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RESEARCH ARTICLE

Interplay between plasma oxidative status, cortisol and coping styles in wild alpine marmots, *Marmota marmota*

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SUMMARY

Variation in how individuals cope behaviourally and physiologically with stressors is widespread and can have a significant impact on life-history traits and fitness. Individual coping styles are characterised by differential behavioural and adrenocortical reactivity to various challenges. As stress hormones can affect the production of reactive chemical species and the antioxidant status, individuals with different coping styles may differ also in oxidative status. Field studies on wild mammalian populations are few in number and none so far has simultaneously tested the relationship between coping style, adrenocortical reactivity and oxidative status in the same individuals. We measured individual variation in coping styles along a proactive–reactive continuum together with variation in baseline and stress-induced plasma oxidative damage, plasma non-enzymatic antioxidant capacity and cortisol in wild alpine marmots, *Marmota marmota*. Confirmatory path analysis revealed that different coping styles are accompanied by different baseline and stress-induced plasma oxidative statuses. Our findings also highlight the potential role of cortisol as a mediator of such differences.

Key words: coping style, personality, oxidative stress, cortisol, open-field test, mammals.

INTRODUCTION

Individual animals differ in the way they cope with stress along an axis polarised at the two extremes by proactive and reactive responses (Koolhaas et al., 1999; Koolhaas et al., 2010). Proactive individuals are generally bold, superficial explorers and are active and aggressive. Reactive individuals exhibit low levels of activity and aggression, and explore a novel environment thoroughly. Differences in coping styles have been considered to reflect consistent behavioural differences among individuals (Sih et al., 2004; Groothuis and Carere, 2005; Réale et al., 2007). There is increasing evidence that such individual variation in behaviour has important ecological and evolutionary consequences (e.g. Carere and Eens, 2005; Sih et al., 2004; Réale et al., 2007). It is generally thought that inter-individual variation is maintained because, although certain behavioural phenotypes do better than others in terms of fitness under some environmental conditions, an opposite pattern can emerge under different conditions (e.g. Sih et al., 2004; Carere et al., 2010). Furthermore, recent studies have suggested that selection should have favoured integrated sets of behavioural, physiological and morphological traits along with variation in life-history strategies (Sih et al., 2004; Wolf et al., 2008; Biro and Stamps, 2008; Careau et al., 2008b; Réale et al., 2010). In other words, these integrated neuro-physiological and behavioural sets of traits may be part of more general life-history strategies with proactive individuals showing high growth rates, early sexual maturity and short lifespans, and reactive individuals being characterised by a slower pace of life (Biro and Stamps, 2008; Careau et al., 2008b; Wolf et al., 2008; Réale et al., 2009; Réale et al., 2010). Thus, in this context we should expect that selection has also led to some potential coevolution between coping styles and mechanisms involved in life-history trade-offs. Some of these mechanisms involve stress responsiveness modulated by glucocorticoids. When confronted with a challenging situation (e.g. a predator attack or social conflict), proactive individuals respond with a strong sympathetic activation and an increase in noradrenergic stimulation, resulting in a general fight-or-flight behavioural response (for reviews, see Koolhaas et al., 2010; Carere et al., 2010; Coppens et al., 2010). In contrast, reactive individuals respond to a challenge with a strong hypothalamic–pituitary–adrenocortical reactivity (Koolhaas et al., 2010; Carere et al., 2010; Coppens et al., 2010), resulting in a freezing response to the stimulus and an increase in circulating glucocorticoids.

Stress responsiveness is also known to affect the redox physiology. For example, experimental elevations of circulating glucocorticoids, which are hormones associated with coping styles, may increase oxidative damage and alter (increase or decrease) levels of specific antioxidants in vertebrates [see meta-analysis in Costantini et al. (Costantini et al., 2011)]. In addition, reproductive

and physical activities in socio-sexual contexts, which are known to differ among coping styles [e.g. mice *Mus musculus* (Koolhaas et al., 1999) and great tits *Parus major* (Carere et al., 2001; Both et al., 2005; Groothuis and Carere, 2005)], could significantly affect the biomarkers of oxidative status and thus expose individuals to oxidative challenges of different magnitudes (e.g. Alessio, 1993; Costantini et al., 2008b). Oxidative stress results from an imbalance between the production of reactive chemical species and antioxidant defences, in favour of the former with a consequent increase in the rate of generation of oxidative damage (Sies, 1991; Halliwell and Gutteridge, 2007; Costantini and Verhulst, 2009). Oxidative stress may also be defined as a disruption of redox signaling and control (Jones, 2006), which regulate the redox balance and the antioxidant response to oxidative insults.

The importance of oxidative stress as one component affecting the progression of diseases, ageing and health span (*sensu* Salmon et al., 2010) has been recognised for decades (Harman, 1956; Beckman and Ames, 1998; Halliwell and Gutteridge, 2007). However, only recently has it been recognised that oxidative stress may also represent an important modulator of trade-offs between life-history traits in wild populations (Costantini, 2008; Dowling and Simmons, 2009; Monaghan et al., 2009; Costantini et al., 2010). Recent studies have also suggested that differences in oxidative stress physiology may be associated with behavioural differences. In mice selected for different levels of aggression, Costantini et al. (Costantini et al., 2008b) found higher baseline non-enzymatic antioxidant capacity in less aggressive mice. In the same study, body mass corrected for age modulated differences in oxidative status profiles between the two mice lines (i.e. more aggressive and less aggressive). More recently, another study showed that more aggressive mice have higher levels of reactive oxygen species production in granulocytes (Rammal et al., 2010). In agreement with these studies on mice, a recent report on greenfinches (*Carduelis chloris*) showed that: (1) neophobic individuals had higher intermediate oxidative damage compounds [hydroperoxides (ROMs)] and lower plasma non-enzymatic antioxidant capacity than neophilic individuals; (2) fast-exploring individuals had higher plasma non-enzymatic antioxidant capacity and lower lipid peroxidation end-products [malondialdehyde (MDA)] than slow-exploring individuals; and (3) greenfinches with extremely high or low neophobia had lower MDA than intermediate responders (Herborn et al., 2011). Overall, these results highlight the potential differences in oxidative stress threat that could be associated with individuality in coping in natural conditions.

