RESEARCH ARTICLE

Shelter availability, stress level and digestive performance in the aspic viper

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SUMMARY

The lack of shelter can perturb behaviors, increase stress level and thus alter physiological performance (e.g. digestive, immune or reproductive functions). Although intuitive, such potential impacts of lack of shelter remain poorly documented. We manipulated shelter availability and environmental and physiological variables (i.e. access to a heat source, predator attack, feeding status) in a viviparous snake, and assessed sun-basking behavior, digestive performance (i.e. digestive transit time, crude estimate of assimilation, regurgitation rate) and plasma corticosterone levels (a proxy of stress level). Shelter deprivation provoked a strong increase in sun-basking behavior and thus elevated body temperature, even in unfed individuals for which energy savings would have been otherwise beneficial. The lack of heat was detrimental to digestive performance; simulated predator attacks worsened the situation and entailed a further deterioration of digestion. The combination of the lack of shelter with cool ambient temperatures markedly elevated basal corticosterone level and was associated with low digestive performance. This hormonal effect was absent when only one negative factor was involved, suggesting a threshold response. Overall, our results revealed important non-linear cascading impacts of shelter availability on stress-hormone levels, behaviors and physiological performance. These results infer that shelter availability is important for laboratory studies, captive husbandry and possibly conservation plans.

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INTRODUCTION

In most animals, individuals spend a considerable amount of time in refuges, and shelter availability is often essential for the persistence of populations (Wright and Shapiro, 1990; Armstrong and Griffiths, 2001, Souter et al., 2004; Bonnet et al., 2009; Grillet et al., 2010; Lagarde et al., 2012). Shelters provide protection against predators and buffer environmental conditions (Anderson, 1986; Schwarzkopf and Alford, 1996; Sih, 1997; Roper et al., 2001; Berryman and Hawkins, 2006). The benefits of retreat site selection have been examined in species belonging to various lineages [e.g. arachnids, fish, reptiles, mammals and amphibians (Bulova, 2002; Kearney, 2002; Goldsbrough et al., 2004; Kotler et al., 2004; Millidine et al., 2006; Hossie and Murray, 2010; Hossie and Murray, 2011)]. However, previous investigations focused on constraining temperature conditions, i.e. hibernation, aestivation or sun-scorching periods (Seebacher and Alford, 2002; Beck and Jennings, 2003; Lagarde et al., 2002; Lagarde et al., 2012; Cooper and Withers, 2005). Yet many animals intensively use their refuges for reasons other than to escape severe climatic conditions: for instance to sleep, for reproduction or for digestion (Siegel, 2005; Pike et al., 2010; Pike et al., 2011). Consequently, important behavioral and physiological consequences unrelated to overheating or dehydration should be associated with shelter availability, yet this eco-physiological issue is poorly documented.

When they leave their refuge, animals are exposed to challenging abiotic and biotic factors (Sih, 1997; Kearney, 2002; Langkilde et al., 2003; Berryman and Hawkins, 2006). Assessing the consequences of the absence of shelter on stress level, behavior and physiological

performance is thus crucial to understanding how behavioral trade-offs are mediated. Notably, many animals retreat into their shelter between foraging episodes. Such shuttling activity offers an appropriate context in which to test behavioral trade-offs. For example, individuals deprived of a shelter may be forced to undertake displacements to escape unfavorable and/or dangerous conditions, with possible negative consequences on other activities. A lack of shelter may also generate physiological stress, which in turn may perturb immunity, sexual behaviors and possibly many other fitness-related processes (Rabin, 1999; Moore and Jessop, 2003).

To examine these issues, we manipulated shelter availability, ambient temperature, feeding status and predatory threat in a snake species. We assessed the consequences on three major traits: thermoregulation, digestive performance and plasma corticoid levels (Möstl and Palme, 2002; Bassett and Buchanan-Smith, 2007). We measured corticoid hormones because they influence the process of acquisition and allocation of resources, the mobilization of body reserves, metabolism and various behaviors (DeNardo and Sinervo, 1994; Guillette et al., 1995; Romero, 2004).

We expected that snakes would display contrasting behavioral and physiological responses to different environmental conditions. We hypothesized that unfavorable conditions (e.g. lack of shelter, low ambient temperature) would provoke an elevation of plasma corticoid levels and impede digestive performance. By contrast, favorable conditions (presence of a shelter plus high ambient temperature) should be associated with low plasma corticoid levels and high digestive performance. Finally, intermediate situations (combination of favorable and unfavorable factors) should produce intermediate

stress levels and average digestive performance. Overall, these predictions can be expressed through one main hypothesis: environmental conditions should exert an additive effect (either positive or negative) on physiological parameters associated with stress and resource assimilation. Further, we expected state-dependent effects where individual physiological status influences response (Aubret and Bonnet, 2005). Notably, recently fed snakes should exhibit different behaviors than unfed snakes (second hypothesis). Indeed, as digestion requires elevated body temperatures, fed snakes should exhibit a positive thermoregulation shift to select hot microhabitats more often than unfed snakes.

