



Effects of mild wintering conditions on body mass and corticosterone levels in a temperate reptile, the aspic viper (*Vipera aspis*)



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ABSTRACT

Temperate ectotherms are expected to benefit from climate change (e.g., increased activity time), but the impacts of climate warming during the winter have mostly been overlooked. Milder winters are expected to decrease body condition upon emergence, and thus to affect crucial life-history traits, such as survival and reproduction. Mild winter temperature could also trigger a state of chronic physiological stress due to inadequate thermal conditions that preclude both dormancy and activity. We tested these hypotheses on a typical temperate ectothermic vertebrate, the aspic viper (*Vipera aspis*). We simulated different wintering conditions for three groups of aspic vipers (cold: $-6\text{ }^{\circ}\text{C}$, mild: $-14\text{ }^{\circ}\text{C}$ and no wintering: $-24\text{ }^{\circ}\text{C}$) during a one month long period. We found that mild wintering conditions induced a marked decrease in body condition, and provoked an alteration of some hormonal mechanisms involved in emergence. Such effects are likely to bear ultimate consequences on reproduction, and thus population persistence. We emphasize that future studies should incorporate the critical, albeit neglected, winter season when assessing the potential impacts of global changes on ectotherms.

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1. Introduction

Climate change is now clearly established and is predicted to accelerate in the next century (IPCC core writing team, 2014), while it is considered as one of the main driver of biodiversity loss (Sala et al., 2000; Sinervo et al., 2010; Bellard et al., 2012). Predictions regarding the impact of global warming on biodiversity have become a central theme in ecology (see Pereira et al., 2010 for a review) and one essential prerequisite is to understand the proximate mechanisms by which species will respond to environmental changes (Dillon et al., 2010; Pereira et al., 2010; Huey et al., 2012; Seebacher and Franklin, 2012). Notably, clarification of metabolic adaptations and physiological tolerance are essential to better understand macroecological responses to climate change such as shift in species distribution (Chown et al., 2003; Bernardo and Spotila, 2006; Bernardo et al., 2007; Dillon et al., 2010).

Due to their direct dependency on environmental temperature, ectotherms are expected to strongly respond to thermal constraints mediated by climate change (Deutsch et al., 2008; Huey et al., 2012, see Lillywhite, 2013 for a review). Most ectotherms lack the ability to produce significant metabolic heat and their body temperature is driven by ambient conditions. As a consequence, any change in environmental temperature will affect most aspects of their life cycle through body temperature changes (Angilletta, 2009). Accordingly, impacts of global warming have been shown to influence rates of embryonic

development, growth rates, reproduction, activity, maintenance costs, and survival rate (Chamaille-Jammes et al., 2006; Parmesan, 2007; Pörtner and Knust, 2007; Sinervo et al., 2010; Neuheimer et al., 2011; Bestion et al., 2015; Stahlschmidt et al., 2015). Ultimately, climate warming is thought to induce effects on distribution and population persistence (see Parmesan, 2006 for a review). Recent studies have suggested that tropical ectotherms are particularly at risk through increased maintenance costs, overheating and disproportionately reduced activity times (Deutsch et al., 2008; Dillon et al., 2010); while climate change should increase the potential activity time of temperate ectotherms due to latitudinal gradient in thermal breadth and may even enhance their fitness (Parmesan et al., 1999; Buckley et al., 2011, but see Bestion et al., 2015).