In this study, we used confirmatory path analyses (Shipley, 2000) to support, for the first time in a wild mammalian species (the alpine marmot, *Marmota marmota* Linnaeus 1758), the hypothesis that variation in coping styles along a proactive–reactive continuum is causally related to variation in baseline and stress-induced plasma oxidative damage and non-enzymatic antioxidant capacity (OXY). Alpine marmots are social breeders and form groups that defend a territory against neighbours (Perrin et al., 1993). In each group, sub-adult helpers keep growing until 3 years of age, when they disperse and are generally inhibited in their reproduction by dominant, reproductive adults (Perrin et al., 1993). Previous work on the study population (Ferrari, 2010) showed that fast exploration of a novel environment was positively correlated at the individual level with heart rate under restraint, illustrating individual differences in coping style in that population. Here we tested whether individuals varying in their coping styles expressed different levels of ROMs, hydroxynonenal-protein adducts (HNE) and OXY. Firstly, overall we expected that proactive individuals, because they are generally associated with a fast pace of life (Réale et al., 2010), should show a higher level of baseline and stress-induced ROMs and HNE and potentially a higher level of OXY, independent of body mass or age (see Costantini et al., 2008b; Rammal et al., 2010). Secondly, we tested the hypothesis that the relationship between a coping style and plasma oxidative status is modulated by cortisol secretion because stress hormones can induce changes in oxidative damage and antioxidant status through several mechanisms, such as an increase in metabolic rate (Sapolsky et al., 2000), remobilization of non-enzymatic antioxidants among tissues and induced synthesis of antioxidant enzymes through modulation of gene expression (Yoshioka et al., 1994; Atanasova et al., 2009) [see also Costantini et al. (Costantini et al., 2011) for a comprehensive discussion of the links between stress hormones and oxidative stress]. Finally, we tested the hypothesis that body mass and age contribute to explain variation in physiological status because: (1) larger individuals might have higher metabolic needs (Hulbert and Else, 2004; van de Crommenacker et al., 2010), (2) older individuals might be more susceptible to oxidative stress independent of the intensity of cortisol secretion (Beckman and Ames, 1998) and (3) older individuals might decrease cortisol secretion to avoid impairment of reproductive activity (Wingfield and Sapolsky, 2003).

MATERIALS AND METHODS

Study area and population

Alpine marmots are large herbivorous, diurnal, burrow-dwelling rodents inhabiting the high alpine and subalpine meadows in the mountainous regions of Western and Central Europe. Alpine marmots form social groups of up to 20 individuals and are one of the most social species of rodents (Arnold, 1990a; Arnold, 1990b; Perrin et al., 1993). They grow until 3 years of age, when they reach their adult size and mass (Arnold, 1990a; Arnold, 1990b). Individuals reach sexual maturity between their second and third summer.

The study was carried out in Orvielles (Valsavarenche, Aosta, Gran Paradiso National Park, northwestern Italian Alps, 45°34′N, 7°11′E). Marmots included in this study were live-trapped between May and July 2008 and 2009 (see Table 1 for descriptive statistics and sample size) using Tomahawk traps (150×30×30 cm, Tomahawk Live Traps, Hazelhurst, WI, USA) and horse fodder (Omolene, Purina, Gray Summit, MO, USA) as food bait. The study complied with Canadian law regarding animal experiments (Comité Institutionnel de Protection des Animaux, protocol no. 615).

Capture operations and blood sampling

Once trapped, each marmot was put inside a handling bag and transported to the working area within 17±5 min (mean ± s.d.). At their arrival, we first sampled 0.3 ml of venous blood from the leg into a heparinated tube. Using a stethoscope, we measured heart rate by counting the number of heartbeats during 15 s (2 min on average). We tagged animals using a unique PIT tag (Bayer Animal Coder, Bayer S.p.a., Milan, Italy), two plastic 5 cm long coloured ear tags (Minirototag, Ghislandi & Ghislandi, Bergamo, Lombardy, Italy) with different colour combinations, and fur bleaching (using Modus bleach, Aosta, Italy) to help in visual recognition at a distance (mean ± s.d.=177.15±64.88 m). We then weighed and took biometric measures of the captured marmots. Overall, the post-bleeding operations lasted on average 7±2 min, which refers to the pre-restraint interval. Animals were not allowed to recover from transportation before the open-field (OF) test was performed.

Table 1. Descriptive statistics for the main traits tested in alpine marmots over the 2 years of the study at Orvielles

	2008		2009	
Trait	*N*	Mean ± s.d.	*N*	Mean ± s.d.
Body mass (g)	19	3.24±0.71	23	2.74±0.75
Coping style index	19	–0.55±0.98	23	0.45±1.03
Age (years)	19	2.74±0.65	23	1.96±0.77
CORT (ng ml^{-1})	19	19.74±7.70	9	31.9±12.46
ROMs (mmol l^{-1} H_2O_2 equiv.)	19	8.67±1.98	23	8.14±1.88
OXY (mmol l^{-1} HOCl neutralised)	19	442.3±66.0	23	577.4±50.6
HNE (μg ml^{-1})	n.a.	n.a.	23	3.33±0.66

The coping style index was calculated from the information on heart rate under restraint and activity/exploration in an open-field test (see Materials and methods for more details). Values of physiological variables refer to the pre-restraint state. CORT, cortisol; HNE, hydroxynonenal-protein adducts; n.a., not available; OXY, non-enzymatic antioxidant capacity; ROMs, hydroperoxides.

