RESEARCH ARTICLE

Two stressors are worse than one: combined heatwave and drought affect hydration state and glucocorticoid levels in a temperate ectotherm

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ABSTRACT

Heatwaves and droughts are becoming more intense and frequent with climate change. These extreme weather events often occur simultaneously and may alter organismal physiology, yet their combined impacts remain largely unknown. Here, we experimentally investigated physiological responses of a temperate ectotherm, the asp viper (*Vipera aspis*), to a simulated heatwave and drought. We applied a two-by-two factorial design by manipulating the daily temperature cycle (control versus heatwave) and the water availability (water available versus water deprived) over a month followed by exposure to standard thermal conditions with *ad libitum* access to water. Simulated heatwave and water deprivation additively increased mass loss, while water deprivation led to greater plasma osmolality (dehydration). Mass gain from drinking after the treatment period was higher in vipers from the heatwave and water-deprived group, suggesting that thirst was synergistically influenced by thermal and water constraints. Heatwave conditions and water deprivation also additively increased baseline corticosterone levels but did not influence basal metabolic rate and plasma markers of oxidative stress. Our results demonstrate that a short-term exposure to combined heatwave and drought can exacerbate physiological stress through additive effects, and interactively impact behavioral responses to dehydration. Considering combined effects of temperature and water availability is thus crucial to assess organismal responses to climate change.

KEY WORDS: Combined stressors, Dehydration, Physiological stress, Temperature, Water availability

INTRODUCTION

The vulnerability of organisms to climate change depends on their capacity to maintain homeostasis when they face multiple environmental changes (Huey et al., 2012; McEwen and Wingfield, 2010; Williams et al., 2008). In particular, combined climate stressors can challenge the maintenance of energy and water balance in wild animals, and alter their physiology (Gunderson et al., 2016; Rozen-Rechels et al., 2019; Sokolova, 2013). Individuals may cope with changes in climate conditions over months or years through the acclimation of their physiological traits (Gunderson et al., 2017; Seebacher et al., 2015). However, climate is also characterized by extreme climatic events (ECE; Ummenhofer and Meehl, 2017) that can challenge homeostasis over periods of days or weeks and may reduce survival (Buckley and Huey, 2016; Zhang et al., 2018). Documenting the energy and water balance regulation mechanisms of organisms exposed to ECEs is thus crucial to assess their capacity to cope with current and future climate conditions (Sergio et al., 2018; van de Pol et al., 2017).

In temperate ecosystems, climate change is leading to more intense and frequent summer droughts and heatwaves over some geographic areas (Rahmstorf and Coumou, 2011; Stillman, 2019; Trenberth et al., 2014). These ECEs can last a few days or weeks and often occur simultaneously given the strong relationship between temperature and rainfall (Hao et al., 2019; Liu et al., 2009). This leads to combined changes in air temperature and water availability at a fast pace, from days to weeks (Ummenhofer and Meehl, 2017), challenging the regulation of both body temperature and water balance in wild animals (Riddell et al., 2019; Rozen-Rechels et al., 2019). The negative effects of drought on energy and water balance are likely to be exacerbated by heatwave stress because energy expenditure and water loss both increase at higher temperatures (Albright et al., 2017). Yet, the combined effects of high temperature and water constraints on energy and water balance regulation mechanisms remain largely unknown. To understand these mechanisms, it is necessary to disentangle the independent effects of each climate variable and examine their possible interactions, which generally calls for experimental approaches (Brusch et al., 2020b; Padda et al., 2021). Manipulative studies are needed because thermal and water constraints could either (i) synergistically impact physiology through interactive effects on mechanisms involved in the regulation of heat and water exchange (Riddell et al., 2018; Rozen-Rechels et al., 2019), or (ii) additively alter physiology through independent effects on different response pathways (Brusch et al., 2020b). Our ability to predict physiological challenges for terrestrial ectotherms is highly dependent on our understanding of these mechanistic interactions between heat and water balance regulation mechanisms (reviewed in Rozen-Rechels et al., 2019).

Terrestrial ectotherms rely on behavioral and physiological changes to maintain their optimal body temperature and minimize their energy expenditure and water loss (Angilletta, 2009; Pintor et al., 2016; Rozen-Rechels et al., 2019). In response to challenging and unpredictable situations such as ECEs, the secretion by the

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hypothalamo-pituitary–adrenal (HPA) axis of corticosterone (CORT), the main glucocorticoid in reptiles, is a major component of the stress response that can modulate shifts in whole-organism physiology and behavior (Angelier and Wingfield, 2013; Wingfield, 2013; Wingfield et al., 1998). At baseline levels, CORT is an important regulator of the energy budget and basal metabolism (Dupont et al., 2019; Landys et al., 2006; Lorioux et al., 2016). When ectotherms are chronically exposed to warm temperatures, baseline CORT levels can increase in line with a higher metabolism and energy expenditure, which helps divert resources towards increased foraging, enhanced locomotion and elevated gluconeogenesis (de Bruijn and Romero, 2018; Jessop et al., 2016). Yet, chronic elevation of baseline CORT levels can lead to deleterious physiological effects (e.g. reduced performance, suppressed immune response; Berger et al., 2005; Sapolsky et al., 2000) and notably can generate oxidative stress (Costantini et al., 2011). This may cause long-term physiological dysfunction and is considered as a major process for lifespan shortening in ectotherms (Burraco et al., 2020; Zhang et al., 2018). In response to acute life-threatening events (e.g. predation, thermal extremes), the rapid secretion of CORT (stress-induced CORT levels) can further allow short-term resource allocation toward physiological and behavioral responses that promote immediate survival, such as more alert behaviors or escape movements (Landys et al., 2006; Palacios et al., 2012; Vitousek et al., 2019). However, chronic exposure to environmental stressors can influence these acute stress responses. It can induce an 'emergency' life history stage associated with increased stress-induced CORT levels because of an enhanced sensitivity of the HPA axis (Dupoué et al., 2013; Narayan et al., 2012). Alternatively, chronic stressors can downregulate the HPA axis responsiveness to acute stressors, which leads to reduced stress-induced CORT levels (Narayan and Hero, 2014; Rich and Romero, 2005). Hence, how exposure to warmer temperatures during ECEs affects stress-induced CORT levels remains to be elucidated in terrestrial ectotherms.