Although the hypothesis that temperate ectothermic vertebrates are expected to benefit from climate warming is supported by empirical data (Chamaille-Jammes et al., 2006; Clarke and Zani, 2012; Huang et al., 2013; Stahlschmidt et al., 2015, but see Bestion et al., 2015), very few studies have focused on the impacts mediated by climate change during the overlooked, yet critical, winter season (Zani, 2008; Williams et al., 2015). Winter is a key season for terrestrial ectothermic vertebrates, as it may represent more than half of their time budget and have long-lasting consequences on other life-history stages (Williams et al., 2015). During this season, low temperatures impede normal activity, cellular metabolism and thus major physiological functions (e.g. locomotion, digestion). Therefore, ectotherms stay inactive for several months, secluded in thermally buffered den (Ultsch, 1989). This period is an important facet of ectotherms life history for at least three reasons. First, the selection of appropriate shelter is critical to

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avoid low temperatures and associated mortality risks. Second, for most of these species, reproduction occurs immediately following emergence; which suggests that the changes in neurophysiology that characterize reproduction are likely to occur and thus to be triggered during winter “dormancy” (Lutterschmidt and Mason, 2009). Finally, during this period food intake is absent and specific mechanisms are triggered to optimise energy conservation (Patterson and Davies, 1978; Hailey and Loveridge, 1997; Tattersall and Ultsch, 2008). In turn, individuals can mobilize these body reserves to fuel both emergence and early reproductive mechanisms upon emergence (Chen et al., 2013) despite reduced feeding opportunities.

In cold and temperate areas, current climatic alterations, and especially the increase of surface temperature occurs partly through the elevation of the annual minima (DeGaetano, 1996; Easterling et al., 1997; Easterling et al., 2000), thereby producing overall warmer winter, and thus milder overwintering conditions (Zani, 2008; Williams et al., 2015). Mild winters are expected to greatly influence the responses of terrestrial ectotherms to climate change (Williams et al., 2015). Notably, patterns of resource utilization are likely to be affected by warmer winter temperature through elevated metabolic rates, while resource acquisition is limited. Therefore, milder winters are expected to decrease body condition upon emergence, which, in turn would affect crucial life-history traits, such as winter and spring survival and reproduction (Irwin and Lee, 2000; Zani, 2008; Sorvari et al., 2011; Zani et al., 2012). Independently from energetic considerations, inappropriate overwintering conditions (out of an appropriate thermal range) may also affect reproduction through the disruption of the neurophysiological mechanisms that trigger reproduction upon emergence (Lutterschmidt, 2012). Eventually, non-optimal overwintering temperature may cause a state of chronic stress where an individual cannot be active because temperature is too low, but can neither be dormant because temperature is too high. Corticosterone (CORT), both involved in energy use and response to stress in terrestrial ectotherm, has previously been shown to increase at non-optimal body temperatures (Li et al., 2011; Dupoué et al., 2013; Telemeco and Addis, 2014, but see Sykes and Klukowski, 2009). Importantly, high circulating levels of CORT are known to negatively interact with animal long-term survival (Breuner et al., 2008; Goutte et al., 2010) or reproduction (reviewed in Bonier et al., 2009). Taken together these elements suggest that mild wintering conditions due to current climate change could negatively affect temperate ectothermic vertebrates, through altered patterns of energy conservation and direct (neurophysiological mechanisms, chronic stress) and indirect (through reduced body conditions) effects of suboptimal temperatures on animal fitness.

In this study, we examined the influence of overwintering temperature on patterns of mass loss and baseline CORT upon emergence in a typical temperate ectothermic vertebrate, the asp viper (*Vipera aspis*). We experimentally simulated different wintering conditions for three groups of asp vipers (cold: $\sim 6^\circ\text{C}$, mild: $\sim 14^\circ\text{C}$ and no wintering: $\sim 24^\circ\text{C}$) during a one month long period. Specifically, we expected body mass loss during dormancy to be positively related to temperature. Upon emergence, we also predicted baseline CORT to be abnormally high in the mild wintering temperature group because of chronic stress.

2. Materials and methods

2.1. Study species and husbandry

The asp viper (*V. aspis*) is a typical small sized (~ 50 cm) temperate western palearctic snake species. Individuals are active from early March to the end of October, and rely on sit-and-wait foraging mode to accumulate vast amounts of energy before reproduction (capital breeding), resulting in low reproductive frequency (Naulleau and Bonnet, 1996; Bonnet et al., 1999). From October to late February individuals seclude in thermally buffered underground refugia where they

attain a relatively low and constant body temperature ($\sim 5\text{--}7^\circ\text{C}$) (Duguy, 1962).

