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SHORT COMMUNICATION

Oxidative stress in a capital breeder (*Vipera aspis*) facing pregnancy and water constraints

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ABSTRACT

The physiological mechanisms underlying the 'cost of reproduction' remain under debate, though oxidative stress has emerged as a potential candidate. The 'oxidative cost of reproduction' has received considerable attention with regards to food and antioxidant availability; however, the limitation of water availability has thus far been neglected. In this study, we experimentally examined the combined effect of pregnancy and water deprivation on oxidative status in a viviparous snake (*Vipera aspis*), a species naturally exposed to periods of water and food deprivation. We predicted a cumulative effect of pregnancy and dehydration on oxidative stress levels. Our results support the occurrence of an oxidative cost of reproduction as we found higher oxidative damage levels in pregnant females than in non-reproductive individuals, despite an up-regulation of antioxidant defences. Surprisingly, water deprivation was associated with an up-regulation of antioxidant defences, and did not increase oxidative damage, either alone or in combination with reproduction.

KEY WORDS: Cost of reproduction, Oxidative shielding, Dehydration, Oxidative damage, Antioxidant, Water deprivation

INTRODUCTION

Life-history theory predicts that reproductive investment may compete with individual survival and/or future reproductive events, a trade-off well known as the 'cost of reproduction' (Stearns, 1992). However, the proximate physiological determinants of this cost remain under debate (Metcalfe and Monaghan, 2013; Zhang and Hood, 2016). Several studies have suggested oxidative stress as one potential physiological mediator of this life history trade-off (Monaghan et al., 2009; Stier et al., 2012; Metcalfe and Monaghan, 2013; Speakman et al., 2015). Oxidative stress is defined as the imbalance between reactive oxygen species (ROS) production and antioxidant defence, leading to oxidative damage. ROS are unstable molecules that can damage DNA, phospholipids and proteins, which may eventually lead to accelerated ageing and senescence (Speakman et al., 2015). Theoretically, oxidative stress might occur in breeding individuals as a result of increased ROS production linked to high metabolic rate (but see Stier et al., 2014a)

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or to a reallocation of resources from antioxidant defences towards reproductive investment (Speakman et al., 2015). Accordingly, some studies have shown that pregnancy increases oxidative stress levels in humans and other mammalian species (Al-Gubory et al., 2010; Agarwal et al., 2012). However, recent findings in viviparous species also suggest that pregnant females may increase antioxidant levels and reduce oxidative damage to protect their developing embryos from oxidative stress exposure, a phenomenon referred to as oxidative shielding (Blount et al., 2016; Vitikainen et al., 2016).

The physiological costs of reproduction have been extensively studied with regard to limitations in energy and time resources. However, other resources, such as water, have been relatively overlooked. Water is a vital resource that cannot be stored by most animals, and water access can be naturally limited in certain environments or during specific periods of the year. Dehydration rapidly impairs survival and reproduction (Marquis et al., 2008; McKechnie and Wolf, 2010) and species may use diverse water-saving strategies in response to water limitation (e.g. decreased activity, metabolic depression, decreased skin permeability). Oxidative stress is one mechanism leading to membrane destruction and cell death during dehydration (França et al., 2007). However, the cumulative effects of reproduction and water restriction on oxidative stress have thus far not been investigated.

Pregnancy imposes important resource demands, in terms of both energy and water requirements (Lourdais et al., 2015). In a previous study, we showed that pregnant female aspic vipers (*Vipera aspis*) compete with their embryos during periods of water deprivation (Dupoué et al., 2015). This intergenerational trade-off for water could result in a mother–offspring conflict, with negative impacts on female and/or offspring fitness (Dupoué et al., 2015). To gain more insight on this hypothesis, we investigated the combined effects of pregnancy and water deprivation on oxidative stress levels. We predicted that water deprivation would accentuate the potential oxidative cost of reproduction and/or limit the occurrence of oxidative shielding.