Behavioural test

We ran a 3 min long OF test. The OF test provides a measure of activity/exploration in a novel environment (Boon et al., 2007; Martin and Réale, 2008; Boyer et al., 2010; Montiglio et al., 2010). The OF arena consisted of a square playpen (94.5×102×80 cm, width×length×height) that was covered on the four sides and at the bottom with white plastic panels and on the top with a plastic net of large mesh. In this way the marmot had no direct contact with the external environment apart from the sky. Tests were conducted only in stable weather and not in rain or strong wind. The marmot was gently pushed inside through a door and its behaviour was video recorded while all the operators were sitting silently and hidden from the animal. The behaviour of the marmots was then scored using the software The Observer (Noldus Information Technology, Leesburg, VA, USA) and we calculated the percentage of the time spent (measured in seconds) performing a specific behaviour, which we then used for the following analyses. To score the videos, we adapted an ethogram used in the same behavioural tests with another species [red squirrels (Boon et al., 2007)]. We identified four main different behaviours, defined as follows: walking, the marmot walks or runs inside the arena (this also includes animals that were constantly moving their head to look around); destroying, the marmot digs and bites the floor, the sides of the box or the lid of the net; up posture, the marmot is in a rearing position or climbs against the walls (it also includes animals that were constantly moving their head looking around while climbing); and immobility, the marmot stands or lies with four paws on the ground, and does not move its head. Once the test was finished, the marmot was gently pushed out from the same door into a bag. At the end of the test, we collected a second blood sample and recorded the heart rate of the marmot. The animal was then transported back to the site of capture, where it was released. The entire capture operation for each individual lasted on average 32±20 min. In the text, we will refer to this stage as restraint sampling.

Blood samples were kept at +4°C until centrifugation, which happened within the next 48 h (70% of samples were centrifuged within 24 h). Physiological measures did not differ between samples centrifuged within or after 24 h (Mann–Whitney *U*-test, $U \geq 2.0$, $P \geq 0.11$). Plasma samples were then stored at –20°C until cortisol and oxidative stress analyses were performed (within a few months from collection). It is unlikely that our sampling and storage procedures significantly impacted the physiological measures in plasma, although we cannot definitively exclude the possibility that sampling and storage may have introduced some noise. In support of our approach, Cavalleri and co-workers (Cavalleri et al., 2004) showed that storage of human sera between –30 and –80°C, including repeated freeze–thaw cycles, causes a 4% decrease in levels of ROMs as measured by the d-ROMs assay (which tests the derivatives of ROMs) only after 2 years of storage. Notably, they also showed that the ROMs are stable in blood samples maintained at 4°C at least until 48 h, and the basic radical activity, if any, is not sufficient to induce a significant change in ROMs levels. Celi et al. (Celi et al., 2010) also showed that ROMs and OXY are stable in horse blood conserved at 4°C at least until 24 h. van de Crommenacker et al. (van de Crommenacker et al., 2011) stated on the basis of their own unpublished results that storage of goose plasma at –18°C for 6 months did not affect ROMs or OXY. Finally, high stability of cortisol in human blood, plasma or saliva was reported at sampling and storage procedures comparable to ours (e.g. Kley and Rick, 1984; Garde and Hansen, 2005).

Evaluation of plasma oxidative status

Three biomarkers were used to evaluate the plasma oxidative status (i.e. both the oxidized and antioxidant components) of marmots. Haemolysed samples were removed from the analyses. In the samples collected in 2008, we determined plasma ROMs (plasma hydroperoxides), which represent intermediate products of the oxidative cascade, and OXY. Given the promising preliminary results obtained in 2008, we also measured the same biomarkers in the samples collected in 2009 together with HNE, which represents the end-products of the oxidative cascade. All these biomarkers have been used to evaluate the plasma oxidative status in several vertebrate groups [mammals (Brambilla et al., 2001; Brambilla et al., 2002; Costantini et al., 2008b; Maki et al., 2009; Hindle et al., 2010); birds (Costantini and Dell'Omo, 2006; Costantini et al., 2008a); and reptiles (Costantini et al., 2009; Isaksson et al., 2011)]. They provide information on different traits of the redox physiology of the organism, hence allowing us to obtain a better estimate of the plasma oxidative status.

Measurement of plasma ROMs

Plasma ROMs (mostly hydroperoxides) are intermediate oxidative damage compounds derived from several molecules, such as lipids, proteins and nucleotides. Plasma ROMs may reflect damage in other tissues; for example, a study on rats showed that serum hydroperoxides may correlate with those occurring in liver, spleen, heart and kidney [$r \geq 0.66$ (Argüelles et al., 2004)]. In addition, a study on rabbits showed that serum hydroperoxides may reflect lipid peroxidation biomarkers in muscle [$r=0.73$ (Oriani et al., 2001)], and a study on humans showed that serum hydroperoxides can correlate with serum 8-isoprostanes [$r=0.68$ (Lubrano et al., 2002)]. Plasma ROMs were measured by colorimetric determination using

the d-ROMs assay (Diacron International, Grosseto, Italy) according to previous studies (e.g. Costantini and Dell'Omo, 2006; Costantini et al., 2008b). The absorbance was read with a microplate reader (Multiskan Spectrum, ThermoFisher, Vantaa, Finland) at a wavelength of 490 nm. Similar results were obtained if readings were carried out at 505 nm. Plasma levels of ROMs are expressed as mmol l^{-1} H_2O_2 equivalents. Measurements were run in duplicate and a mean value was used in the analyses (intra-assay CV=4.38%). Hereafter we will use ROMs to refer to pre-restraint plasma ROMs and ΔROMs to refer to the change in ROMs after restraint and the OF test.