In this study, we used adult, captive-born snakes from a colony raised at the Centre d'Etudes Biologiques de Chizé (CEBC) since 2009. Individuals were maintained in plastic boxes ($30 \times 16 \times 10$ cm) containing a shelter and a water bowl with water ad libitum. A thermal gradient ($18\text{--}40^\circ\text{C}$) was created by a heating cable located at one side of the cage. Snakes were fed with mice once every two weeks.

2.2. Experimental design

We exposed non reproductive snakes to ecologically relevant ambient temperature (T_a) simulating different wintering conditions. Vipers were moved to a transparent plastic box ($35 \times 25 \times 12.5$ cm) and transferred in a climatic chamber (Vötsch Industrietechnik, VP 600, Balingen, Germany). The cold treatment ($T_a = 5.7 \pm 1.7^\circ\text{C}$) was designed to mimic the temperature in typical winter den (Duguy, 1962; Shine and Bonnet, 2009). The control treatment ($T_a = 24.0 \pm 0.5^\circ\text{C}$) corresponded to an optimal temperature selected by active snakes that are not engaged in any specific activity (e.g., digestion, skin sloughing or reproduction, Ladyman et al., 2003). Finally, we subjected a group of vipers to a mild winter treatment ($T_a = 13.9 \pm 0.4^\circ\text{C}$). This temperature was selected to simulate warmer winter conditions due to elevation of annual minima.

Snakes were randomly assigned within sexes to a treatment (Table 1). At the onset of experiment, females were heavier and had lower CORT levels than males (both $p < 0.02$, Table 1), but within sexes, snake body mass and CORT levels were similar between treatments (both $p > 0.7$, Table 1).

The experiment was conducted between December and January 2013 consistent with natural biological cycle of snakes (see above). However, in order to limit putatively deleterious effects (i.e., strong body mass loss in the control group), we limited the duration of the experiment to one month (35 ± 2 days). Two weeks before exposure to wintering treatment, all snakes were acclimatized to the control treatment (no wintering, $T_a = 25^\circ\text{C}$). Temperature of each climatic chamber was recorded using miniature temperature data logger (Thermochron iButtons, Dallas Semiconductor, Dallas, TX, USA), to control for potential deviations. Snakes were kept unfed during the experiment to avoid potential effect of contrasted feeding activity between treatments on body mass and CORT levels. In order to mimic the conditions of a typical winter den, drinking water was not available.

2.3. Physiological parameters

2.3.1. Body mass

We measured the change of body mass over exposure to wintering treatment as an index of change in snake body reserve (Lourdais et al., 2002a, 2002b). Initial and final body masses were measured at the onset and the end of the exposure to wintering treatment respectively.

2.3.2. Baseline CORT

We measured the changes in baseline CORT level as an indicator of putative chronic stress (Wingfield et al., 1998; Sapolsky et al., 2000; Landys et al., 2006). Blood samples were collected in a random order at the onset and the end of the treatment. Within less than 4 min (mean \pm SE: 3.35 ± 0.12 min), we collected blood samples ($100 \mu\text{l}$) via cardiocentesis using a 1 ml syringe with a 27-gauge heparinized needle. CORT levels were not related to handling time ($r^2 = 0.0008$, $p = 0.78$, $n = 98$), and were therefore considered to be representative of “baseline CORT” levels. Immediately after collection, blood from each sample was placed into a 0.675 ml microcentrifuge tube and centrifuged for 3 min at 3000 rpm. The plasma was separated, collected and stored at -28°C . Plasma CORT concentrations were then determined at the CEBC by following a well-established radioimmunoassay protocol

Table 1
Body mass and CORT values at the beginning of the experiment.