Osmoregulation involves physiological pathways that are likely to be interrelated with the responses to temperature constraints (Rozen-Rechels et al., 2019). Indeed, baseline CORT is also presumed to have mineralocorticoid actions and therefore may contribute to osmoregulation (McCormick and Bradshaw, 2006). In response to water deprivation, baseline CORT can increase (Brusch et al., 2020a; Dupoué et al., 2016) and might be involved in changes in energetic pathways tightly linked to dehydration, such as a higher protein catabolism and muscle wasting, which can allow for bound-water reallocation when drinking water is limited (Brusch et al., 2018; Dezetter et al., 2021b). At the same time, dehydration can lead to rapid changes in oxidative status depending on its effects on oxidative damage and oxidative defense mechanisms, which seem to vary in reptiles across species and even according to physiological states within the same species (Brusch et al., 2020b; Dezetter et al., 2021b; Dupoué et al., 2020; Stier et al., 2017). Chronic water restriction can also lead to higher stress-induced CORT levels, potentially reflecting an enhanced reactivity to acute stressors during dehydration, which may compensate for behavioral inactivity and impaired locomotor performance in dehydrated organisms (Dupoué et al., 2014; Moeller et al., 2017). Therefore, one hypothesis is that the combined exposure to warmer temperatures and water deprivation during ECEs will lead to an interactive and synergistic increase in baseline CORT, metabolism and oxidative stress. As the duration of exposure to an ECE gets longer, chronic exposure to warmer temperatures and water deprivation may however induce a down-regulation of CORT

secretion and a decrease in metabolic rate as an energy- and water-saving strategy (Dupoué et al., 2018b; Muir et al., 2007). In long-lived ectotherms such as the snakes investigated here, these acclimation responses take place over the span of months (Dezetter et al., 2021a) and it remains to be investigated whether they are likely to occur or not within the typical duration of ECEs (i.e. days or weeks).

Herein, we experimentally examined the physiological response to temperature and water constraints during a simulated ECE in a temperate ectotherm, the asp viper, *Vipera aspis* (Linnaeus 1758). We used climatic chambers to manipulate independently daily thermal cycles (control versus heatwave) and access to drinking water (water available versus water deprived) in a two-by-two factorial design lasting 1 month, followed by a re-exposure to standard thermal conditions with *ad libitum* access to water. We hypothesized that higher temperatures combined with water deprivation should synergistically challenge homeostasis during a simulated ECE. We tested the following predictions. First, combined heatwave temperatures and water deprivation will interact to lead to greater physiological dehydration (increased body mass loss, higher increase in plasma osmolality) and thirst levels (higher post-treatment water intake). Second, these combined constraints will also synergistically lead to greater energy mobilization (increase in plasma CORT levels and in metabolic rate), which will further induce oxidative stress (higher levels of oxidative damage relative to anti-oxidant defense).

MATERIALS AND METHODS
Study species
The asp viper is a medium sized viviparous snake distributed over southwestern Europe (Lourdais et al., 2002b). Ambient temperatures drive activity of this heliothermic ectotherm (Lourdais et al., 2004; Luiselli et al., 2018; Rugiero et al., 2013), and thermal and water constraints have a strong influence on its behavior and physiology, notably during reproduction (Dezetter et al., 2021a; Dupoué et al., 2015a,b; Lorioux et al., 2013). The asp viper displays a relatively small home range and is strongly exposed to local habitat constraints (Aragón et al., 2010; Zwahlen et al., 2021). Basking and microhabitat selection behaviors allow vipers to thermoregulate while minimizing water loss during the day (Dupoué et al., 2015b; Guillon et al., 2014; Lourdais et al., 2017, 2013). However, this species faces both extreme temperature and limited access to water during summer heatwaves and droughts. The frequency and intensity of these events have increased in recent times over the range distribution of asp vipers (Bador et al., 2017; Spinoni et al., 2018). The asp viper can tolerate water deprivation over weeks, even during water-demanding stages such as pregnancy (Dupoué et al., 2018a; Lourdais et al., 2017, 2015). Water shortage involves flexible changes in energy and water resource allocation, changes in corticosterone secretion and an up-regulation of oxidative defense in adult females, especially during pregnancy (Dupoué et al., 2016; Stier et al., 2017). This species relies on sporadic feeding events to accumulate energy reserves and can fast over a period of several months (Lourdais et al., 2002a). We used 47 non-reproductive adult snakes (27 females and 20 males) from a long-term captive colony originating from western France, born and raised in laboratory conditions at the Centre d'Etudes Biologiques de Chizé, France (Dezetter et al., 2021a). Snakes were used to seeing humans and to being handled and restrained as part of a routine animal care protocol. All experimentation procedures with animals listed in this study were approved by an independent ethical committee (Apafis#18149-2018122010137623_v2).

Experimental design and treatments

We exposed individuals to one of four treatments in a two-by-two factorial design manipulating thermal conditions (control versus heatwave thermal treatment) and access to drinking water (water available versus water deprived). We applied the treatments for 33 days (hereafter called the treatment period), which corresponds to a moderate ECE duration within the range distribution of the asp viper in Western Europe (Perkins et al., 2012; Sutanto et al., 2020). On day 34, all individuals were provided with water *ad libitum* and transferred to standard maintenance thermal conditions (see below).

We designed ecologically relevant daily thermal cycles with different mean and variance of temperatures (Colinet et al., 2015; Morash et al., 2018) to mimic either a moderate summer heatwave (hereafter HW thermal treatment) or standard summer weather conditions (hereafter control thermal treatment, see Fig. 1). During the daytime in natural conditions, the asp viper uses micro-habitats to behaviorally thermoregulate at a preferred temperature close to 30°C (Lorioux et al., 2013). At night in natural conditions, body temperature fluctuates with the ambient temperature of the underground shelters used by these snakes (Lourdais et al., 2013). Higher ambient temperatures induced by a moderate heatwave will lead to an extended time spent at the preferred temperature and to higher night-time body temperatures (Blouin-Demers and Weatherhead, 2001; Davy et al., 2017). We thus applied a daytime body temperature of 30°C in both thermal treatments but with different durations during the daytime (3 versus 12 h of daytime in the control versus the HW thermal treatment, respectively). We also applied different night-time temperatures and durations (18 versus 26°C in the control versus the HW thermal treatment, see Fig. 1).

In the water-available treatment, individuals were provided with drinking water *ad libitum* in a glass bowl, whereas individuals in the water-deprived treatment had no access to drinking water during the treatment period. Air humidity is tightly linked to air temperature and respiratory or cutaneous water loss rates increase when the deficit in water vapor pressure is higher (Dupoué et al., 2015b; Woods and Smith, 2010). Thus, we maintained a similar water vapor pressure deficit (WVPD) of ca. −1 kPa in all treatments (Fig. 1), which corresponds to a minimally desiccant environment

for vipers (Dupoué et al., 2015b). To achieve this, relative humidity was set at 75% at 30°C ambient temperature, 70% at 26°C, and 50% at 18°C.