Measurement of plasma HNE

The HNE 4-hydroxynonenal is an end-product derived from decomposition of lipid hydroperoxides. This molecule is capable of binding to proteins and forming stable adducts, also termed advanced lipid peroxidation end-products. These modifications of proteins by HNE cause both structural and functional changes of oxidised proteins. The OxiSelectTM HNE-His adduct ELISA assay (Cell Biolabs, Inc., San Diego, CA, USA) was used to quantify the plasma concentration of HNE-His protein adducts. Plasma samples were diluted 1:2000 in 1× Dulbecco's phosphate buffered saline in order to generate a protein content, as measured by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA), not higher than 10 μg ml^{-1}. The assay was performed following the manufacturer's instructions. Absorbance was read after 3 min from the addition of stop solution using a microplate reader (Thermo Scientific Multiskan Spectrum) at a wavelength of 450 nm. Values are expressed as μg HNE-protein adducts ml^{-1}. Measurements were run in duplicate and a mean value was used in the analyses (intra-assay CV=3.99%). Hereafter we will use HNE to refer to pre-restraint HNE measures and ΔHNE to refer to the change in HNE after restraint and the OF test.

Measurement of OXY

OXY was measured by a colorimetric determination assay using the OXY-adsorbent test (Diacron International). The contribution of uric acid to plasma antioxidant capacity as measured by the OXY-adsorbent test is low (r_S=0.29, N=10, P=0.21), hence, unlike other methods (e.g. trolox equivalent antioxidant capacity or ferric reducing ability of plasma assay), the OXY test does not overemphasise the contribution of uric acid. The procedure was carried out according to previous studies (e.g. Costantini and Dell'Omo, 2006; Costantini et al., 2008b). The absorbance was measured with a microplate reader (Thermo Scientific Multiskan Spectrum) at a wavelength of 490 nm. Similar results were obtained if readings were carried out at 505 nm. Values are expressed as mmol l^{-1} HOCl neutralised. Measurements were run in duplicate and a mean value was used in the analyses (intra-assay CV=7.09%). Hereafter we will use OXY to refer to pre-restraint plasma antioxidant capacity and ΔOXY to refer to the change in OXY after restraint and the OF test.

Measurement of plasma cortisol

Plasma cortisol concentration was determined using commercially available solid phase ^{125}I RIA kits (Canine Serum Cortisol, Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, USA) validated for marmots according to National Committee for Clinical Laboratory Standards guidelines. The analyses were made according to the manufacturer. The samples were read (1 min) using a cell gamma counter with an NaI (Tl) detector (Cobra II, Perkin Elmer Italia, Monza, Italy). In a preliminary validation of the method by dose–response analyses, the sensitivity was 1.9 ng ml^{-1}, and the intra- and inter-assay CVs were 5 and 6.2%, respectively. Here we used measures of cortisol prior to any manipulation (hereafter referred to as CORT) and the change in cortisol between the first and the second blood sample occurring after restraint and the OF test (hereafter referred to as ΔCORT).

Statistical analyses

To investigate the relationships between behavioural variables recorded during the OF test, we first ran an explorative principal component analysis (PCA) on the percentage of time spent in the different four behaviours (see Behavioural test, above, for a description of the behaviours considered) scored during the OF test. The first component highlighted a division between behaviour variables related to activity and immobility as shown by the loadings of each variable on the first PC (walking, –0.46; destroying, –0.29; up posture, –0.51; immobility, +0.67). Thus, in the following analyses we used the sum of the percentages of all the activity-related behaviours (i.e. walking, up posture and destroying) as an index of activity during the OF test: a high positive score of the index reflects an individual that is highly active during the OF test.

Our index of coping style was then obtained by using the first component of a PCA including heart rate during restraint and the index of activity (loadings of the two variables, +0.79). A high positive value of coping style reflected a proactive individual, showing high values for both heart rate under restraint and activity/exploration in the open field. We took two measures of heart rate at any capture/manipulation of a marmot. Heart rate did not change significantly between the first and the second measure of heart rate after the OF test, and here we present data on the first measure of heart rate.

Two separate confirmatory path analyses (Shipley, 2000) (see below) were used to test for the causal relationships between the measured variables. In a first model, we tested the causal relationships among coping style, body mass, age, and pre-restraint values of CORT, ROMs and OXY. In a second model, we tested the causal relationships among coping style, body mass, age and ΔCORT, ΔROMs and ΔOXY. Linear mixed-effect models (not shown) were first used to explore the relationships between the variables in order to select the predictors to be included in the confirmatory path analysis. Sex and sampling date were not significantly correlated with any variables, and thus were not included in the path analyses. HNE data were only collected in 2009 and measures of HNE and CORT in the same individuals were available for only nine marmots. Therefore, we ran bivariate or partial correlation analyses to test for the links between HNE and the other variables. Comparison of HNE between males and females was carried out using the Mann–Whitney U-test.

Confirmatory path analysis tests the likelihood of *a priori* models of causal relationships between the variables (Shipley, 2000). The way the variables are causally linked together imposes constrains on the covariance structure of the model. The fit of the hypothesised constraints can thus be tested by comparing the predicted patterns of conditional independence between the variables that is implied by the hypothesised causal model with the actual patterns of conditional dependence and independence in the data. We did this using the d-sep test developed by Shipley (Shipley, 2000). At the basis of this test, there is a basis set of d-separation claims, which describes all the predicted patterns of conditional independence (Shipley, 2000). We tested each hypothesised conditional independence of type $X\_ \perp\!\!\!\perp \_Y \mid \{A,B,...\}$ by fitting linear models of $A,B,...+X$ on Y and calculated the probability (p_i) that the partial

Table 2. Comparison of different nested path models with links between age, body mass (BM), coping style (CS) and pre-restraint values of oxidative damage (ROMs), plasma antioxidant capacity (OXY) and cortisol (CORT) in alpine marmots

Model	Link	C	d.f.	P	ΔC	$P_{\Delta C}$
1	Best model	12.47	12	0.41		
2	CS–OXY	12.01	10	0.28	0.45	0.79
3	CS–ROMs	11.40	10	0.33	1.07	0.58
4	BM–CS	10.40	10	0.41	2.07	0.35
5	BM–OXY	10.03	10	0.44	2.44	0.30
6	BM–CORT	12.08	10	0.27	0.39	0.82
7	CORT–ROMs	9.13	10	0.52	3.34	0.19