	Males			Females		
	Cold (N = 8)	Mild (N = 8)	Control (N = 8)	Cold (N = 8)	Mild (N = 8)	Control (N = 9)
BM (g)	87.3 ± 5.9	84.4 ± 5.5	88.5 ± 5.5	127.5 ± 8.3	132.8 ± 7.0	119.5 ± 9.8
CORT (ng · ml ⁻¹)	14.4 ± 2.4	19.7 ± 4.8	11.3 ± 2.4	7.7 ± 2.9	6.7 ± 1.9	11.4 ± 3.4

(see Lormée et al. (2003) for details). Samples were run in two assays (intra-assay variation: 7.07%, inter-assay variation: 9.99%).

We only bled snakes in the morning (from 08:30 to 11:30), to limit the effect of the time of the day on CORT concentrations, since CORT could be subjected to circadian variations in snakes (Dupoué et al., 2013). The day before sampling, we re-exposed all snakes to the control treatment ($T_a = 25^\circ\text{C}$) because we were interested to investigate putative effects of the treatment upon emergence. In addition, this allowed to facilitate blood sampling (which is difficult at low temperature because of low heart rate and blood flow) and to control for the influence of temperature on CORT levels (see Dupoué et al., 2013).

3. Results

3.1. Body mass

We found a significant effect of the treatment on adjusted mass loss (ANCOVA on the body mass change with treatment and sex as the factors and the initial body mass as the covariate, temperature effect: $F_{2,44} = 6.60$, $p = 0.003$, Fig. 1). Body mass loss was significantly lower in the cold treatment (Fisher's LSD, $p < 0.007$) and similar in the two warmer treatments (Fisher's LSD, $p = 0.48$, Fig. 1).

3.2. Baseline CORT levels

We found an effect of the treatment, the sex and their interaction on the change in baseline CORT levels (ANCOVA on the CORT change with treatment and sex as the factors and initial baseline CORT as the covariate, temperature effect: $F_{2,42} = 39.27$, $p < 0.0001$, sex effect: $F_{1,42} = 7.89$, $p = 0.007$, and their interaction: $F_{2,42} = 4.23$, $p = 0.02$, Fig. 2). Baseline CORT levels were significantly higher in the cold treatment (Fisher's LSD, $p < 0.0001$), and within this treatment, males had significantly higher baseline CORT levels than females (Fisher's LSD, $p < 0.002$).

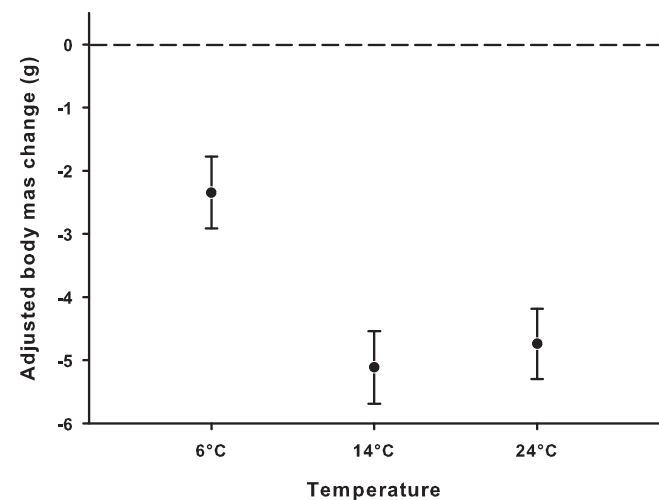


Fig. 1. Body mass change (adjusted to initial body mass) throughout the experiment for snakes maintained under contrasted wintering conditions (cold: $5.7 \pm 1.7^\circ\text{C}$, mild: $13.9 \pm 0.4^\circ\text{C}$ and no wintering: $24.0 \pm 0.5^\circ\text{C}$).