MATERIALS AND METHODS

We studied the aspic viper, *Vipera aspis* (Linnaeus 1758), a viviparous snake species from western Europe. As a typical capital breeder (Bonnet et al., 1998), females accumulate and store energy for 2–3 years in order to sustain the energy requirements of reproduction. Food intake is reduced or sometimes absent during pregnancy (2–3 months) (Lourdais et al., 2002). Maternal effort during pregnancy is mainly dedicated to behavioural thermoregulation and to the acquisition of enough water to support embryonic development (Lourdais et al., 2015).

In May and June 2012, we caught 58 females (29 pregnant and 29 non-reproductive) from neighbouring sites in western France (for full methodological details, see Dupoué et al., 2015). For each reproductive state, females were evenly and randomly assigned to

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either a control (*n*=30) or a water-deprived group (*n*=28). Control females had access to water *ad libitum*; however, water was removed for 20 days in the water-deprived group, at mid-gestation (full details are given in Dupoué et al., 2015). We previously showed that this water-deprivation treatment accentuated dehydration (measured through changes in plasma osmolality) and physiological stress (measured through baseline corticosterone level) in pregnant females without impairing reproductive performance or offspring phenotype (Dupoué et al., 2015, 2016). No food was provided during the experiment. Room temperature was maintained at 20°C and additional heat was provided at one end of the cage, with a 75 W heating lamp, from 10:00 h to 15:00 h. At the end of the water-deprivation treatment, blood samples (~150 µl) were taken from control and water-deprived females via cardiocentesis. Blood was centrifuged at 2000 *g* for 3 min, plasma was separated from red blood cells (RBCs), and samples were subsequently stored at −28°C until laboratory analysis. Experiments were performed in accordance with laws and rules relating to the conservation and welfare of the animals (permit no. A 79-001, Préfecture des Deux-Sèvres).

We evaluated a total of eight oxidative stress markers in plasma or RBCs (see Table 1). RBCs in non-mammalian vertebrates are fully functional (i.e. possess a nucleus and mitochondria, in contrast to mammalian RBCs) and can be used to gather information on several physiological mechanisms including cellular oxidative stress and mitochondrial function (Stier et al., 2013, 2015a, 2017). Oxidative stress markers measured in RBC lysate were normalised by the protein content of the sample, because of the difficulty of standardising the amount of RBCs being used. Protein content of the samples was determined using a Pierce™ BCA protein assay (Thermo Scientific, USA).

Non-enzymatic antioxidant capacity

We evaluated the non-enzymatic antioxidant capacity of plasma (diluted 1:100) and RBC lysate (diluted 1:750) using the OXY-adsorbent assay (Diacron International, Italy) following the manufacturer's protocol. Non-enzymatic antioxidant capacity is expressed as mmol HClO neutralised l^{-1} for the plasma and as µmol l^{-1} HClO neutralised mg^{-1} of protein for RBCs. Intra-individual coefficient of variation based on duplicates was 5.49±0.74%.

Total and oxidised glutathione

Total glutathione (tGSH) and oxidised glutathione (GSSG) content of RBC lysate (diluted 1:60) was determined using the DetectX® Glutathione fluorescent detection kit (Arbor Assays, Ann Arbor, MI, USA), following the manufacturer's protocol. Glutathione

(GSH) plays a key role in many biological processes, including the protection of cells against oxidation. We evaluated tGSH content (as an indicator of antioxidant protection), the amount of GSSG and the ratio GSSG:tGSH (where the proportion of oxidised glutathione is an indicator of the pro-oxidant power buffered by the glutathione system). Values were expressed as mmol GSH or GSSG mg^{-1} protein, and as a ratio of GSSG/tGSH (where 0 indicates that no glutathione was oxidised, and 1 indicates that all glutathione was oxidised), respectively. Intra-individual coefficient of variation based on duplicates was 3.74±0.61%.