Details on the model selection procedure are presented in the Materials and methods. C is the Fisher C-statistic (Shipley, 2000) used to test the fit of the data with the model. A P-value >0.05 indicates a fit of the data with the model. ΔC is the difference in the C-statistic between the best-fitting model and the nested model. A significant P-value for this difference ($P_{\Delta C}$) indicates a better fit of the nested model.

regression coefficient associated with X is zero (i.e. the effect of X on Y conditional on A,B,...). The overall test of the basis set is given by the Fisher C-statistic, where C=–2Σ[ln(p_i)], which is distributed as a χ^2 variate with 2k degrees of freedom (where k is the number of independence tests in the basis set) if all predicted conditional independencies hold in the data. The path model is considered to fit the data when the C-value is not significant (P>0.05) (Shipley, 2000; Shipley, 2004). A path model is nested within another when the parameters fixed to zero in the first model are a subset of the fixed parameters in the second model (Shipley, 2000). It is possible to test the significance of adding a causal link between two variables within the basis model by determining the probability of observing the change in the Fisher C-statistics of two nested models, with and without the tested link (ΔC=C_{model1}–C_{model2}). C follows a χ^2 distribution, with Δd.f.=d.f.$_{model1}$–d.f.$_{model2}$ (Shipley, 2000). The basis model is rejected in favour of the nested model when the probability associated with C is lower than the chosen significance level (α=0.05). We compared non-nested models using a likelihood-ratio approach (Royall, 1997) as exemplified by Shipley (Shipley, 2004). We standardized path coefficients of continuous variables and calculated means and standard error of the factorial variable age every time we found a dependence link to age in the model (Tables 4, 5). In this analysis we tested the following *a priori* hypotheses: (1) proactivity should be negatively related with CORT (Koolhaas et al., 1999), thus we tested whether coping style affected CORT; (2) proactivity (i.e. high values of the coping style index) should be positively and directly linked to both ROMs and OXY, independent of body mass or age (see Costantini et al., 2008b; Rammal et al., 2010); (3) the relationship between a coping style and plasma oxidative status (i.e. ROMs and OXY) should be modulated by CORT secretion, CORT potentially decreasing OXY and increasing ROMs (Costantini et al., 2011); and (4) because we expect that age and body mass could affect any of these variables, we included all the links between these two variables and coping style, OXY, ROMs, CORT, ΔOXY, ΔROMs and ΔCORT.

All models were run using R version 2.13.0 (R Development Core Team, 2008). Year was included as a random factor in all models. Data from 29 marmots were included in both path models, but the sample size was reduced to 18 when testing the links with cortisol.

RESULTS

Path analysis

The best-fitting path model for pre-restraint values (model 1 in Table 2; Table 4, Fig. 1) suggests that the positive relationship between proactivity and OXY was modulated by CORT, which was higher in more proactive animals. We also found an increase of ROMs with age, higher levels of OXY in subadult marmots than yearling and adult marmots, higher levels of CORT in subadult marmots than yearling and adult marmots, a decrease of pro-activity with age, an increase of body mass with age, a positive relationship between ROMs and body mass, and a negative relationship between OXY and ROMs (Fig. 1). The correction of physiological variables for the time elapsed from the capture to first bleeding (residuals of a linear regression of a physiological variable on time) did not change the fit of our path model shown in Fig. 1 (results not shown). Although we cannot conclude that pre-restraint values properly reflect baseline values, especially for plasma CORT, which starts to increase after a few minutes from capture, we can safely exclude that our bleeding procedure have biased our results.

The best-fitting path model for restraint-induced changes of physiological variables in relation to age, body mass and coping style (model 1.1 in Table 3; Table 5, Fig. 2) suggests that the restraint induced a higher increase in ROMs and OXY in more proactive marmots. We found that the increase in ROMs was higher in younger marmots and, in agreement with the model for pre-restraint values, that proactivity and body mass decreased and increased, respectively with age. The change in plasma CORT was associated with age, with sub-adults having a higher increase in CORT than adults.

Variation in HNE

Pre-restraint values of plasma HNE were strongly correlated with post-restraint values (r=0.85, N=20, P<0.001). Pre-restraint values of plasma HNE were also positively correlated with ROMs (r=0.55,

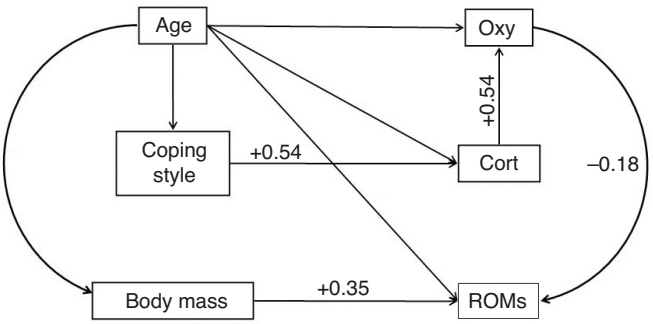

Fig. 1. Best-fitting path model of relationships between coping style, age, body mass, and pre-restraint values of cortisol (CORT), plasma oxidative damage (ROMs) and plasma antioxidant capacity (OXY) in alpine marmots. Standardized path coefficients are reported above the causal links, whereas for the relationships including the categorical variable age, we calculated unstandardized means ± s.e.m., shown in Table 4.