Males and females were randomly assigned to treatments, with similar sex ratios in each treatment. We manipulated temperature cycles while keeping a constant WVPD by using four climatic chambers (Vötsch VP 600, Balingen, Germany) controlled with SIMPATI software (v.2.06 Vötsch). Individuals were housed individually in plastic boxes (35×25×13 cm) with a PVC shelter and an artificial grass carpet as substrate. Therefore, our experimental design did not allow for behavioral thermoregulation and microclimate selection. While postural changes remained possible, we expect these individual responses to be of limited relevance in our highly controlled laboratory conditions. The photoperiod followed that of natural daylight cycles (ca. 12 h:12 h). Prior to the treatment period, individuals were housed for a month inside the climatic chambers for acclimation to general maintenance conditions with *ad libitum* access to drinking water and a daily thermal cycle intermediate between that of the control and HW thermal treatments (6 h of daytime at 30°C and a night-time temperature of 22°C) with a similar WVPD of ca. −1 kPa. We did not feed the snakes during this pre-experimental period and the experimental period in order to avoid confounding effects of digestion and energy intake on physiology (Stahlschmidt et al., 2015).

Variables collected and parameters measured
Changes in mass

Body mass changes provide a reliable proxy of hydration state in the absence of food intake in squamate reptiles (Dupoué et al., 2014). We therefore weighed (±0.1 g) all individuals every 3 days during the treatment period and further weighed them at the end of day 34. We used the mass change between day 33 and day 34 as a proxy for post-treatment water intake (minus water loss). In the groups where water was available, 3 individuals did not drink and kept losing mass from day 33 to day 34, leading to negative values as a result of evaporative water loss. Post-treatment water intake could not be assessed for one individual in the control and water-deprived group because it defecated on day 34.
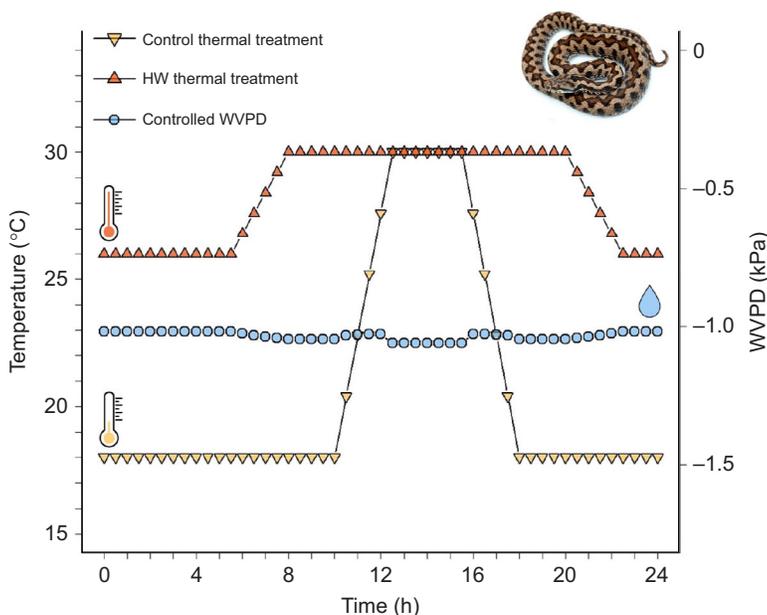

Fig. 1. Experimental design. Daily temperature cycle (left axis) applied in each thermal treatment (control; simulated heatwave, HW); and controlled water vapor pressure deficit (WVPD) set at approximately −1 kPa (right axis) for both treatments. These conditions were applied to a sample of 47 non-reproductive adult asp vipers of both sexes. To mimic ecologically relevant thermal conditions, we manipulated both mean temperature and the amplitude of thermal variability (control: mean of 20.7°C, amplitude of 12°C; simulated HW: mean of 28.4°C, amplitude of 4°C). Photo credit: copyright Matthieu Berroneau.

Blood parameters

We collected blood samples from each individual at the onset (day 0) and at the end (day 33) of the treatment period to assess plasma osmolality, baseline and stress-induced plasma CORT concentration as well as two standard markers of oxidative status (concentration of reactive oxidative metabolites, ROMs; and antioxidant capacity, OXY). We sampled individuals in a random order by sex and experimental treatment. We used a 1 ml syringe and a heparinized 29-gauge needle to collect all blood samples (150 μl) through cardiocentesis. We used repeated blood sampling by cardiocentesis without anesthesia, following ethical and methodological guidelines for small snakes (Beaupre et al., 2004; Bonnet et al., 2020; Köbölkuti et al., 2009). This method causes only minimal histological changes of limited clinical significance and low levels of pain similar to those from percutaneous blood collection in mammals (Isaza et al., 2004). It was approved by an independent ethical committee (Apafis#18,149-2018122010137623_v2) and performed by a trained person. Blood was immediately centrifuged at 2000 g, plasma was separated from red blood cells and samples were stored at −28°C until laboratory analysis.

In order to measure baseline and stress-induced CORT levels, we applied a capture and restraint stress protocol standardized for the asp viper (Dupoué et al., 2016; Lorioux et al., 2016). To measure baseline CORT levels, we collected the first blood sample (body temperature of 30°C) immediately upon removing the snake from its cage (mean±s.d. blood sample duration 3.4±1.4 min; Romero and Wikelski, 2001). Blood sampling was carried out of sight of the other individuals to avoid disturbance, and handling was gently conducted to minimize defensive responses that could influence baseline values. No correlation was found between baseline CORT levels and blood sample duration (all $P>0.05$; Table S2), supporting the assumption that our assays quantified 'true' baseline values (Tylan et al., 2020). To promote a stress response, snakes were then placed in a transparent plastic box (35×25×13 cm) without any shelter for 1 h (64.0±2.6 min) at a controlled temperature (25±0.1°C). Blood was then sampled a second time (body temperature of 25°C) to measure stress-induced CORT levels. We used plasma aliquots from the first blood sample to measure plasma osmolality and markers of oxidative status.

Plasma CORT concentration (ng ml^{-1}) was determined from 50 μl aliquots using a radioimmunoassay protocol (see Dupoué et al., 2016, for details). Samples were run in six assays (intra-assay variation: 10%, inter-assay variation: 14.6%). We measured plasma osmolality from 10 μl aliquots using a vapor pressure osmometer (±3 mOsm kg^{-1}; Vapro2, Elitech group). We used d-ROMs colorimetric kits on 4 μl plasma aliquots (MC003, Diacron International; coefficients of variation: intra-plate, 1.5%; inter-plate 1.4%) to measure the activity of organic hyperoxides (ROMs, mg H$_2$O$_2$ dl^{-1}) as an index of oxidative damage. Further, we used OXY-absorbent test kits (MC435, Diacron International; coefficients of variation: intra-plate, 0.5%; inter-plate 9.8%) to assess the non-enzymatic ability of 2 μl aliquots of diluted plasma samples (1:100) to neutralize an oxidant attack from hypochlorous acid (OXY, μmol HClO ml^{-1}) as an index of oxidative defense (Costantini, 2016, 2011).