Glutathione peroxidase and superoxide dismutase antioxidant activities

The glutathione peroxidase (GPx) enzyme uses GSH as a reductant to scavenge deleterious hydrogen peroxide. The enzymatic activity of GPx in RBC lysate (diluted 1:30) was measured using the RANSEL kit (Randox Laboratories, Crumlin, UK) following the manufacturer's protocol. GPx activity was expressed as U g^{-1} protein. Intra-individual coefficient of variation based on duplicates was 9.09±1.15%.

Superoxide dismutase (SOD) is involved in the first step of the antioxidant enzymatic cascade catalysing the dismutation of superoxide radical into oxygen and hydrogen peroxide. The enzymatic activity of SOD in RBC lysate (diluted 1:1500) was measured with the RANSOD kit (Randox Laboratories) following the manufacturer's instructions. This test quantifies *in vitro* the kinetics of inhibition of superoxide formation resulting from SOD antioxidant activity. SOD activity was expressed as U mg^{-1} protein. Intra-individual coefficient of variation based on duplicates was 12.45±2.02%.

Protein carbonyl content

Protein carbonylation occurs when carbonyl groups are introduced into proteins by reactions with free radicals or lipid peroxidation products. The carbonyl content of plasma samples (diluted to 1 mg protein ml^{-1}) was quantified using the method of Levine et al. (1990). First, nucleic acids were removed by precipitation with streptomycin sulphonate (15 min at room temperature) and centrifugation at 12,000 *g* for 10 min. Protein carbonyls were then derivatised to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH) for 1 h at room temperature. The pellet was precipitated with cold 20% trichloroacetic acid and then washed three times with a 1:1 solution of cold ethanol:ethyl acetate. The pellet was finally re-suspended in 350 µl of 6 mol l^{-1} guanidine hydrochloride. The absorbance of the samples was read at

Table 1. Description of the eight oxidative stress markers measured in aspic viper blood (*N*=58) and results from the GLMs testing the effects of reproductive state, water treatment and their interaction on oxidative stress levels

	Oxidative stress measure			Statistical effect	
Marker	Type	Sample type	Reproductive state	Water treatment	Reproductive state×water treatment
OXY	Non-enzymatic antioxidants	Plasma	**P<NR****	**WD>C***	n.s.
		RBCs	**P>NR***	n.s.	n.s.
SOD	Antioxidant enzyme	RBCs	**P>NR***	n.s.	n.s.
GPx	Antioxidant enzyme	RBCs	n.s.	n.s.	n.s.
tGSH	Endogenous non-enzymatic antioxidant	RBCs	n.s.	**WD>C***	n.s.
GSSG	Glutathione having neutralised ROS	RBCs	n.s.	n.s.	**‡**
GSSG:tGSH ratio	Oxidative attack buffered by the glutathione system	RBCs	**P>NR***	n.s.	n.s.
Protein carbonyl	Oxidative damage	Plasma	**P>NR***	**WD<C‡**	n.s.

SOD, superoxide dismutase; GPx, glutathione peroxidase; tGSH, total glutathione; GSSG, oxidised glutathione. Significant and marginal effects are in bold (n.s. non-significant, ‡P≤0.1, *P≤0.05, **P<0.01) and the direction of the effect between groups is indicated using < and > symbols. P, pregnant; NR, non-reproductive; C, control; WD, water deprived.

370 nm and the mean absorbance of control tubes (incubated with 0.1 mol l^{-1} HCl instead of DNPH) was then subtracted. We used the extinction coefficient of DNPH (0.022 µmol l^{-1} cm^{-1}) to calculate protein carbonyl content, which was expressed as nmol mg^{-1} protein. Intra-individual variation based on duplicates was 14.03 ±2.93%.