Snakes are suitable organisms with which to test these hypotheses. Most species are secretive and individuals spend considerable amounts of time sheltered (Bonnet and Brischoux, 2008). They usually leave their refuge to feed on large prey items, and they tend to cease moving during long digestive episodes. Therefore, foraging and digestion can be easily distinguished and monitored (by comparison, these processes overlap in herbivorous animals, rendering analyses complicated). Digestion speed depends on body temperature and is often associated with prolonged phases of sun basking (at least in temperate climates), which increases predation risk (Garland and Arnold, 1983; Beck, 1996); consequently, there is a conflict between rapid digestion associated with sun exposure and survival provided by the refuge (Sih, 1997). The experiments presented in this study are largely based on this trade-off.

MATERIALS AND METHODS
Ethics statement
The authors attest their adherence to National Institutes of Health standards. No snake was mistreated and the mice were euthanized following rapid cervical dislocation. Permits to collect the snakes were issued by the DIREN Poitou-Charentes (no. 09/346/DEROG). All experiments were performed under permits A79-001 and 79-157 issued by the 79 district.

Study species
Previous studies provided important background (Saint Girons, 1957; Bonnet et al., 1999; Bonnet et al., 2000; Bonnet et al., 2001; Bonnet et al., 2002; Bonnet, 2011; Ladyman et al., 2003; Zuffi et al., 2009; Michel and Bonnet, 2010). Aspic vipers [*Vipera aspis* (Linnaeus 1758)] feed mainly on rodents and digestion is impeded when body temperature falls below 15°C (Naulleau, 1983). In the field, animals are active under ambient temperatures ranging from 10°C to more than 30°C (depending upon cloud cover). Vipers shuttle between periods of heliotheria [i.e. 'sun-basking behavior to warm up the body' (Bailly, 1894)] and periods sheltered in their refuge; this shuttling activity is a central element of their thermoregulation.

The snakes involved in the experiment were captured in the vicinity of the laboratory (between spring 2003 and autumn 2008; forest of Chizé, 46°08′49″N, 0°25′31″W). Prior to the experiment the individuals were maintained in captivity (during at least 3 months) in standard boxes (42×34×19 cm, length×width×height), with artificial grass as substratum, clean water *ad libitum*, a thermal gradient of 20–35°C and a roof-tile as a refuge. All the snakes accepted their food regularly (dead mice approximately every 2 weeks). The experiments were performed during the normal activity period of the species (May–August).

Experimental design
We performed two main experiments, and to obtain comparable data between them we used a single modular system (Fig. 1). We built
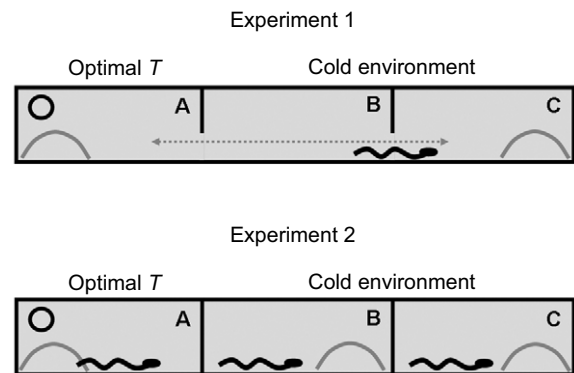

Fig. 1. Ten large boxes with three compartments (A, B and C) were used to test microhabitat selection by the vipers (top configuration; Experiment 1), and the physiological consequences associated with such selection (bottom configuration; Experiment 2). The dashed arrow means that the snake can move freely between the compartments (top), the absence of the arrow means that the snake is restrained to one compartment (bottom). The circle represents a heat source (50 W halogen lamp). Optimal *T* means that the snake has access to a wide range of ambient temperatures during the day phase (compartment A only; see Materials and methods for details); the cold environment was permanently set to 16°C (compartments B and C). The gray curved line represents shelters (roof tiles). The shelters were available or not. In Experiment 1, zero, one (compartment C) or two shelters (compartments A and C) were placed in the large box. In Experiment 2, zero or one shelter was placed in each compartment.

10 three-compartment cages fitted with artificial grass substratum (total length 150 cm, 50×30×20 cm per compartment) and with a small door (Ø 5 cm) between adjacent compartments. When the doors were open, the snakes could freely move across the compartments (Experiment 1). When the doors were shut, the snakes were restricted to one compartment (Experiment 2). In one compartment (A, Fig. 1) we placed a 50 W halogen lamp to create a heat source (i.e. mimicking solar radiation). Roof-tiles were used as shelters. The cages were placed in a temperature-controlled room set at 16°C (14 h of daylight: 09:00–21:00 h). Ambient temperatures were measured with data loggers (iButtons Thermochron, San Jose, CA, USA). The temperature of the shelter and the surface body temperature of the snakes were measured from a distance (20–30 cm) using a three-dot infrared laser thermometer (Raytek MX2, Fotronic Corporation, Melrose, MA, USA; measurement diameter 19 mm, range –30 to 900°C, precision ±1°C, 250 ms per reading; see supplementary material Fig. S1). Surface and internal body temperatures are highly correlated in reptiles (Lagarde et al., 2012). The front of the cages was transparent; hence the observer and the snakes could see each other.