4. Discussion

4.1. Body mass loss

According to our first prediction, we found that mass loss was influenced by temperature. We found the lowest body mass loss in the colder treatment, which indicates that low winter temperatures are associated with low metabolic rate and therefore energy conservation (Patterson and Davies, 1978; Hailey and Loveridge, 1997; Irwin and Lee, 2003; Tattersall and Ultsch, 2008). Unexpectedly, we found that snakes maintained in mild wintering condition ($\sim 14^\circ\text{C}$) lost, on average, as much mass as the control (active, $\sim 24^\circ\text{C}$) snakes (Fig. 1). Clearly, this result indicates that mild wintering temperature resulted in higher metabolism that translated in significant energy use (Patterson and Davies, 1978; Hailey and Loveridge, 1997). This pattern of body mass loss under mild wintering conditions can have very strong implications. First, reduced body condition has been identified as a direct cause of mortality in wintering and emerging snakes (Hirth, 1966; Shine et al., 2001). Emergence involves a need to revive basic organismal functions, and emaciated organisms may not be able to sustain such an energy demand (Irwin and Lee, 2003; Zani et al., 2012). Second, in the asp viper, both sexes are typical capital breeders that rely on previously accumulated reserves to fuel reproduction and reproductive behaviours (Naulleau and Bonnet, 1996; Aubret et al., 2002). In males, abdominal fat stores are the sole source of energy during the sexual vernal anorexia (Bonnet, 1996; Vacher-Vallas, 1997). Reproductive effort is adjusted to their body reserves and males in higher body condition tend to have higher levels of circulating testosterone (Aubret et al., 2002). This reliance on previously stored reserves to fuel current reproduction is even more marked in females which accumulate vast amounts of energy stores during several years prior to reproduction (Naulleau and Bonnet, 1996). Feeding activity is highly reduced during reproduction (Bonnet et al., 2001; Lourdaïs et al., 2002a, 2002b). In our study, snakes wintering under mild conditions have lost an average of 5 g over the duration of our experiment. Over the whole duration of a

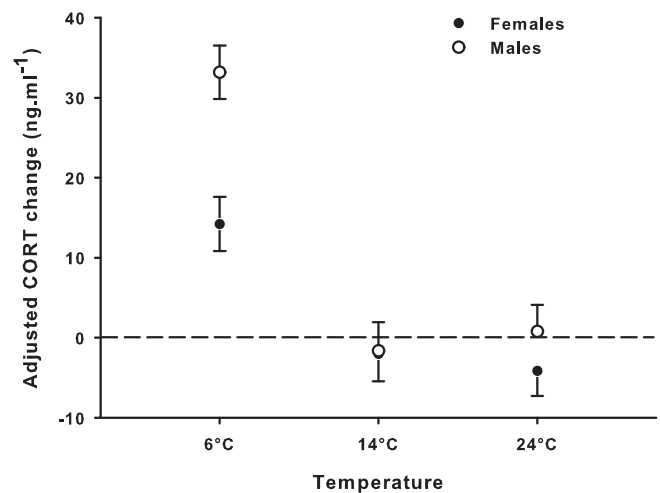


Fig. 2. Baseline CORT change (adjusted to initial baseline CORT) throughout the experiment for snakes maintained under contrasted wintering conditions (cold: $5.7 \pm 1.7^\circ\text{C}$, mild: $13.9 \pm 0.4^\circ\text{C}$ and no wintering: $24.0 \pm 0.5^\circ\text{C}$).

typical winter (e.g., ~5 months), this may represent a mean value of 25 g (i.e. respectively 20% and 30% of the initial body mass of females and males) of body reserves lost to fuel reproduction. Clearly, such a loss of previously stored reserves should greatly alter reproductive performances in both males and females. Although greater mass loss rates were predictable at mild temperature (Irwin and Lee, 2000, 2003; Hahn and Denlinger, 2007; Muir et al., 2013), the magnitude of such loss was unexpected, especially at a temperature (~14 °C) markedly below thermal preferences. It is plausible that although snakes were maintained at low temperature, their activity level may have been sufficient to induce significant energy use, and thus mass loss. Overall, our results suggest that the inverse thermal compensation involved to save energy at low temperature (Patterson and Davies, 1978) cannot be achieved at mild wintering conditions. On the contrary, it is plausible that mild wintering conditions induced a thermal compensation that resulted in higher metabolism and significant energy use (Patterson and Davies, 1978; Hailey and Loveridge, 1997).