Resting metabolic rate

To investigate energy-saving responses, we measured the rate of oxygen consumption ($\dot{V}_{O_2}$, ml h^{-1}) as a proxy for resting metabolic rate (RMR), 5 days prior to the start of the treatment period, on day 14 and on day 28. We used a closed-system respirometry setup (for details, see Supplementary Materials and Methods) to assess $\dot{V}_{O_2}$ for animals at rest at 25°C in randomized trials by sex and experimental treatment. Individuals were inactive when $\dot{V}_{O_2}$ was measured. We used a temperature-controlled test chamber in which we placed each individual 2 h prior to each measurement in order to stabilize the test temperature and avoid exploratory behaviors. In the event that the snake was active or defecated during the run, we repeated the trial until standard conditions were reached.

Statistical analyses

All analyses were performed using R software version 3.6.3 (holding the windsock, http://www.R-project.org/). We used linear models fitted with the *lm* function of the *stats* package, and linear mixed effects models fitted with the *lme* function of the *nlme* package (https://CRAN.R-project.org/package=nlme). For model selection, we started with full models and used stepwise removal of insignificant variables and interactions using standard and marginal *F* tests for linear and mixed models, respectively (*anova*, package *stats*). We checked that residuals of our models fulfilled a Gaussian distribution using Shapiro–Wilk tests and had homogeneous variances across treatments. Statistical differences between treatment groups were further explored using pairwise *post hoc* Tukey tests (*emmeans*, package *emmeans*; https://CRAN.R-project.org/package=emmeans).

Before testing for the effects of temperature and water treatment on changes in mass, blood parameters and RMR, we built linear models to confirm that there were no differences among groups at the beginning of the experiment. We built independent linear models for each variable with the initial values as the dependent variable, and thermal and water treatment and their interaction as explanatory factors. To confirm that baseline CORT was not dependent on blood sample duration in each treatment, we built four independent linear models (i.e. one for each treatment group) with baseline CORT as the dependent variable and blood sample duration as the explanatory variable.

Effects of temperature and water treatment on body mass were tested with a linear mixed effect model for repeated measurements with body mass as the dependent variable, and thermal treatment, water treatment, time (i.e. days on which individuals were weighed) and the interactions between thermal treatment, water treatment and time as explanatory factors. Individual identity was set as a random factor. To assess the effects of experimental treatment on the water intake subsequent to the end of the water deprivation period, we built a linear model with post-treatment water intake as the dependent variable, initial body mass as a linear covariate, and sex, thermal treatment, water treatment, time and the interactions between thermal treatment, water treatment and time as explanatory factors.

To further investigate the effects of thermal and water treatment on changes in blood parameters, we built five independent linear models with the changes in each physiological trait as the dependent variable (change in: plasma osmolality, baseline CORT, stress-induced CORT, ROMs or OXY), the initial value of each physiological trait as a linear covariate, and sex, thermal treatment, water treatment and the interactions between these three parameters as fixed factors. To investigate linear changes in RMR in response to the thermal and water treatments, we built a linear mixed effect model for repeated measurements with $\dot{V}_{O_2}$ as the dependent variable, body mass as a linear covariate, and sex, thermal treatment, water treatment, time and the interactions between thermal treatment, water treatment and time as explanatory variables. We log-transformed $\dot{V}_{O_2}$ to meet the assumptions of homoscedastic and

normal residuals. Individual identity was included as a random factor.

We investigated the relationship between body mass loss and changes in baseline CORT and in plasma osmolality using two independent linear models with body mass loss as the dependent variable, initial body mass as a linear covariate and either change in baseline CORT or change in plasma osmolality as an explanatory variable. We further examined the relationship between baseline CORT and plasma osmolality across both sampling periods using a linear regression, grouping all individuals together regardless of treatment. To investigate the relationship between post-treatment water intake and plasma osmolality, we built two independent linear models with water intake as the dependent variable, initial body mass as a linear covariate, and either change in plasma osmolality or final plasma osmolality as a covariate. Similarly, we investigated the relationship between post-treatment water intake and body mass loss using a linear model with water intake as the dependent variable, initial body mass as a linear covariate, and body mass loss as a

covariate. We also investigated the relationship between water intake and baseline CORT using similar linear models with either change in baseline CORT or final baseline CORT as a covariate. These analyses allowed us to examine how physiological and behavioral responses correlate with each other.

RESULTS
Effects of thermal and water treatment on body mass change and thirst

At the beginning of the experiment, individuals had similar body mass between treatments (all $P>0.05$; Table S1). Unsurprisingly, vipers lost body mass during the manipulation (time: $F_{1,514}=77.5$, $P<0.001$; Fig. 2A). The thermal treatment (thermal treatment×time: $F_{1,514}=40.9$, $P<0.001$) and the water treatment (water treatment×time: $F_{1,514}=83.6$, $P<0.001$) additively influenced body mass loss during the treatment period (Fig. 2A; Table S3). Water-deprived vipers lost body mass at a faster rate than vipers in the water-available treatment groups, and vipers also lost body mass at a
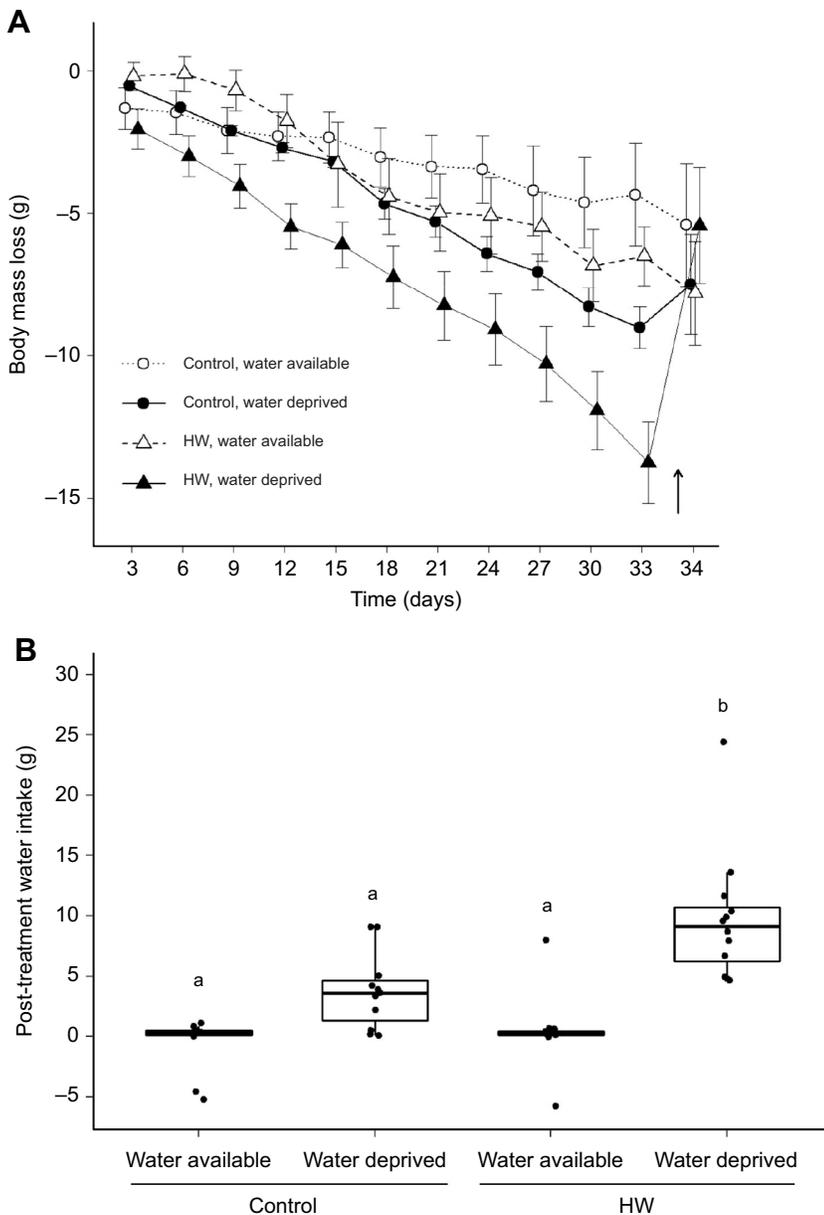