Experiment 1
We first assessed the behavioral trade-off between safety and sun basking. More precisely, we focused on the impact of shelter availability and feeding status on heliotheria. Each snake was placed in a three-compartment cage, and thus in a 16–40°C thermal gradient during the day (Fig. 1). Two compartments, A and C, were fitted with or without a shelter in four treatment combinations. (1) No shelter available: the snake was always visible and could select either the hot compartment (A) or one of the two cold compartments (B or C, Fig. 1). (2) One shelter was placed in the cold compartment (C) at the opposite of the hot spot (A). The snake could select the hot–unsheltered (A), the cold–unsheltered (B) or the cold–sheltered compartment (C). (3) Two shelters were provided: one in the hot

Table 1. Characteristics of the snakes involved in Experiments 1 and 2

Sex	Age class (N)	SVL (cm)	Mass (g)
Experiment 1			
Female	Juvenile (4)	26.7±0.8	14.4±1.9
	Sub-adult (6)	43.7±4.6	66.0±21.5
	Adult (6)	59.1±6.5	180.7±66.2
Male	Juvenile (4)	26.6±0.6	13.2±0.9
	Sub-adult (6)	43.1±3.7	45.4±3.2
	Adult (6)	51.8±2.0	94.4±10.5
Experiment 2			
Female	Juvenile (18)	27.1±3.8	15.2±8.7
	Sub-adult (12)	48.0±9.1	75.8±41.0
	Adult (18)	56.2±6.8	161.2±67.5
Male	Juvenile (16)	26.7±5.9	15.0±10.6
	Sub-adult (10)	42.4±4.4	47.7±9.9
	Adult (14)	52.0±4.6	100.0±24.1

Data are means ±1 s.d.
SVL, snout–vent length.

compartment and one in the opposite cold compartment. The snake could select the hot–sheltered, the cold–sheltered or the cold–unsheltered compartment. (4) The medium compartment (B) was never provided with a shelter to better assess selection; indeed, the snakes were forced to cross it to find a heat source and/or shelter in compartments A and/or C.

To obtain comparative data, we also tested other situations. Notably, the snakes (fed or unfed) were given the choice between hot–sheltered and cold–unsheltered compartments, or sheltered and unsheltered compartments without any heat source. However, these situations do not entail any major conflict (hence, they were partly beyond the scope of the present study). Indeed, terrestrial snakes prefer to remain sheltered rather than to stay in the open and they prefer a warm place rather than a cold one. For simplicity, these specific results, although useful to test our protocol, will be briefly presented and referred to as checking tests.

We used 32 snakes in this first experiment, balancing sex and age ratios, and encompassing a wide range of body sizes (Table 1). At least 2 weeks after their last meal, unfed snakes were randomly allocated to one of the four situations described above (following a random order). The snake was gently placed in the medium compartment of the cage one night before the beginning of records. It was observed every hour between 09:05 and 20:05 h during two subsequent days (36 h). Its position and attitude (e.g. coiled, stretched, moving, motionless) were noted (N=12 observations per day, total number of observations per snake: N=24). Then, we performed again the same experiment with the same recently fed snakes. We offered a mouse to each snake [mouse mass represented 25±2% of snake mass; all individuals accepted the meal except during skin shedding (a relatively rare event), hence the slight variations in sample sizes] and we placed the snake into the cage immediately after the mouse was swallowed.

Overall, most of the snakes were tested either fed or unfed, and for each digestive status they experienced four different situations [four combinations of shelter(s)]. We collected a total of 4752 focal observations. In addition, we collected 816 focal observations for the checking tests on a random subsample of 25 snakes.

Experiment 2
In the second experiment, we examined the consequences associated with the selection of one of the three compartments available to the snakes in the first experiment. Thus, we shifted from measuring

heliotheria (i.e. sun basking behavior) and sheltering behaviors to measuring digestive performance and stress levels.

For 2 weeks, we imposed the four combinations of heat source and shelter availability to recently fed snakes: cold–unsheltered, cold–sheltered, hot–unsheltered and hot–sheltered. The doors of the boxes were then shut, for a total of 30 separate compartments (Fig. 1). Half of the compartments were fitted with a shelter and half were not (following a random procedure); water was provided in each compartment. The heat source was available during the diurnal phase in the 10 hot compartments; the 20 other compartments remained cold all the time (16°C). We placed one snake per compartment during 16 consecutive days and the compartments were inspected three times per day (09:00, 13:00 and 17:00 h). The feces were rapidly collected, weighed and dried (see below).

First, we tested 88 recently fed snakes (32 vipers from the first experiment plus 56 vipers; Table 1) immediately after the prey was swallowed. The snakes were weighed twice: before feeding and 16 days later.

Second, we analyzed the impact of repeated simulated predator attacks on recently fed snakes. We randomly selected and tested 56 vipers (among the 88 available). We followed the same procedure as above but every day we touched each snake with a leather glove (10 times, total duration 15 s, standard movements, same observer; the glove was attached to a 50 cm rod), and this was repeated three times per day (09:00, 13:00 and 17:00 h). The snakes subjected to the simulated attack displayed typical defensive behaviors (e.g. hissing, striking). As above, the snakes were monitored for 16 days.

Third, we assayed plasma corticoid levels in unfed vipers (N=63 vipers large enough for blood sampling) that experienced the same treatment as above, but without predatory attack simulations. These vipers were then not involved into any experiment for at least 2 weeks before being tested. They were not fed and not attacked to limit the potential confounding effects of digestion and predatory threat on basal plasma corticosterone level. Indeed, our objective was to assess the impact of shelter and heat source availability on endocrine stress levels. All individuals were blood sampled 10 days after the beginning of the test. This duration was selected to limit the effect of initial handling stress (i.e. placing snake into the box) and to offer the snakes enough time to 'adapt' to the experimental conditions.

