$, $P = 0.011$). Males with digesting material in the stomach tended to exhibit higher GLUC compared to all the other categories (fig. 4; post hoc tests: $0.011 < P < 0.148$). Second, we found a significant association between DF antipredator tactic and CORT. Yet this effect was obtained when sex ($F_{1,38} = 5.830$, $P = 0.021$) or feeding status ($F_{1,36} = 5.759$, $P = 0.022$) was included as a factor (see the appendix for additional tests). Post hoc tests suggested that most of these effects were driven by the strong difference

observed between males exhibiting DF and those that did not ($P = 0.021$), but not by females ($P = 0.401$; fig. 5).

Discussion

The most salient results are that capturing free-ranging dice snakes triggered a strong GLUC and CORT response characterized by two phases: a rapid increase followed by a long-lasting plateau. These results suggest that the HPA system of the snakes responded vigorously but then reached its limits. The prolonged plateau

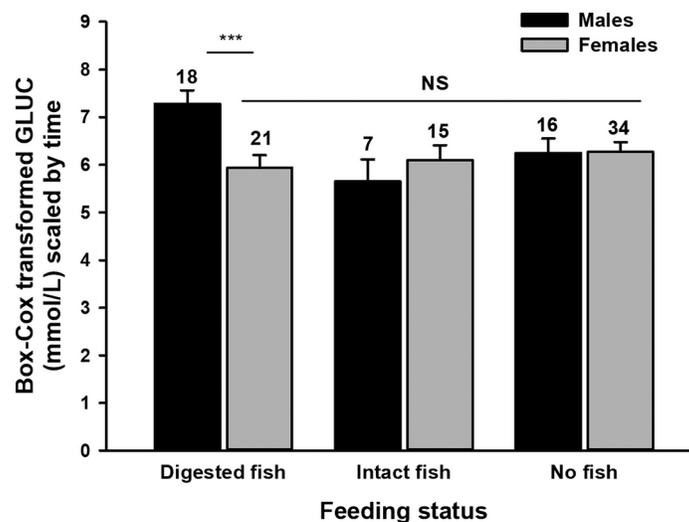


Figure 4. Effect of sex and feeding status (i.e., snakes with digested fish, intact fish, or without prey in the stomach; X-axis) on mean blood glucose (GLUC) of dice snakes. Mean values (\pm SE and sample size) have been scaled by the number of seconds elapsed since capture to take into account the effect of time. Values were Box-Cox transformed before analyses. A significant interaction between sex and feeding status was found ($P < 0.02$). Significant post hoc tests were exclusively observed when comparing GLUC of males with digested fish in the stomach against digesting females or males with intact fish ($0.011 < P < 0.040$, indicated with asterisks).

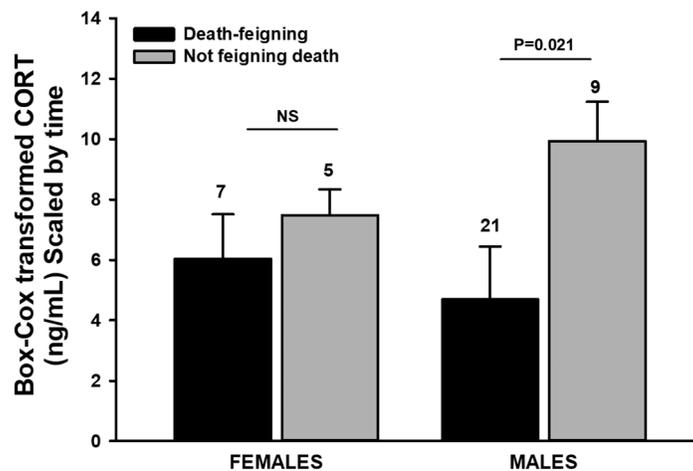


Figure 5. Effect of sex on mean corticosterone (CORT) level (scaled by time) of dice snakes that exhibited death-feigning behavior or that did not exhibit such behavior at capture. Mean values (\pm SE and sample size) are shown. Values were Box-Cox transformed before analyses. Significant post hoc tests were observed in males only. NS = not significant.

(several hours) indicates a saturation of physiological capacities, at least in terms of classical plasma biomarkers of stress (many were not investigated). This finding might be implemented in field studies, minimizing the time that animals are restrained, especially for sensitive stages like gravid females. Unexpectedly, stress responses were not influenced by phenotypic traits, with the noticeable exception of DF antipredator behavior. Below, we discuss methodological and biological aspects of these outcomes.