Statistical analyses were conducted using SPSS 20.0. We examined oxidative stress levels in relation to reproductive state, water treatment and their interaction using general linear models (GLMs). We also used GLMs to examine oxidative stress levels (in pregnant females only) in relation to reproductive effort (measured as litter size or litter mass; for details, see Dupoué et al., 2015), water treatment, and their interaction. Factors with $P>0.10$ were

sequentially removed from the final models starting with the interactions. We previously checked with a standard model selection procedure that our model selection process was appropriate (see Table S1).

RESULTS AND DISCUSSION

Reproductive state significantly influenced the levels of five out of the eight oxidative stress markers measured (Table 1, Fig. 1). Pregnant females exhibited a lower plasma non-enzymatic antioxidant capacity compared with non-reproductive females (Fig. 1A), while RBC non-enzymatic antioxidant and SOD levels were higher in pregnant females (Fig. 1B,G). The GSSG:tGSH ratio (i.e. the proportion of glutathione oxidised) as an indicator of
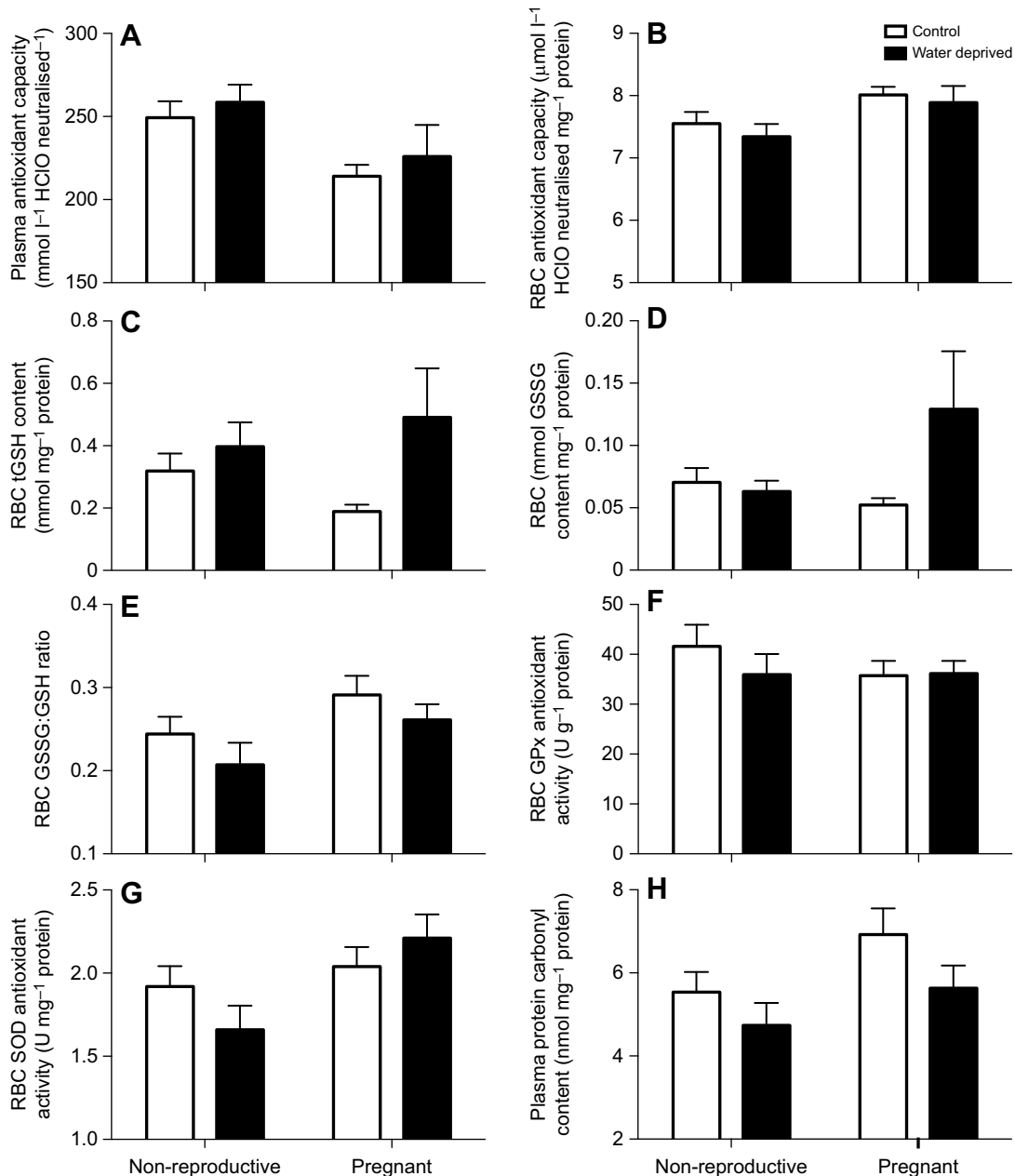