Digestive performance
Experiment 2 provided a sufficient amount of time (16 days) to monitor digestion: the production of feces requires 1 week on average (Michel and Bonnet, 2010). We considered the date for the first feces as an index of transit time; we also counted the other feces and noted their date of production.

We used a crude estimate of assimilation rate: the proportion of ingested prey in relation to snake body mass variation. For example, a 20 g mouse inducing a 4 g increase in snake mass after digestion provides a value of 20% (for details, see Michel and Bonnet, 2010). We emphasize that such a calculation does not provide accurate values of digestion efficiency (e.g. other factors such as metabolism, energy content of feces and specific body composition are involved), but is a useful index for inter-individual or inter-group comparisons.

Several individuals regurgitated their prey. Snakes tend to regurgitate when threatened or exposed to unfavorable ambient temperatures (Greene, 1988; Naulleau, 1983). In these instances, the date of regurgitation was noted and the vomited items were examined and weighed (fresh and dry mass). In the case of regurgitation, to estimate the proportion of the prey digested (not assimilated) by the snake we divided the dry mass of the regurgitated prey by the estimated dry mass of the corresponding ingested mouse.

This index reflects the proportion of the prey wasted relative to that digested (note that actual calculation of digestive efficiency requires passage through the gut). The dry masses of vomited prey were obtained after 2 weeks in a 60°C autoclave. The estimated dry mass of the ingested prey was derived from the linear correlation between fresh and dry masses measured in a range of euthanized mice [body mass ranged from 2.8 to 55.0 g; N=7, r^2=0.99, P<0.01; estimated dry mass=0.0098(fresh mass)+0.3072]. In several cases, snakes that regurgitated did not produce any feces, generating some variations in the sample size. Importantly, the strong variations in the crude estimate of digestive performance caused by different timing of regurgitation (see below) were more crucial to assess digestive efficiency than any other sources of variation [e.g. presence of a heat source (Michel and Bonnet, 2010)]; therefore, we essentially focused on this key factor.

Hormonal assays

Basal corticosterone levels were obtained from blood samples collected less than 5 min after handling. We obtained 100–500 μl of blood per snake *via* intracardiac punctures (<5% of total body mass, using 30 G needles). This technique is routinely employed in snakes (Bonnet et al., 1994; Bonnet et al., 2001; Fauvel et al., 2012). The blood was immediately centrifuged (3 min at 10,000 g), and the plasma was separated and stored at −25°C until assay.

Hormonal assays were performed at the Centre d'Etude Biologique de Chizé. Plasma concentrations of corticosterone (Mathies et al., 2001; Moore et al., 2000; Palacios et al., 2011) were determined using radioimmunoassay. The mean extraction rate of the steroids from the plasma was 97.3±5.2%, and was always greater than 89% (N=28 tests); intra- and inter-assay coefficients of variation remained lower than 4% and the sensitivity of the assay was 1.9 pg per tube. Cross-reactions with other steroids (using the same protocol) were as follows: androstenedione (<0.1%), compound S (7%), cortisol (0.1%), 11-deoxy-corticosterone (0.1%), progesterone (7%) and testosterone (<0.1%). Plasma corticosterone levels were not significantly correlated with snake body mass (r^2=−0.005, P=0.424).

Analyses

During checking tests and the first experiment, the vipers were observed discontinuously. In a preliminary study, we tested (and validated) this approach through continuous monitoring of 20 snakes for 12 h: we placed each snake in the central compartment of the box (B, Fig. 1), the doors were open, and we noted all the subsequent movements. We found no difference between continuous observations and a single observation per hour. Indeed, the snakes moved slowly to select a compartment, and then remained in the same place for prolonged periods. Thus the 1 h sampling over 2 days (N=24 records per individual) captured all the displacements between the compartments. For unknown reasons, the snakes almost never stayed in the middle compartment B: the mean score was negligible (<1 compared with a possible maximal value of 24). This means that the vipers were essentially observed either in compartment A or in compartment B. Consequently, we simply considered the number of observations in compartment A (the heat source was turned on in this compartment, except during several checking tests), and the resulting score (0–24) was expressed as the heliotheria score (i.e. sun basking score). Different observers were involved, but we did not detect any observer effect. Indeed, the snakes remained motionless most of the time during observations.

In all experiments, the mean characteristics of the snakes (body size, body mass, body condition and age class) were not different
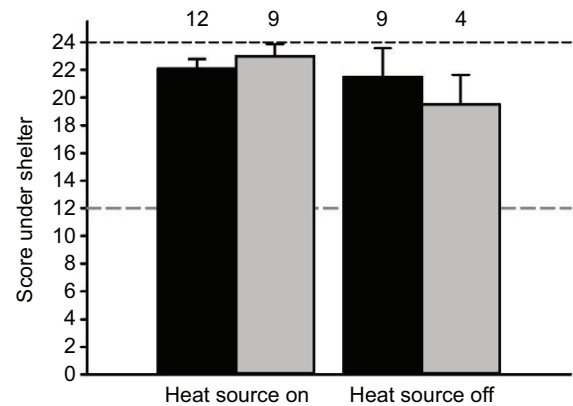