Fig. 2. Effect of treatments on body mass and thirst of asp vipers. (A) Effect of thermal and water treatment on changes in body mass during the manipulation (mean±s.e.m., control and HW treatment under water-available and water-deprived conditions). Body mass values were adjusted to initial body mass. The arrow indicates the end of the treatment period and access to *ad libitum* water for all vipers. (B) Effect of thermal and water treatment on post-treatment water intake. The top and bottom of the boxes represent the upper and lower quartiles, the horizontal line within the box represents the median and the whiskers represent the 5th and 95th percentiles. Points are individual data records (control and water available $n=11$; control and water deprived $n=12$; HW and water available $n=12$; HW and water deprived $n=12$). The different letters indicate significant differences among treatment groups based on Tukey *post hoc* tests of contrasts.

faster rate in the HW than in the control treatment. There was no interactive effect of thermal and water treatments on body mass loss (thermal treatment×water treatment×time: $F_{1,513}=0.1$, $P<0.74$).

Unlike body mass, post-treatment water intake was influenced by the interaction between the thermal treatment and water treatment ($F_{1,42}=5.3$, $P=0.01$; Table S4). Individuals exposed to combined HW and water deprivation drank plentiful amounts of water and rapidly gained mass whereas water-deprived snakes from the control thermal treatment only slightly recovered body mass, at a similar level to snakes from the water-available groups (see Fig. 2B). Sex had no influence on the pattern of body mass loss or on the post-treatment water intake (all $P>0.05$).

Changes in blood parameters in response to thermal and water treatment

At the beginning of the experiment, there was no initial difference in blood parameters between treatments (all $P>0.05$; Table S1). Changes in plasma osmolality were dependent on initial plasma osmolality ($F_{1,41}=25.3$, $P<0.001$) and on the water treatment ($F_{1,41}=10.0$, $P=0.002$), but were not influenced by thermal

treatment ($F_{1,41}=0.07$, $P=0.79$; Table S5). Plasma osmolality increased in water-deprived vipers (mean±s.e.m. 17.4±4.8 mOsm kg^{-1}) throughout the experiment as expected if vipers were more dehydrated, while it remained stable (-0.8 ± 3.4 mOsm kg^{-1}) in vipers from the water-available treatment (Fig. 3A, Table 1), as expected if they maintained a normosmotic state.

Changes in baseline CORT levels were influenced by initial baseline CORT levels ($F_{1,42}=50.7$, $P<0.001$) and sex ($F_{1,42}=5.7$, $P<0.02$), but we also found additive effects of both thermal ($F_{1,42}=4.8$, $P=0.03$) and water treatment ($F_{1,42}=7.7$, $P=0.008$) on changes in baseline CORT (Fig. 4, Table 1; Table S5). The change in baseline CORT was greater on average in vipers from the HW treatment than in controls, and in water-deprived vipers than in the water-available groups. Changes in stress-induced CORT levels were dependent on initial stress-induced CORT levels ($F_{1,43}=20.1$, $P<0.001$) and the thermal treatment ($F_{1,43}=6.2$, $P=0.02$), but not on the water treatment ($F_{1,43}=0.3$, $P=0.61$). Stress-induced CORT levels (Table 1) increased in vipers from the HW thermal treatment (mean±s.e.m. 17.1±7.7 ng ml^{-1}) while it decreased in vipers from the control thermal treatment (-10.3 ± 7.8 ng ml^{-1}; Fig. S1).
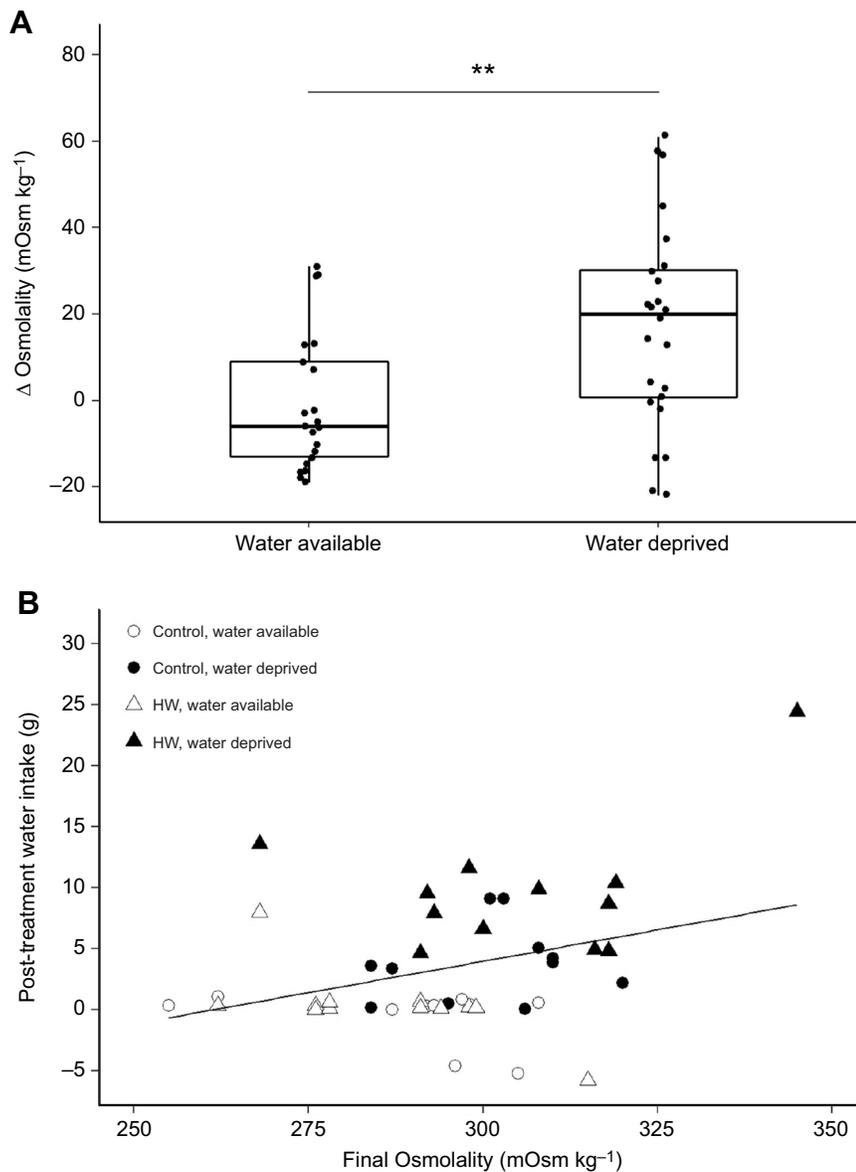