4.2. Corticosterone levels

Contrarily to our prediction, we did not detect higher baseline CORT levels in the mild wintering temperature group. Indeed, baseline CORT in this group was similar to those assessed in the control group (Fig. 2), which could indicate that mild wintering temperature did not trigger a state of chronic stress due to inadequate thermal conditions (i.e. for which an individual cannot be active because temperature is too low, but can neither be dormant because temperature is too high). However, several studies have shown that CORT increases at non-optimal body temperatures (Li et al., 2011; Dupoué et al., 2013; Telemeco and Addis, 2014, but see Sykes and Klukowski, 2009).

Although we cannot rule out that mild wintering treatment did not trigger a state of stress in our experiment, two alternative explanations could also explain this result. First, we imposed a relatively short wintering period (1 month) in order to limit body mass loss in the control group (25 °C). This duration may have been too limited to produce a state of chronic stress in the mild wintering treatment group. Although this hypothesis seems relatively unlikely (i.e., CORT levels increase much more rapidly in other studies, see Dupoué et al., 2013 for instance), future studies could usefully explore the impact of mild wintering treatment on CORT levels in snakes maintained in experimental conditions for a longer period of time. Second, because we were interested in the processes occurring upon emergence, and because blood sampling is impaired at low temperature, experimental snakes were re-exposed to warm (~25 °C) temperature one day before the final sampling. It is plausible that this short exposure to warmer temperature allowed clearance of putative excess CORT levels and such potential mechanisms needs further investigations.

Although we did not detect higher baseline CORT in the mild wintering temperature group, we did detect a strong increase in CORT levels in the cold group (Fig. 2). While our experiment does not allow teasing apart whether this increase occurred over the duration of the treatment, or represents a peak linked to return to warm conditions, previous studies suggest the latter is more likely (Lutterschmidt and Mason, 2009; Lutterschmidt, 2012). Indeed, CORT is known to be linked with energy demands (e.g., Astheimer et al., 1992) and the increase we detect suggests that CORT secretion may be linked to the resource mobilization occurring at the onset of emergence to revive basic organismal functions (Lutterschmidt and Mason, 2009). Interestingly, we found that males display a stronger peak in baseline CORT upon emergence than females (Fig. 2), suggesting that physiological and/or behavioural changes linked to reproduction may be triggered by CORT levels upon emergence (Lutterschmidt and Mason, 2009; Lutterschmidt, 2012). If this hypothesis is true, then the absence of baseline CORT surge under mild wintering condition may negatively affect the setting up of some aspects of reproductive physiology and/or behaviour, at least in males (Lutterschmidt and Mason, 2009; Lutterschmidt, 2012). Such an

indirect and unexpected effect of mild wintering condition on subsequent reproductive physiology and behaviour, and especially the role of CORT as a potential mediator, will require further investigations (Lutterschmidt and Mason, 2009; Lutterschmidt, 2012).

More generally, we found that altered (mild) winter temperatures can strongly affect wintering temperate ectotherms (Williams et al., 2003; Zani, 2008; Zani et al., 2012; Muir et al., 2013). Most notably, the fitness of these organisms can be influenced both through proximate (reduced body condition and altered physiology upon emergence) and ultimate (influence on reproduction) effects. Such disruption of reproduction may ultimately affect population persistence (Parmesan, 2006). We emphasize that future studies should incorporate the critical, albeit neglected, winter season when assessing the potential impacts of global changes on ectotherms (Williams et al., 2015).

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