Fig. 2. Mean score expressing the time spent by the snakes in the compartment fitted with a shelter (on a scale of 0–24 observations per snake, *y*-axis). A heat source placed above the shelter was either turned on or turned off (*x*-axis). The snakes were unfed (black bars) or recently fed (gray bars), and they could move freely between the three compartments (Fig. 1). Means are expressed ±1 s.e.m.; the numbers indicate the number of snakes involved in each situation. The dashed lines indicate the maximal theoretical value (dark gray) and the absence of selection (light gray). Note that several snakes were placed in more than one situation (total number of snakes=25).

between the different experimental situations (or compartments) (P>0.40 in all comparisons). Most vipers were tested repeatedly; however, at least 1 week elapsed between subsequent experiments, and none of the snakes presented any sign of disorder. Our experiments were not (or weakly with blood sampling) invasive and the snakes were maintained under appropriate conditions. Thus, the impact of one experiment on the next remained undetectable. For conciseness, we do not present all the results (e.g. all sex effects were non-significant). This study did not aim to investigate the thermal biology of the aspic viper; thus, we simply report broad relevant descriptive thermal data. We used non-parametric tests (e.g. Friedman ANOVA for repeated measures on the same individual) to assess behavioral scores and contingency tables for comparisons of the occurrence of certain events among groups (e.g. regurgitation). We used parametric tests (e.g. ANOVA *F*-test) to compare continuous variables; the homogeneity of variance assumption was not violated. Pseudo-replicates were discarded from calculations. Statistical analyses were performed with Statistica 7.1 (StatSoft, Tulsa, OK, USA).

RESULTS
Experiment 1: microhabitat selection

The checking tests showed that the snakes markedly preferred the compartment fitted with a shelter (heat source either turned on or off); all tests provided a mean value (±s.d.; as in subsequent results) greater than 19.5 (21.9±3.9) with no difference between experimental situations (χ^2=0.00, d.f.=3, P=1.00; Fig. 2). These expected (confirmatory) results enabled us to examine the behavior of the snakes placed under more challenging situations.

When no shelter was available, unfed snakes selected the hot compartment A (i.e. heat source turned on) most of the time (Fig. 3). The mean heliotheria score (20.2±3.8) was close to the maximal theoretical value (24). The addition of a shelter in the cold compartment (C) attracted an important proportion of individuals under this refuge. When two shelters were available, we observed an intermediate situation: many snakes shifted to hot compartment with
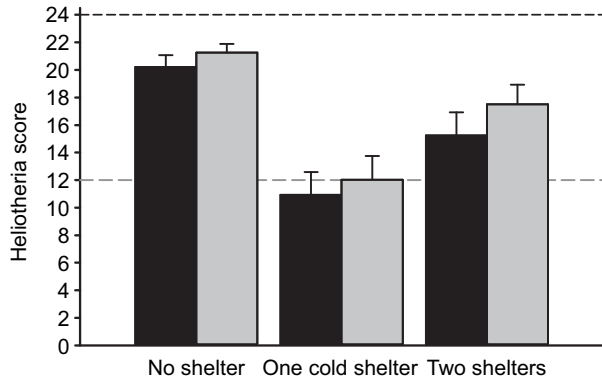
Fig. 3. Heliotheria scores (i.e. microhabitat selection) of 32 snakes placed under three experimental situations (Experiment 1, Fig. 1). Values are means ± 1 s.e.m. The snakes were unfed (black bars) or recently fed (gray bars). The dashed lines indicate the maximal theoretical value (dark gray) and the absence of selection (light gray). Heliotheria score was calculated as the number of times (out of a total of 24) the snake was observed under the heat source.
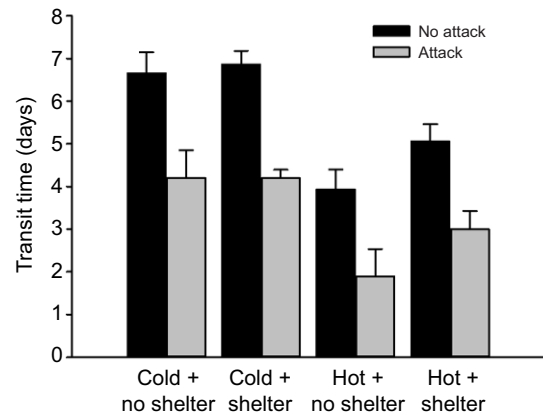

Fig. 4. Mean transit time (time elapsed between the ingestion of the prey and the production of the first feces) of the snakes placed into four different experimental situations (Experiment 2, Fig. 1). The snakes were not disturbed (black bars, N=88) or were harassed through simulation of predator attacks (gray bars, N=56).

a shelter (A); the others selected the cold compartment with a shelter (C). These differences were significant (Friedman ANOVA with different individual scores as the repeated dependent variables, N=28, d.f.=2, χ^2=14.04, P<0.001). Snakes with recently ingested prey exhibited a similar pattern of microhabitat selection (Fig. 3). Although not significant, this later result was nonetheless very close to conventional significance (Friedman ANOVA, same design, N=28, d.f.=2, χ^2=5.82, P=0.054). Recalculating a single score per snake irrespective of the feeding status (averaging fed and unfed scores displayed in Fig. 3) supported the above patterns observed in unfed and fed snakes (Friedman ANOVA, N=32, d.f.=2, χ^2=25.07, P<0.001).