Fig. 3. Effect of treatments on plasma osmolality. (A) Effect of water treatment on changes in plasma osmolality (water available $n=23$; water deprived $n=24$) in asp vipers. The top and bottom of the boxes represent the upper and lower quartiles, the horizontal line within the box represents the median and the whiskers represent the 5th and 95th percentiles. Points are individual data records. Asterisks indicate significant differences among treatment groups (**$P<0.01$) based on Tukey *post hoc* tests of contrasts. (B) Relationship between post-treatment water intake and final plasma osmolality (control and water available $n=11$; control and water deprived $n=12$; HW and water available $n=12$; HW and water deprived $n=12$). The line represents the best regression curve.

Table 1. Summary statistics of the physiological parameters measured for individuals at the onset and at the end of the treatment period

Time of measurement	Variable	Control		Heatwave	
		Water available	Water deprived	Water available	Water deprived
Onset – day 0	Osmolality (mOsm kg^{-1})	286.20±4.21	284.50±4.70	289.45±4.22	284.83±5.88
	Baseline CORT (ng ml^{-1})	20.23±3.61	12.47±1.94	14.73±3.03	16.75±2.98
	Stress-induced CORT (ng ml^{-1})	63.01±14.23	79.21±17.66	55.48±8.00	75.77±18.03
	ROMs (H$_2$O$_2$ dl^{-1})	7.90±1.56	5.53±0.81	7.57±1.19	8.36±1.37
	OXY (µmol HClO ml^{-1})	269.70±21.94	264.59±20.69	283.96±18.77	268.55±22.55
End – day 33	Osmolality (mOsm kg^{-1})	289.30±5.50	298.67±3.86	285.00±4.73	305.50±5.64
	Baseline CORT (ng mL^{-1})	9.77±1.05	12.27±2.21	11.23±2.45	20.36±3.33
	Stress-induced CORT (ng ml^{-1})	50.19±11.53	68.23±9.99	89.42±17.22	78.92±12.40
	ROMs (H$_2$O$_2$ dl^{-1})	5.98±1.40	5.14±1.55	4.95±0.79	4.74±0.84
	OXY (µmol HClO ml^{-1})	279.02±25.39	257.12±18.98	230.41±18.05	257.38±24.08

Data are means±s.e.m. for the different treatment conditions: control and heatwave with or without water (available or deprived). CORT, corticosterone; ROMs, reactive oxidative metabolites; OXY, antioxidant capacity.

In contrast, treatments had no effects on changes in plasma ROMs (thermal treatment: $F_{1,42}$=1.4, P=0.25; water treatment: $F_{1,42}$=0.2, P=0.62) and OXY concentrations (thermal treatment: $F_{1,42}$=1.58, P=0.21; water treatment: $F_{1,42}$=0.002, P=0.96). Instead, the changes in ROMs and OXY were dependent on sex (ΔROMs, sex: $F_{1,42}$=6.5, P=0.01; ΔOXY, sex: $F_{1,42}$=12.2, P=0.001) and on initial values of ROMs ($F_{1,42}$=33.6, P<0.001) and OXY ($F_{1,42}$=46.4, P<0.001).

Changes in RMR
There was no initial difference in RMR between treatments (all P>0.05; Table S1). As expected, $\dot{V}_{O_2}$ scaled positively with body mass ($F_{1,92}$=15.1, P<0.001). However, RMR was not influenced by thermal treatment (thermal treatment×time: $F_{1,92}$=1.2, P=0.28) or by water treatment (water treatment×time: $F_{1,92}$=0.1, P=0.72; Table 2; Table S6).

Correlations between variables
Body mass loss during the experiment was positively correlated with changes in baseline CORT ($F_{1,44}$=6.9, P=0.01; Fig. S2) but not with changes in plasma osmolality (marginal effect; $F_{1,42}$=3.9, P=0.05; Table S7). The baseline CORT levels were not correlated with plasma osmolality across both sampling sessions ($F_{1,44}$=2.2, P=0.15; Table S8). Water intake after the end of the experiment was positively correlated with final plasma osmolality ($F_{1,42}$=4.9, P=0.03; Fig. 3B), but not with changes in plasma osmolality during the experiment ($F_{1,42}$=1.4, P=0.24). It was also positively correlated with body mass loss during the experiment ($F_{1,43}$=16.4, P<0.001; Table S7, Fig. S3). Water intake was not correlated with final baseline CORT ($F_{1,43}$=1.4, P=0.24) or with changes in baseline CORT ($F_{1,43}$=2.5, P=0.12).

DISCUSSION
Wild organisms are facing more frequent and intense ECEs, which combine both high ambient temperatures and reduced water availability over days or weeks and may challenge their capacity to maintain homeostasis. Contrary to our initial predictions that combined heatwave temperatures and water deprivation would interactively alter hydration state, glucocorticoids levels and oxidative state, we found mostly additive or single-factor effects of temperature and water treatment on organismal physiology. However, we found an interactive effect on drinking behavior, suggesting a synergistic effect on behavioral responses to dehydration.