Table 3. Comparison of different nested path models with links between restraint-induced changes in oxidative damage (ΔROMs), plasma antioxidant capacity (ΔOXY) and cortisol (ΔCORT) in relation to age, body mass (BM) and coping style (CS) in alpine marmots

Model	Link	*C*	d.f.	*P*	Δ*C*	$P_{\Delta C}$
1.1	Best model	14.81	18	0.67		
2.1	BM–ΔCORT	14.42	16	0.57	0.38	0.83
3.1	CS–ΔCORT	13.23	16	0.65	1.58	0.45
4.1	ΔCORT–ΔROMs	10.40	16	0.84	4.41	0.11
5.1	ΔCORT–ΔOXY	13.89	16	0.61	0.92	0.63
6.1	BM–CS	14.23	16	0.58	0.58	0.75
7.1	BM–ΔROMs	10.11	16	0.86	4.70	0.09
8.1	ΔOXY–ΔROMs	13.02	16	0.67	1.77	0.41
9.1	BM–ΔOXY	12.03	16	0.74	2.78	0.25
10.1	ΔOXY–Age	14.56	16	0.56	0.25	0.88

Details on the model selection procedure are presented in the Materials and methods. *C* is the Fisher *C*-statistic (Shipley, 2000) used to test the fit of the data with the model. A *P*-value >0.05 indicates a fit of the data with the model. Δ*C* is the difference in the *C*-statistic between the best-fitting model and the nested model. A significant *P*-value for this difference ($P_{\Delta C}$) indicates a better fit of the nested model.

N=23, *P*=0.003) and age (*r*=0.38, *N*=23, *P*=0.038), but were not correlated with coping style (*r*=0.08), body mass (*r*=0.20) or OXY (*r*=–0.20). HNE and coping style were still not correlated in partial correlations while controlling for age. ΔHNE was not correlated with any other variable in bivariate or partial correlation models. Males and females did not differ in HNE (adjusted *Z*=0.93, *P*=0.36) or ΔHNE (adjusted *Z*=–1.28, *P*=0.21).

DISCUSSION

In the present study, we show for the first time in a wild mammal (alpine marmot) that individual coping style can be associated with differences in pre-restraint or acute stress-induced blood oxidative status. Marmots with a more proactive coping style had higher baseline levels of OXY, through a positive action of plasma CORT on OXY (see Fig. 1). Coping style was, however, not associated with two measures of oxidative damage in plasma, ROMs and HNE. Our second path model, which included changes in blood oxidative status induced by an acute stressor (i.e. a 30 min restraint and an OF test), further suggests that more proactive marmots experienced a higher increase in ROMs in agreement with our predictions, but also an unexpected higher increase in OXY. In contrast to pre-restraint status, changes in CORT under restraint were not associated with coping style, ROMs or OXY. Correlative analyses also show

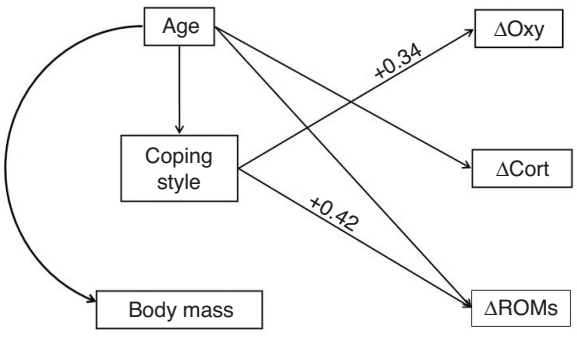

Fig. 2. Best-fitting path model for relationships between coping style, age, body mass, and restraint-induced changes in cortisol (ΔCORT), plasma oxidative damage (ΔROMs) and plasma antioxidant capacity (ΔOXY) in alpine marmots. Standardized path coefficients are reported above the causal links for all the relationships, whereas for the relationships including the categorical variable age, we calculated unstandardized means ± s.e.m., shown in Table 5.

that HNE was not associated with coping style, whereas it showed a significant and positive correlation with ROMs and age.

According to previous results on the relationship between glucocorticoids and coping styles (Koolhaas et al., 1999; Koolhaas et al., 2010; Øverli et al., 2007; Coppens et al., 2010), we predicted that proactive individuals should show both a lower baseline level of CORT and a lower production of CORT under restraint than reactive individuals. Our results did not support these predictions. Most previous studies on the topic have been done using artificial selection experiments. In natural conditions, though, an individual's social experience may affect its behavioural profile and stress responses to subsequent challenges (Jansen et al., 2010; Sachser et al., 2010). For example, proactive marmots may be involved more frequently than reactive marmots in territory defence activities or in agonistic interactions with dominant individuals in the colony. This may increase their basal CORT level in a way that masks the basal variation caused by differences in coping styles under more controlled environmental conditions.

Our expectations of the link between coping styles and oxidative status were only partially supported; although a more proactive coping style was related to higher pre-restraint OXY values, we did not find any link between coping style and pre-restraint ROMs. Herborn et al. (Herborn et al., 2010) recently found that fast explorer (i.e. proactive) individual greenfinches also show higher values of OXY, and did not exhibit a link between exploration and ROMs. Isaksson et al. (Isaksson et al., 2011) show that males' aggressive phenotype is positively associated with OXY in White's skink (*Egernia whitii*). In contrast, in a previous study on two captive lines of mice selected for different levels of aggression – long attack latency (LAL), reactive; and short attack latency (SAL), proactive – Costantini et al. (Costantini et al., 2008b) found that baseline levels of serum antioxidant capacity (measured by the OXY test as in the present study) were higher in less aggressive (LAL) mice. This finding seems contrary to what we found in wild marmots because LAL mice display a reactive coping strategy with non-social environmental challenges (Benus et al., 1991; Koolhaas et al., 1999). Once again, discrepancies between these different studies may be caused by differences in treatments among them. LAL and SAL mice come from artificial selection lines, which represent the two most extreme phenotypes/genotypes of the range existing in the original population, and they are maintained under optimal and standard husbandry conditions in the laboratory. In contrast, the marmots tested represent the whole range of variation existing in a natural population. For example, LAL and SAL mice included in

Table 4. Unstandardized mean and standard error of each relationship (represented in Fig. 1) between continuous response variables [coping style, body mass, and pre-restraint values of plasma antioxidant capacity (OXY), oxidative damage (ROMs) and cortisol (CORT)] and the explanatory variable age class

Response variable	Explanatory variable	Coefficient	s.e.m.
Coping style	S	0.73	0.33
	A2	0.50	0.31
	A3	–0.48	0.21
Body mass	S	1.94	0.17
	A2	2.92	0.16
	A3	3.37	0.11
OXY	S	505.72	25.72
	A2	598.49	24.40
	A3	488.43	16.45
ROMs	S	6.33	0.55
	A2	8.71	0.52
	A3	9.03	0.35
CORT	S	16.10	6.63
	A2	33.71	3.54
	A3	21.33	2.21

S, intercept in young; A2, intercept in sub-adults; A3, intercept in adults.