Fig. 1. Plasma and red blood cell markers of oxidative stress in pregnant and non-reproductive female aspic vipers having *ad libitum* access to water (white bars) or being deprived of water for 20 days (black bars). (A) Plasma non-enzymatic antioxidant capacity. (B) Non-enzymatic antioxidant capacity of red blood cells (RBCs). (C) Total glutathione (tGSH) content of RBCs. (D) Oxidised glutathione (GSSG) content of RBCs. (E) Proportion of glutathione oxidised in RBCs (GSSG:GSH ratio). (F) Glutathione peroxidase (GPx) antioxidant activity of RBCs. (G) Superoxide dismutase (SOD) antioxidant activity of RBCs. (H) Plasma protein carbonyl content. Data are means±s.e. and statistical results of GLMs are presented in Table 1 (N=58).

Table 2. Summary results of GLMs testing the effects of water treatment, litter mass (i.e. an indicator of reproductive effort) and their interaction on oxidative stress parameters

Oxidative stress measure	Water treatment	Litter mass	Water treatment×litter mass
Plasma non-enzymatic antioxidant capacity	P=0.024	n.s. (P=0.42)	n.s. (P=0.44)
RBC non-enzymatic antioxidant capacity	n.s. (P=0.70)	n.s. (P=0.14)	n.s. (P=0.34)
RBC tGSH	n.s. (P=0.10)	n.s. (P=0.36)	n.s. (P=0.36)
RBC GSSG	n.s. (P=0.14)	n.s. (P=0.32)	n.s. (P=0.32)
RBC GSSG:tGSH	n.s. (P=0.58)	n.s. (P=0.49)	n.s. (P=0.48)
RBC GPx	n.s. (P=0.91)	n.s. (P=0.91)	n.s. (P=0.42)
RBC SOD	n.s. (P=0.60)	n.s. (P=0.73)	n.s. (P=0.54)
Plasma protein carbonyl	n.s. (P=0.15)	n.s. (P=0.53)	n.s. (P=0.43)

Abbreviations as in Table 1. We found qualitatively the same results using other proxies of reproductive effort such as litter size or residual litter mass/size after accounting for female body size effect on fecundity (data not shown).

oxidative attack and oxidative damage to protein were also higher in pregnant females (Fig. 1E,H). This suggests that despite an increase in SOD and RBC non-enzymatic antioxidants, pregnant females were suffering more from oxidative stress, as demonstrated by their higher proportion of oxidised glutathione and oxidative damage to their proteins. As such, our results support the occurrence of an oxidative cost of reproduction in the aspic viper, rather than a potential oxidative shielding (Blount et al., 2016; Vitikainen et al., 2016). However, the up-regulation of the endogenous antioxidant enzyme SOD suggests that pregnant individuals may be under selection to minimise oxidative stress exposure, which would be in accordance with the oxidative shielding hypothesis. Diet-acquired antioxidant molecules may be important for enabling efficient oxidative shielding and avoiding oxidative damage. However, typical capital breeders such as the aspic viper might be short on such compounds during pregnancy as they often cease to feed during this time (Lourdais et al., 2002). This hypothesis is partly supported by the decrease in plasma non-enzymatic antioxidant capacity we found in pregnant females, as most non-enzymatic compounds found in plasma are diet acquired (Cohen et al., 2007). Experimentally supplementing pregnant and non-reproductive females with dietary antioxidants could provide further important insights regarding this hypothesis.