Changes in body mass, plasma osmolality and drinking behavior indicated that heatwave temperatures and water deprivation resulted in a significant physiological dehydration. Body mass loss throughout the treatment period was additively increased by heatwave temperature and water deprivation. Surprisingly, changes in plasma osmolality were affected only by water deprivation, and not by the heatwave treatment, contrary to recent findings in gravid lizards using a fairly similar experimental design (Brusch et al., 2020b). In addition, body mass loss was correlated with the increase in baseline CORT but not with the increase in plasma osmolality. Altogether, this suggests that the enhanced body mass loss at higher temperatures probably reflected mostly changes in energy expenditure and catabolism to support the higher pace of life in a warmer environment instead of being a consequence of physiological dehydration per se (Burraco et al., 2020; CaraDonna et al., 2018).

In vipers from the combined heatwave and water-deprived treatment, there was a sharp mass gain during the post-experimental re-hydration period, which suggests significant levels of dehydration-induced thirst following the joint manipulation of temperature and water availability (Dezetter et al., 2021b; Dupoué et al., 2014). To our knowledge, this study is the first to demonstrate an interactive effect of high temperature and water deprivation on drinking behavior, a central behavioral trait for water balance regulation, especially in snakes (Edwards et al., 2021; Lillywhite et al., 2019). This result suggests that the combined exposure to droughts and heatwaves in natural habitats of ectotherms can synergistically alter their hydroregulation behavior despite additive effects on hydration state. Exploratory analyses hint at potential explanations for this contradictory finding. Indeed, we observed that the post-treatment water intake was positively correlated with final plasma osmolality, but not with changes in plasma osmolality or with glucocorticoid levels. Thus, thirst was linearly related to physiological dehydration on average (Fig. 3B). Yet, the water-deprived snakes in the simulated heatwave treatment drank more than those from the control thermal treatment, which suggests that the combined effects of temperature and water deprivation generated non-additive changes in another critical physiological trait controlling drinking behavior (Edwards et al., 2021; Lillywhite et al., 2012). More studies are needed to disentangle the interactive effects of temperature and water availability on thirst.

We further found that heatwave temperatures and water deprivation additively increased the baseline CORT levels measured in snakes at their preferred body temperature. Higher baseline CORT levels in response to the heatwave treatment may reflect an altered physiological state due to a higher energy expenditure in the warmer climate (DuRant et al., 2008; Jimeno et al., 2018). This contrasts with some previous findings in
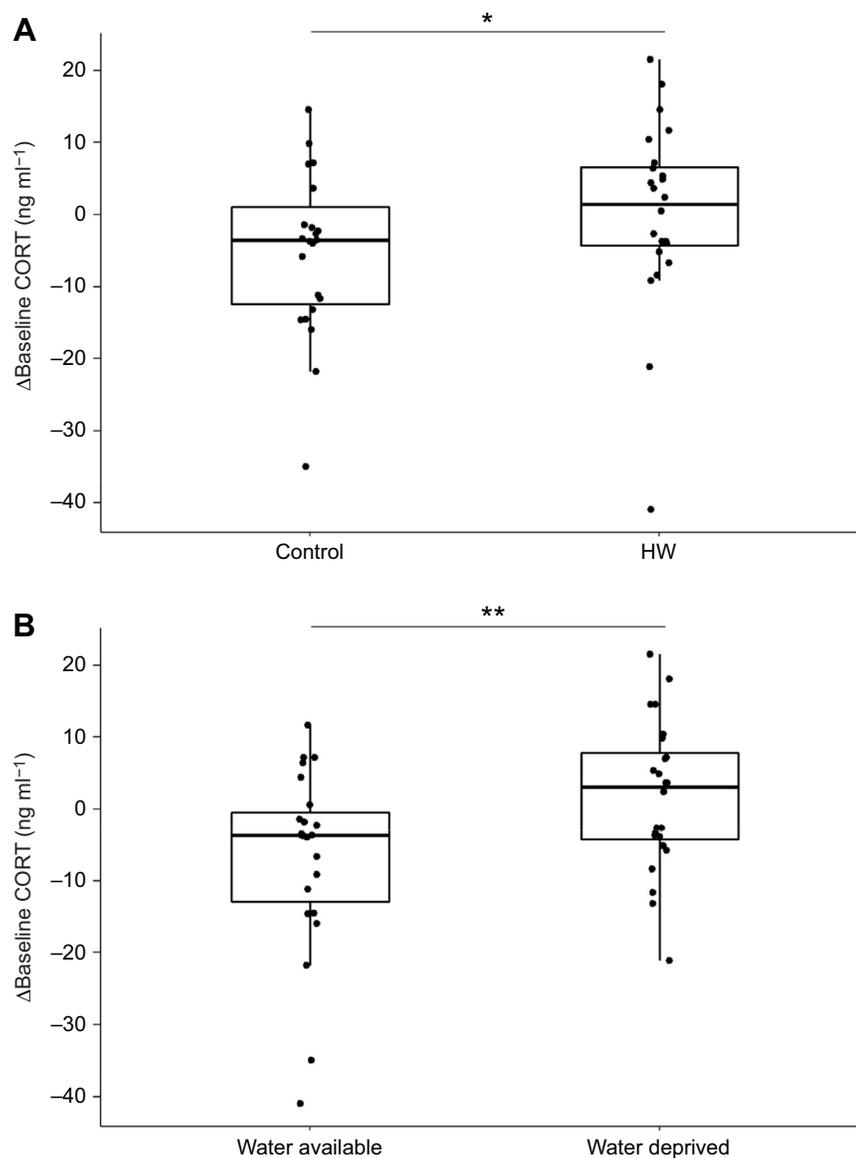
A

B

Fig. 4. Additive effects of thermal and water treatment on baseline corticosterone (CORT) plasma concentration. (A) Control (n=23) and HW (n=24). (B) Water available (n=23) and water deprived (n=24). The top and bottom of the boxes represent the upper and lower quartiles, the horizontal line within the box represents the median and the whiskers represent the 5th and 95th percentiles. Points are individual data records. Asterisks indicate significant differences among treatment groups (*P<0.05 and **P<0.01), based on Tukey *post hoc* tests of contrasts.

ectotherms, in which lower baseline CORT levels at a higher environmental temperature were associated with a reduction in activity and interpreted as an energy-saving mechanism (Dupoué et al., 2018b; Jessop et al., 2016). Higher baseline CORT levels in response to dehydration can modulate water balance regulation through the mineralocorticoid actions of CORT (Brusch et al., 2020a; McCormick and Bradshaw, 2006). However, baseline

Table 2. Summary statistics of the resting metabolic rate (RMR) measured on individuals at three time points during the experimental period

Time of measurement	Control		Heatwave	
	Water available	Water deprived	Water available	Water deprived
Day − 5	6.27±0.67	6.40±0.75	7.71±1.28	6.19±0.68
Day + 14	4.76±0.48	5.54±0.79	7.21±1.36	5.05±0.52
Day + 28	6.23±0.93	5.46±0.80	6.69±1.49	4.81±0.52

RMR ($\dot{V}_{O_2}$, ml h^{-1}) is given as the mean±s.e.m. for the different treatment conditions: control and heatwave with or without water (available or deprived).