Table 5. Unstandardized mean and standard error of each relationship (represented in Fig. 2) between continuous response variables [coping style, body mass, and restraint-induced changes in oxidative damage (ΔROMs) and cortisol (ΔCORT)] and the explanatory variable age class

Response variable	Explanatory variable	Coefficient	s.e.m.
Coping style	S	0.87	0.44
	A2	0.50	0.31
	A3	–0.59	0.26
Body mass	S	1.86	0.20
	A2	2.92	0.14
	A3	3.48	0.12
ΔROMs	S	0.60	0.46
	A2	–0.62	0.33
	A3	–0.54	0.28
ΔCORT	S	n.a.	n.a.
	A2	7.05	2.01
	A3	2.36	1.68

S, intercept in young; A2, intercept in sub-adults; A3, intercept in adults. n.a., not available.

the study by Costantini et al. (Costantini et al., 2008b) were naïve concerning resident–intruder tasks (age: 38–64 days old), whereas marmots tested had totally different social experiences prior to the analyses. These multiple effects of social experiences and life-history trajectories may make it difficult to find a pattern in wild populations that is consistent with the ones found in controlled laboratory experiments. However, studies in the wild are essential to understand the link between oxidative status and fitness in wild animals.

We also expected that differences in coping styles could reflect differences in oxidative status *via* their link with CORT secretion. Accordingly, we found that the only way coping style was related to pre-restraint OXY was through its link with CORT. A recent meta-analysis on studies where physiological stress was induced by administration of glucocorticoids to evaluate the magnitude of their effects on oxidative stress showed that stress hormones increase oxidative stress only after several days of administration and may upregulate antioxidant defenses in the short term through genomic or non-genomic effects (Costantini et al., 2011). Although we did not measure whether restraint caused an increase in metabolic rate, it is reasonable to expect that the changes observed may also be associated with an increase in metabolism, and a proactive style is generally characterised by a higher metabolic rate than a reactive one (Careau et al., 2008a; Careau et al., 2011; Biro and Stamps, 2010). Furthermore, metabolic activity is responsible for most of the production of reactive chemical species (e.g. free radicals) (Halliwell and Gutteridge, 2007). Even if the association between metabolic rate and oxidative stress is not proportional in vertebrates, an increase in metabolic rate associated with the stress response to our restraint protocol might reasonably explain some of the increase in production of ROMs. A recent study on captive pigeons shows a positive correlation between oxygen consumption and ROMs (van de Crommenacker et al., 2010). Coping style, however, did not predict pre-restraint levels of HNE or its change caused by the stress of restraint. ROMs (i.e. hydroperoxides) are intermediate molecules of peroxidative reactions and derive from oxidation of lipids, proteins and nucleic acids (Halliwell and Gutteridge, 2007). In contrast, HNE is an end-product of lipid peroxidation that mainly derives from biotransformation of lipid hydroperoxides (Halliwell and Gutteridge, 2007). HNE can form adducts with proteins, which are damaged as a consequence. Pre-restraint levels of plasma ROMs and HNE were positively correlated; however, the restraint-induced changes of both molecules were not. These data could suggest that the acute response of plasma oxidative status to an unpredictable stressor was reflected only by changes in early compounds coming out from the oxidative cascade. We do not know whether a longer exposure to a stressor would also have been able to induce a visible change in end-products of the oxidative cascade, nor do we know whether a 30 min stressor is long enough to generate visible changes in HNE, which can emerge long after the end of the stressor exposure. Further studies are needed to analyse the dynamics of the response of different components of the oxidative status to unpredictable stressors in relation to the individual coping style.

The higher increase in ROMs under restraint observed in more proactive marmots was accompanied by an increase in OXY. This suggests that marmots responded to the stressor, remobilizing certain types of antioxidants among tissues and/or upregulating the syntheses of others. Given that we did not measure specific classes of antioxidants (e.g. enzymes, dietary compounds), we are unable to clearly define which between remobilization and upregulation explained most of the increase in OXY. It is, however, plausible that remobilization of antioxidants played a more significant role because any change in gene expression is unlikely to emerge in such a short time. Our path model allows us to infer that cortisol contributed to some extent to explain variation in pre-restraint OXY. However, cortisol did not contribute to explain the change in ROMs or OXY induced by the restraint. This last result suggests the lack of a possible non-genomic rapid action of cortisol on blood redox status or that a 30 min acute stressor is not long enough to detect such action. Glucocorticoids, such as cortisol, are hormones secreted by the hypothalamic–pituitary–adrenal axis and they promote gluconeogenesis and protein catabolism (Sapolsky et al., 2000; Romero, 2004). Glucocorticoid response may be also associated with acute and chronic changes in redox physiology (Costantini et al., 2011). However, as evidenced by a recent meta-analysis, the overall effect of glucocorticoids on antioxidant activity appears to be tissue, age and situation dependent (Costantini et al., 2011; see also Haussmann and Marchetto, 2010).