Female oxidative status was only moderately influenced by water deprivation, with an increase in two components of the antioxidant defence (plasma non-enzymatic antioxidants and tGSH; Table 1, Fig. 1B,C) and a marginal decrease in oxidative damage to proteins (Table 1, Fig. 1H). Contrary to our prediction, dehydration seemed to trigger a number of antioxidant defence mechanisms, which prevented oxidative stress and even led to a marginal reduction in oxidative damage. Additionally, we found no support for a cumulative effect of reproduction and dehydration on oxidative stress levels (i.e. no significant interaction; Table 1). These results might look surprising given the known impact of dehydration on oxidative homeostasis in humans (Hillman et al., 2011). However, it is likely that resistance mechanisms exist in species naturally facing extended periods of water deprivation, as recently suggested by the increased innate immunity found in response to dehydration in a desert reptile (Moeller et al., 2013). Aspic vipers naturally experience drought periods in their habitat, and this might help to explain their resilience to our experimental water-deprivation treatment in terms of both oxidative stress (this study) and reproductive performance (Dupoué et al., 2015).

Overall, we found no significant relationship between female reproductive effort and oxidative stress markers, either as a main factor or in the interaction with water treatment (Table 2; Fig. S1). This contrasts with a recent meta-analysis in endotherm species,

which reported an overall positive association between reproductive effort and oxidative stress (Blount et al., 2016). The limited power of our study (N=29 pregnant females) and the lack of long-term fitness measures (e.g. survival, annual body growth, future reproductive success) preclude the drawing of a definitive conclusion, but certainly encourage further studies in ectotherm species.

The occurrence of oxidative stress during pregnancy has been characterised in both humans and other mammalian species (Al-Gubory et al., 2010; Agarwal et al., 2012), but remains largely understudied in other taxa, as well as in combination with environmental challenges. In this study, using a viviparous snake species, we showed that pregnancy and water deprivation are associated with changes in oxidative balance. Our results demonstrate that pregnancy induces significant oxidative stress, which may contribute to the cost of reproduction in this species (Bonnet et al., 1999). We also found that compensatory mechanisms exist during periods of water restriction, which may help to prevent a potential cumulative effect of dehydration. Monitoring of maternal and offspring fitness is now required before any long-term or delayed adverse effects of dehydration during pregnancy can be excluded. Measuring telomere length could prove fruitful for future research on the cost of reproduction and dehydration in this species, as short telomeres have been shown to predict lower survival probability in several captive and wild animal species (Stier et al., 2015a). Furthermore, telomeres have been shown to shorten in response to both increased reproductive effort (Reichert et al., 2014) and stressful environmental conditions (Stier et al., 2014b, 2015b, 2016; Meillère et al., 2015; Simide et al., 2016). Additionally, recent work suggests that changes in mitochondrial bioenergetics and ROS production during reproduction are potential key mechanisms underlying the costs of reproduction (Zhang and Hood, 2016). Non-mammalian vertebrates like the aspic viper will offer great opportunities in the future to study such questions in a minimally invasive manner as their RBCs possess functional mitochondria, unlike those of mammalian species (Stier et al., 2013, 2015a, 2017). Finally, it will be important to evaluate the combined effect of water restriction and reproduction on oxidative stress levels in other species, especially in species that are less tolerant to drought than the aspic viper.

Author contributions
A.S. designed and conducted laboratory analyses, analysed the data and wrote the paper. A.D. designed and conducted the experiments, analysed the data and wrote

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