Further comparisons, for example between unfed and fed vipers in one of the three experimental situations, failed to detect any significant effect (P>0.30 in all Wilcoxon tests for paired samples), suggesting that unfed and fed snakes exhibited a similar pattern of microhabitat selection (Fig. 3).

Experiment 2: shelter, heat, predator and physiological performance
Ambient and body temperatures
Mean ambient temperature of the cold compartments was 15.5±0.6°C (range 14.0–17.5°C, N=3450 readings) without significant variations over time; the mean body temperature of the snakes in the cold compartments was similar (15.6±0.3°C, N=212).

By contrast, the ambient temperature in the hot compartments varied markedly, similar on average to the values recorded in the cold compartments during the night (15.7±0.8°C, 14.5–17.5°C, N=360), but ranging over a wide gradient when the halogen lamp was turned on. The temperature measured under the tile when the heat source was turned on averaged 33.1±16.4°C (16.0–52.5°C, N=146, logger positioned on the grass substrate in the middle of the shelter). The mean ambient temperature measured in the corner of the compartment provided with the heat source, but at the opposite of the lamp, was 17±1.8°C (15.0–19.5°C, N=140).

The surface body temperature of the snakes placed in the hot compartment varied between 14 and 35°C and averaged 28.3±6.2°C when the thermal gradient was available (N=46 readings, restricting records from 10:00 to 18:00 h to ensure that the snake had enough time for thermoregulation).

Digestive performance
The presence of a heat source shortened digestive transit time, but the presence of the shelter had no effect on this trait (ANOVA with transit time as the dependent variable, heat source and presence of a shelter as the factors; heat source, $F_{1,84}$=22.07, P<0.001; shelter, $F_{1,84}$=1.94, P=0.167; interaction, $F_{1,84}$=0.93, P=0.338; Fig. 4). We found a similar pattern in snakes subjected to the predator attacks with a significant effect of heat source only (heat source, $F_{1,53}$=5.21, P=0.027; shelter, $F_{1,53}$=1.59, P=0.213; interaction, $F_{1,53}$=0.20, P=0.660; Fig. 4). However, the predator attacks strongly reduced transit time in all cases (Wilcoxon test for dependent variables, Z=4.40, P<0.001, N=56; Fig. 4). We found no significant effect of the experimental situation on the total number of fecal droppings (mean 2.3±1.6, range=1–7).

The longer the prey remained in the stomach before regurgitation the greater the proportion of prey digested (Fig. 5). Regurgitation date had a strong effect on the total number of feces produced (r=0.50, $F_{1,57}$=19.43, P<0.001). The crude assimilation rate was different between the vipers that regurgitated (−8%, N=51) and those that did not (20%, N=28; $F_{1,77}$=17.36, P<0.001).

Regurgitation events were not randomly distributed (observed *versus* a theoretical unbiased distribution; d.f.=3, χ^2=18.20, P<0.001; Fig. 6A). Regurgitation was rarely recorded (N=1) when a heat source was provided, but was frequent under the cold conditions (N=30; d.f.=1, Yates χ^2=17.28, P<0.001), and was twofold more frequent when the snakes were subjected to the absence of both shelter and heat source (10 *versus* 20; d.f.=1, Yates χ^2=5.4, P=0.020). Predator attacks amplified these effects, regurgitation rate increasing from 50% to 75% (d.f.=3, χ^2=11.37, P<0.01; Fig. 6B); again, this predominantly affected the cold snakes with 38 and 1 regurgitations in the cold *versus* hot conditions, respectively (d.f.=1, Yates χ^2=21.82, P<0.001). Importantly, the difference between the snakes deprived from shelter *versus* those provided with a shelter was cancelled out when the snakes were harassed: they all lost their prey at the same high rate (d.f.=1, Yates χ^2=0.06, P=0.805; Fig. 6B). Analyses of regurgitation dates supported these trends (Fig. 6). The snakes under the most unfavorable conditions tended to regurgitate sooner [the mean regurgitation date was 7.7±3.8 days in non-attacked (N=30) *versus* 4.6±3.1 days in attacked snakes (N=38), respectively]. Eleven snakes that regurgitated in two experimental situations enabled us to further test this effect on the same individuals; the
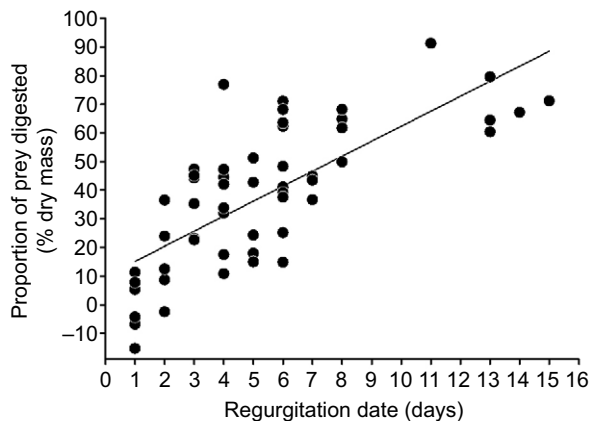
Fig. 5. Proportion of the mass of the prey regurgitated *versus* digested as a function of the number of days the prey remained in the stomach (r=0.77, P<0.001).

predator attack simulation precipitated regurgitation (Wilcoxon tests for paired samples, Z=2.80, N=11, P=0.005).