Previous studies where birds or mammals were subjected to a restraint protocol lasting from 30 min to 18 h have found both significant and insignficant changes in average levels of oxidative damage or antioxidants [e.g. mammals (Gümüslü et al., 2002; Sahin et al., 2004) and birds (Cohen et al., 2007; Costantini et al., 2007; Costantini and Lipp, 2010)]. These studies also found variation among individuals in the response to the restraint, which, in light of our results, could have been due partly to inter-individual variation in coping style.

According to the oxidative stress (or free radical) theory of ageing (Finkel and Holbrook, 2000), we might expect to observe changes in blood oxidative status with age in our cross-sectional sample of marmots. Our path analysis actually shows that OXY had an inverted-U relationship with age, being higher in subadult marmots than in yearlings or adults. In contrast, ROMs and HNE increased with age, being higher in adults. However, younger marmots experienced a higher increase of ROMs induced by the restraint. Nussey et al. (Nussey et al., 2009) found that wild Soay lambs (*Ovis aries*) had significantly higher plasma levels of lipid peroxidation compounds (MDA) than adult females, but MDA was not associated with individual age within the adult female cohort. In contrast, Hindle et al. (Hindle et al., 2010) found an increase in antioxidant enzymes and lipid hydroperoxides with age in wild-caught shrews. Vázquez-Medina et al. (Vázquez-Medina et al., 2011) found that wild adult female hooded seals (*Cystophora cristata*) had a higher muscle production of superoxide and activity of antioxidant enzymes than young, whereas the level of oxidative damage to lipids, proteins or DNA did not differ.

Overall, the evidence in favour of or against the oxidative stress theory of ageing in wild animals is mixed, so it is premature to make general conclusions. Moreover, several limitations of our data set, common to data collected in wild populations, do not permit us to conclude that changes in blood oxidative status are a signal of senescence. First, our data are cross-sectional, which makes it impossible to separate within-individual ageing patterns from between-individual heterogeneity. Therefore, variation in any trait due to phenotypic plasticity or individual life-history strategies cannot be distinguished from the effect of selection on certain phenotypes, and spurious associations between a phenotypic trait and age might arise because of a differential survival among phenotypes (van de Pol and Verhulst, 2006). Second, levels of oxidative stress in young marmots may have been affected by growth patterns, which could demand a raised metabolism that would be expected to increase the oxidative challenge for the individual (Alonso-Alvarez et al., 2007; Nussey et al., 2009), as well as the timing of maturation of antioxidant defences (Davis and Auten, 2010) and naivety in coping with unpredictable stressors, which could explain the higher increase in ROMs experienced by young marmots during the restraint. Finally, reproductive activity could have contributed to generate variation in oxidative status of adult marmots. Our data do not allow us to infer which of these scenarios is the most plausible or whether there are other explanations. Similarly, the inverted-U relationship between cortisol and age is hardly interpretable according to our cross-sectional data. It could be that older marmots have less cortisol baseline secretion than younger marmots, possibly to avoid inhibition of reproduction (Wingfield and Sapolsky, 2003). This explanation seems to be supported by our second path model (Fig. 2, Table 5) because subadults had a higher increase in cortisol than adults during our restraint regime. Alternatively, that pattern may be explained by a natural increase in cortisol with age within each marmot coupled with a higher mortality of individuals with a higher level of cortisol for older marmots. Therefore, future efforts should be addressed to evaluate the mechanisms underlying variation in oxidative status and cortisol of marmots. Longitudinal studies are especially needed to determine whether the temporal variation in oxidative damage, antioxidant status and cortisol we observed is a real signal of senescence or reflects flexible adjustments of the physiological system to the current phase of life cycle, and whether their variation can predict survival.

A further result of our path models is that larger marmots appeared to have higher baseline ROMs. Body mass, however, did not correlate with HNE or OXY. In another study, body mass did not predict levels of lipid peroxidation (MDA) compounds in wild female Soay sheeps (Nussey et al., 2009). Given the positive correlation between ROMs and oxygen consumption (van de Crommenacker et al., 2010), the effect of body mass on ROMs could suggest higher metabolic intensity in larger animals (Hulbert and Else, 2004). The increase in ROMs with age therefore happens directly and indirectly through the higher levels of ROMs in larger individuals. Future studies are needed to assess the possible metabolic and oxidative costs associated with body mass, possibly taking into account the individual body composition.

Finally, a major question is whether the differences between coping styles in baseline and stress-induced plasma oxidative status have consequences for the individual life-history strategy and fitness. Maintaining high baseline levels of antioxidants, as observed in proactive marmots, could expose them to higher energetic costs needed to synthesise endogenous antioxidants, as well as to increase the dietary intake of exogenous antioxidants. Moreover, the higher responsiveness of the redox system to acute stressors could expose proactive individuals to further costs (e.g. energy consumption and tissue damage). We do not have any data on differential survival associated with coping styles in marmots and so we cannot make any inferences. Although neophobic, shy laboratory rats with increased basal corticosterone levels throughout life have a 60% higher chance of death compared with neophilic, bold individuals (Cavigelli and McClintock, 2003), we cannot exclude the possibility that the physiological changes we observed in marmots have significant survival effects on these animals.

We do acknowledge that our analyses of oxidative status are limited to blood and so any generalizations to other tissues are hard to draw. However, a recent meta-analysis on effects of glucocorticoids on oxidative stress shows that the effect size on blood, although lower than that on brain or liver, is significantly high (Costantini et al., 2011). Further studies on marmots are needed to evaluate the longer-term association between coping style and oxidative status, the change in other components of oxidative status with the use of more specific measures (e.g. antioxidant enzymes, aconitase inactivation and nitrotyrosine), and the fitness costs associated with variation in oxidative status and stress response.

In conclusion, our study shows that differences in baseline and stress-induced oxidative status, at least in the plasma, can be detected between different coping styles in a wild mammal species. Our findings also highlight the potential role of cortisol as a modulator of differences in baseline values. Further studies are needed to better understand the causes and ecological consequences of oxidative status differences between coping styles and whether such differences also emerge in tissues other than blood.

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