Usefulness of an Accurate Description of the Stress Response

In most studies, two time intervals were used to assess acute stress responses in wild snakes (Sykes and Klukowski 2009; Holding et al. 2014). CORT and GLUC have been monitored over 3 d with nine time intervals in the common garter snake (Gangloff et al. 2017). In this study we used eight time intervals over 2 d. The procedures used in these two studies were relatively similar and allowed observers to clearly separate the rise phase from the prolonged plateau. Further, both studies revealed the time lag between GLUC (early) and CORT (late) profiles. These similarities suggest a general pattern in snakes that has several implications. First, investigations performed with only two sampling points cannot fully describe the shape (rise and saturation) of the stress response, while comparisons among groups, seasons, populations, or species should take into account these dynamic aspects. Second, detailed assessment of acute stress responses is useful to comply with the three Rs (replacement, reduction, and refinement) principle (Russell and Burch 1959). For example, our results suggest that 0–30-min or 0–60-min timings might often be appropriate in snakes to assess acute response and to determine plateau value, rendering unnecessary the maintenance of individuals in captivity for longer periods.

GLUC versus CORT

GLUC and CORT responses were broadly similar but not fully identical, however. The GLUC response occurred earlier than the

CORT response (fig. 2; appendix; Gangloff et al. 2017). Likely, the rapid massive release of GLUC in the bloodstream was caused by an almost immediate release of catecholamines (unmeasured here). The plateau of GLUC was then likely carried by the following surge of glucocorticoids; this possible “relayed release” of two types of hyperglycemic hormones may explain the offsetting, albeit overlapping, GLUC versus CORT patterns.

In this study, to assess stress response, GLUC was as useful as CORT (i.e., time patterns were broadly similar). Glucometers offer advantages in the field to reduce the impact on individuals and on the environment: 5 μ L of blood is sufficient, and immediate acquisition of the results permits the minimization of sample size, logistical costs, and waste (e.g., vials).

Possible Deleterious Effects of Capture Stress

The term “stress” can refer to any change in the behavior or physiology of an animal (e.g., pupillary dilatation, accelerated heart rate, elevation of CORT) caused by internal or external agents; thus, it includes both harmless and deleterious effects (Koolhaas et al. 2011; Romero et al. 2015). A more rigorous approach involves precise categorization, where harmful stress is characterized by a chronic allostatic overload (distress) that should be distinguished from adaptive episodic allostatic responses (eustress; McEwen and Wingfield 2003). More recently, the reactive scope model defines four steps, with a progressive aggravation of the physiological status of the animal coping with increasing intensity (or duration) of stressors (Romero et al. 2009). Only the fourth stage (i.e., homeostatic failure) can entail short-term pathological effects. Otherwise, no deleterious effect is expected from short-term acute stress response once the tension is alleviated.

In this study, GLUC and CORT plateaus observed in dice snakes suggest that individuals produced a strong response. Harmful consequences were unlikely; however, high GLUC or CORT requires prolonged periods of time to induce pathologies (Sterlemann et al.

2008), and while most snakes were released in fewer than 4 h since capture, five individuals were released after 17 h. A long-term study of a large sample of sea snakes subjected to comparable treatments (handling, temporarily captivity) revealed a lack of effect on survival, for example (Fauvel et al. 2012).

Influence of Phenotype and Physiological Status

Sex differences and reproduction influence CORT, respectively, in marine iguanas (Neuman-Lee and French 2017) and in western diamondback rattlesnakes (Taylor et al. 2004). In the dice snakes, we found very limited effects of sex (i.e., via DF) and no effect of reproductive status or color morph. This finding is inconsistent with previous studies (Moore et al. 2000; Cartledge and Jones 2007; Palacios et al. 2012) and with our initial assumptions. Strong interindividual variations may have blurred the influence of the factors tested. Several effects not previously described were nonetheless observed.

Sustaining a strong, long-lasting stress response (hours) requires energy. Individuals with larger resources should respond more strongly. An experiment in garter snakes showed that stress alters energy use in a manner dependent on the energy state of the animal (Neuman-Lee et al. 2015). Interestingly, dice snakes with digesting material in the stomach and sampled during the plateau (incidentally, mostly males) exhibited significantly higher GLUC, suggesting that incoming resources partly sustained the GLUC response (fig. 4). Including fed and unfed snakes may have induced the large standard errors during the plateau compared to CORT, despite a larger sample size for GLUC (fig. 2A).

The lower CORT response of DF snakes fits well with the physiological characteristics of thanatosis (Gregory and Gregory 2006). Stress hormones exert strong tonic and stimulatory effects; lower CORT values may thus facilitate DF. The motionlessness and marked bradycardia of DF requires a neurophysiological shutdown of the stimulatory effects of capture stress, perhaps through a partial inhibition of the sympathetic nervous fibers that drive the adrenal medulla. Yet to be expressed, DF must overrule the elevation of stress hormones. This effect was more prominent in males than in females, suggesting that predation impacts the sexes in different ways favoring sex-specific tactics.

Future research should include other factors (e.g., diel rhythms, seasons, and reproductive cycles; Tyrrell and Cree 1998; Moore et al. 2001; Taylor et al. 2004; Lutterschmidt and Mason 2010) and compare populations that undergo different predator pressures to examine whether (and how) stress physiology participates in local adaptation (Bradshaw 2017; Sparkman et al. 2018).

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