Plasma corticosterone levels

Vipers provided with a heat source and/or shelter exhibited low basal plasma corticosterone levels (Fig. 7). However, the combined absence of shelter and of heat source was associated with high plasma corticosterone levels, suggesting a chronic stress level (ANOVA with plasma corticosterone concentration as the dependent variable and experimental condition as the factor; interaction, $F_{1,59}$=5.15, P=0.027; Fig. 7).

DISCUSSION

Our results show that the absence of shelter generates marked behavioral and physiological responses that appear to signal an elevation in the organismal stress state of snakes. These responses are expected to have important fitness consequences. For instance, the lack of shelter provoked a strong reduction in energy intake (high regurgitation rate), notably when no heat source was available (an additional factor), or an important waste of energy (metabolism elevation due to the high body temperature in unfed individuals) when a heat source was provided. Yet significant impairment of energy intake should be regarded as particularly detrimental to reproduction in snakes (Bonnet et al., 2002). These results support our main hypothesis that environmental conditions should exert an additive effect on physiological parameters associated with stress and resource assimilation. We acknowledge that this conclusion was derived from different experiments rather than from a direct measure of physiological stress *per se* on food intake. However, the same individuals underwent different experiments, and thus provided strong support for the suggested relationship.

In vipers deprived of a heat source, regurgitation rate was more pronounced when no refuge was available (Experiment 2). In many species (e.g. humans, birds), individuals vomit under stressful conditions or when threatened (Camilleri et al., 1986; Peterson et al., 1987; Siegel-Causey and Hunt, 1981; Vogel, 1950). Stress-induced vomiting can be beneficial because it may deter predators, relax physical burden and increase escape capacities. However, the negative value of crude assimilation rate recorded in the snakes that regurgitated (−8%) was greater than expected, as unfed (i.e. starving) snakes have been shown to display only –0.5% of body mass loss under similar conditions (Michel and Bonnet, 2010). This revealed
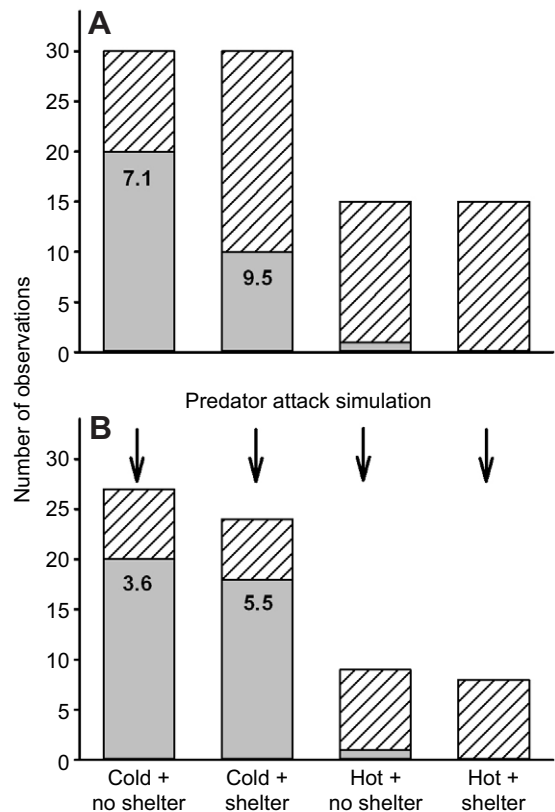

Fig. 6. Number of snakes that regurgitated their prey (gray) relative to the number of snakes that did not (hatched) in four experimental situations (Experiment 2). The number indicates the mean date of regurgitation. The snakes were not disturbed (A) or were harassed through simulation of predator attacks (B).

a new stress-induced cost, possibly as a result of the loss of body fluids and/or the energy demand to initiate digestion (McCue, 2006). Low ambient temperature represented a negative factor (Regal, 1966), but it was insufficient in isolation to trigger a negative effect equivalent to the lack of shelter and heat in combination. Indeed, most cold snakes with a shelter (67%, Fig. 6) successfully digested their prey and eventually gained mass. In support of this, we found a strong correlation between regurgitation date and digestion rate. We also observed a normal crude assimilation rate in the cold snakes that did not regurgitate [18%, compared with 17% in Michel and Bonnet (Michel and Bonnet, 2010)]. In other words, most cold but sheltered snakes digested normally, albeit slowly. This enabled us to discard a possible regurgitation reflex due to putrefaction of the prey and suggests that stress was a key factor.