CORT levels were not correlated with plasma osmolality. The baseline secretion of CORT, a mediator of energy reserve mobilization, was involved in a higher protein catabolism, which releases bound water that can be re-allocated to sustain individual water requirements in dehydrated snakes (Brusch et al., 2018). Unfortunately, we did not quantify muscle wasting and protein catabolism in this study. We also found that heatwave temperatures led to increased stress-induced CORT levels at a standard body temperature of 25°C, in congruence with the positive relationship often observed between stress-induced CORT and body temperature in other studies (Claunch et al., 2017; Racic et al., 2020). One potential interpretation is that exposure to higher temperatures triggered a more alert state in snakes. As a result, vipers may be better able to rapidly increase their CORT secretion in response to an additional stressor when chronically exposed to heatwave temperatures. This interpretation is in line with the idea that warmer environments may induce more active and bolder behaviors correlated with a faster pace of life behavioral syndrome (Goulet et al., 2018, 2017). However, unlike some previous studies (Dupoué et al., 2014; Moeller et al., 2017), we found no effect of water

deprivation on stress-induced CORT levels. This suggests that, contrary to heatwave temperature, water deprivation does not translate into an increased secretion of CORT when an additional stressor occurs (Brischoux et al., 2020). Although the reasons for this discrepancy between studies remain unclear, we note that the duration of the experiment and dehydration levels may not have been strong enough to trigger such effects (e.g. water deprivation lasted 33 days in our experiment versus 50 days in Dupoué et al., 2014).

Contrary to our initial predictions, oxidative state was not influenced by heatwave temperature or water availability. More specifically, changes in plasma markers of oxidative damage (ROMs) and of non-enzymatic antioxidant capacity (OXY) were not affected by heatwave temperatures or water deprivation. This is surprising because heatwave temperatures led to higher baseline CORT levels and to increased body mass loss, both suggesting higher catabolism that should produce oxidative damage (Costantini et al., 2011). Our results also contrast with the pattern of oxidative stress responses to acute exposure to high temperatures generally observed in experimental studies on vertebrates (Ritchie and Friesen, 2021). However, Costantini (2019) cautioned that responses of oxidative stress markers are often dependent on the kind of tissues and specific biological markers examined. Another study in a snake species found that a simulated heatwave of mild magnitude led to reduced oxidative damage (Stahlschmidt et al., 2017), possibly through hormetic responses (i.e. beneficial effects of a stressor at low doses; Costantini et al., 2010). Further work is thus needed to investigate how the duration and magnitude of ECEs may influence the effects of temperature on oxidative damage. In addition, recent studies on reproduction effort in pregnant asp vipers and in adult European common lizards (*Zootoca vivipara*) found a general pattern of increased oxidative damage and up-regulation of antioxidant defenses in response to water deprivation using similar markers to those used in our study (Brusch et al., 2020b; Dupoué et al., 2020; Stier et al., 2017). These and our results probably indicate that water deprivation effects on oxidative status are context dependent and possibly tied to reproductive costs (Metcalfe and Monaghan, 2013). For example, the oxidative status of asp vipers might be more sensitive to physiological dehydration during pregnancy, over a longer time period or with a stronger intensity of drought and heat.

In congruence with oxidative state, RMR did not change in response to heatwave temperatures and water deprivation. Interestingly, we found persistent individual differences that may reflect individual physiological syndromes (Dezetter et al., 2021a). Snakes did not reduce their RMR as they would if they acclimated to both higher temperatures and water deprivation, limiting both energy expenditure and water loss (Muir et al., 2007; Riddell et al., 2018). Empirical data suggest that the time to fully acclimate is longer for long-lived and large-bodied species than for smaller ectotherms (Kingsolver and Huey, 2008; Rohr et al., 2018). In the asp viper, acclimation typically takes place over the span of months (Dezetter et al., 2021a), which is a similar time scale to that of seasonal changes, rather than ECEs (i.e. days or weeks). Metabolic acclimation responses to ECEs in natural habitats may thus be constrained by the relatively short duration of these events relative to the typical acclimation abilities of large organisms such as snakes.

Ectotherms rely on behavioral means, most importantly microhabitat selection (e.g. full-sun exposure in dry substrates versus shade from vegetation cover) to control their temperature and water balance (Guillon et al., 2014; Rozen-Rechels et al., 2020c; Stahlschmidt et al., 2011). Herein, we investigated physiological

responses to thermal and hydric conditions imposed within highly controlled climatic chambers, where vipers could not bask or select microhabitats. Thus, our study represents a situation where there is limited potential for behavioral regulation, mimicking a degraded and homogeneous habitat. In our experimental design, postural changes (e.g. water-conserving posture such as coiling; Lourdais et al., 2007; Stahlschmidt et al., 2008) remained possible. While no obvious postural changes were observed (M.D., personal observation), this facet clearly deserves further work. In addition, when heterogeneous microhabitats are available, dry-skinned ectotherms may rely on flexible behavioral responses to rapidly buffer the short-term effects of ECEs on their physiological state (Huey et al., 2015; Rozen-Rechels et al., 2020a,b). Joint investigation of these behavioral responses together with information on the physiological status of individuals is needed to better predict the ecological consequences of ECEs in the wild.

In conclusion, our results emphasize the independent effects of temperature and water availability on physiological responses to climate change, but a potential synergistic effect when it comes to behavioral responses (thirst). These effects were found in simulated warm environments with no access to water with mildly desiccant air conditions for asp vipers (i.e. WVPD of approximately −1 kPa; Dupoué et al., 2015b). Yet, summer droughts can also lead to much drier ambient conditions and are likely to reduce the availability of moist habitats in the natural environment (Sutanto et al., 2020), which will increase cutaneous and respiratory water loss rates (Dupoué et al., 2015b). Thus, ECEs in natural environments will involve the combined stressors of low air humidity, water shortages and high ambient temperatures and are likely to have greater impacts on organismal physiology than those predicted by this factorial experiment (Rozen-Rechels et al., 2019; Todgham and Stillman, 2013). Using further experimental treatments that better disentangle the multidimensional changes of ECEs will provide novel insights in our understanding of the physiological responses of ectotherms. Such studies should also investigate the potential for joint changes in behavioral and physiological strategies (Basson and Clusella-Trullas, 2015; Beever et al., 2017; Fuller et al., 2021; Rohr and Palmer, 2013).

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