The investigations based on corticosterone provided complementary results. The most stressful condition, lack of shelter and heat source, generated chronic elevated corticosterone levels, halfway between mean normal basal and maximal levels observed during acute stress response (Fig. 7). However, our results do not entirely match one of our initial predictions. We expected gradual hormonal effects in response to the accumulation of stressor agents. Rather, we obtained a threshold effect with a sudden massive corticosterone increase when two adverse conditions were combined (note that this effect can nonetheless be considered as additive). Perhaps the combination of cold ambient temperature with the absence of shelter exceeded the snakes' capacity to cope
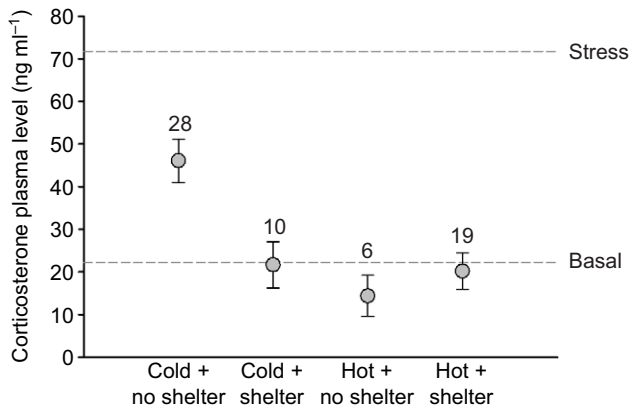
Fig. 7. Mean basal plasma corticosterone concentrations (±s.e.m.) obtained in 63 unfed vipers placed under four experimental situations (Experiment 2). Blood samples were taken in less than 5 min. The dashed lines indicate the mean basal and the mean stress levels obtained in vipers tested for the stress response [i.e. difference between basal and stress-induced corticosterone levels obtained in vipers ~30 min following handling stress (Wingfield, 1994); N=23 vipers (X.B. and C.L.M., unpublished)].

appropriately with stressful conditions, resulting in chronic stress (Bradshaw, 1986; Romero, 2004).

Predatory threat (simulated attacks) further lowered digestive performance, especially under cool conditions: snakes regurgitated at a very high rate (>60%) with no difference between individuals deprived and not deprived of shelter (Experiment 2). In addition, this factor accelerated defecation, another typical defensive behavior (Haas, 1985; Myles, 2002; Weiss, 2006; Tillmann, 2009). These results reinforce the notion that stress induced strong negative physiological consequences.

At first glance, our results may suggest that shelterless snakes provided with a heat source (even individuals subjected to simulated predator attacks) were spared from stress costs. Indeed, they exhibited very low regurgitation rates and low plasma corticosterone levels. However, appearances are deceptive. Indeed, in Experiment 1 (snakes move freely across compartments), the absence of shelter forced almost all snakes to increase their body temperature (and hence energy expenditure) through extensive heliotheria (Fig. 3). This effect was marked in unfed individuals in whom a selection for low temperature to save energy was expected. Our second hypothesis, that snakes should exhibit different behaviors compared with unfed snakes, was thus challenged. As predicted, the snakes with a full stomach exploited the opportunity to shorten digestion via an elevation of their body temperature (Michel and Bonnet, 2010). This pattern conforms to field observations. In natural conditions, recently fed snakes tend to bask intensively under cool ambient temperatures (Beck, 1996; Regal, 1966). However, unfed (or non-reproductive) vipers generally remain hidden in cool places to minimize both metabolic expenditure and predation risks (Bonnet and Naulleau, 1996; Bonnet, 2011). Consequently, in the present study, the fact that most snakes with an empty stomach decided to remain under the heat source most of the time was surprising. At least, we expected that the fed snakes would have exhibited higher heliotheria scores compared with unfed snakes, but this was not the case. The immediate consequence of such marked apparently useless heliotheria was an obligatory waste of energy as the metabolic difference between divergent body temperatures (i.e. 28 versus 16°C) is massive in ectotherms (Andrews and Pough, 1985;

Ladyman et al., 2003). Why did the unfed snakes not select the cool compartment to save energy?

The manipulation of shelter availability suggests that such pronounced heliotheria corresponded to an anti-predator tactic. In the absence of shelter, the snakes situated in a cold compartment exhibited low body temperature (i.e. 16°C ambient or surface body temperature) and they were slow and vulnerable (Heckrotte, 1967). By maintaining high body temperature they considerably improved their defensive potential, as fleeing ability, striking speed and bite efficiency are temperature dependent (Goode and Duvall, 1989; Greenwald, 1974; but see Whitaker et al., 2000). When a shelter was provided (either in the cold or hot compartment), an important proportion of snakes was attracted, whatever the ambient temperature and the feeding status, suggesting that the snakes essentially based their decision on two conflicting options: maintaining a costly high defensive potential (high body temperature) versus using the cold refuge associated with major energy savings.

The main outcome of our study is to emphasize that a crucial characteristic of the habitat, the availability of a shelter, can exert a strong influence on stress status with cascading consequences on major behaviors and energy balance. A recent experiment on fish showed that the absence of refuge increased not only the unsafe perception caused by a predation threat, but also the stress level (indicated by darker skin coloration), metabolic costs and, consequently, total energy budget (Millidine et al., 2006). Thus, two convergent experimental studies carried out on very different organisms (fish and snakes) documented a negative impact due to the absence of shelter associated with a high stress level. The widely acknowledged climatic buffering role of the refuge cannot explain our results. Indeed, in the two studies that specifically investigated this issue (performed in both aquatic and terrestrial environments), shelter availability was experimentally manipulated under controlled ambient conditions (Millidine et al., 2006; present study). In both cases, the absence of shelter stressed the individuals independently from other environmental factors. These results suggest that the feeling of safety associated with the characteristics of the habitat is a key element to understanding habitat selection, thermoregulation and energy budget. If our results are confirmed by further investigations, many laboratory studies should be re-examined. Notably, the behavioral and thermoregulation results from the numerous experiments where the animals were placed into a thermal gradient without any refuge available may well be questionable. More generally, both shelter availability and quality should be integrated as important elements of animal welfare and field management in captivity and conservation studies, respectively.

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