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The effects of weather conditions on oxidative stress, oxidative damage and antioxidant capacity in a wild-living mammal, the European badger (Meles meles)

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Abstract:	Wild-living animals are subject to weather variability that may cause the generation of reactive oxygen species, resulting in oxidative stress and tissue damage, potentially driving demographic responses. Our three-year field study investigated the effects of seasonal weather conditions on biomarkers for oxidative stress, oxidative damage and antioxidant defence in the European badger (Meles meles). We found age-class effects: cubs were more susceptible to oxidative stress and oxidative damage than adults, especially very young cubs in the spring, when they also exhibited lower antioxidant biomarkers than adults. Although previous studies have found that intermediate spring and summer rainfall and warmer temperatures favor cub survival, counter-intuitively these conditions were associated with more severe oxidative damage. Oxidative damage was high in cubs even when antioxidant biomarkers were high. In contrast, adult responses accorded with previous survival analyses. Wetter spring and summer conditions were associated with higher oxidative damage, but also with higher antioxidant biomarkers. Autumnal weather did not vary substantially from normative values and thus effects were muted. Winter carry over effects were partially evident, with drier and milder conditions associated with greater oxidative damage in the following spring, but also with higher antioxidant capacity. Plausibly warmer conditions promoted more badger activity, with associated metabolic costs at a time of year when food supply is limited. Modeling biomarkers against projected climate change scenarios predicted greater future risks of oxidative damage, although not necessarily exceeding antioxidant capacity. This interdisciplinary approach demonstrates that individual adaptive physiological responses are associated with variation in natural environmental conditions.

Abstract

Wild-living animals are subject to weather variability that may cause the generation of reactive oxygen species, resulting in oxidative stress and tissue damage, potentially driving demographic responses. Our three-year field study investigated the effects of seasonal weather conditions on biomarkers for oxidative stress, oxidative damage and antioxidant defence in the European badger (*Meles meles*). We found age-class effects: cubs were more susceptible to oxidative stress and oxidative damage than adults, especially very young cubs in the spring, when they also exhibited lower antioxidant biomarkers than adults. Although previous studies have found that intermediate spring and summer rainfall and warmer temperatures favor cub survival, counter-intuitively these conditions were associated with more severe oxidative damage. Oxidative damage was high in cubs even when antioxidant biomarkers were high. In contrast, adult responses accorded with previous survival analyses. Wetter spring and summer conditions were associated with higher oxidative damage, but also with higher antioxidant biomarkers. Autumnal weather did not vary substantially from normative values and thus effects were muted. Winter carry over effects were partially evident, with drier and milder conditions associated with greater oxidative damage in the following spring, but also with higher antioxidant capacity. Plausibly warmer conditions promoted more badger activity, with associated metabolic costs at a time of year when food supply is limited. Modeling biomarkers against projected climate change scenarios predicted greater future risks of oxidative damage, although not necessarily exceeding antioxidant capacity. This interdisciplinary approach demonstrates that individual adaptive physiological responses are associated with variation in natural environmental conditions.

Key Words: Antioxidant, Climate Change, Eco-physiology, Reactive Oxygen Species, Oxidative Damage, Oxidative Stress, Weather Conditions.

Introduction

When wild animals experience low food availability or disease, changes in their energetic or immune activity can lead to increased metabolic stress, promoting the generation of reactive oxygen species (ROS) (Leeuwenburgh and Heinecke 2001). Although animals produce endogenous antioxidant molecules (e.g. glutathione), or enzymes (e.g. peroxidases; supplemented by their consumption of exogenous dietary antioxidants) to neutralize the unpaired electron in ROS, oxidative stress (OS) and oxidative damage (OD) can occur if antioxidant defences are exceeded (Matés et al. 2002). Consequently, when evolutionarily novel stressors arise from Human Induced Rapid Environmental Change (HIREC; Sih 2013), these can further exacerbate the physiological burden wild-living animals must cope with (Sies 1997), potentially compromising their defence systems.

Weather conditions are often intrinsically linked to food availability, foraging success, thermoregulatory costs and metabolic rates (Kronfeld-Schor and Dayan 2013); conditions likely to influence OS and OD. Furthermore, there can be cumulative 'carry-over effects' (COE; Harrison et al. 2011), where weather conditions in one season affect an individual's subsequent performance, provided it continues to survive. Consequently, if weather becomes increasingly unseasonable, variable and extreme, as predicted under climate change scenarios (Allen et al. (IPCC), 2014; see also Parmesan, Root and Willig 2000), this may exceed species' coping capacities (Smit et al. 2000), with implications for fitness and survival (White 2008).

Here, we undertake an inter-disciplinary approach (White & Ward 2011), using a medium-sized generalist carnivore, the European badger (henceforth 'badger') to explore how OS may operate mechanistically as the currency (*sensu*, Metcalfe and Alonso-Alvarez 2010) through which weather stress can affect eco-physiology (Costantini et al. 2010). We exam how seasonal weather conditions interact with OS (using the ability of red blood cells

(RBCs) to survive a free radical attack *ex vivo*; Kurata et al. 1993; Bize et al. 2008), OD (using lipid peroxidation, Mylonas and Kouretas 1998; via plasma malondialdehyde, MDA, Nielsen et al. 1997), antioxidant capacity (AOX) and enzymatic antioxidant capacity (peroxidise, PER; see Somogyi et al. 2007). This expands on previous research identifying weather-induced macro-demographic responses for this species (inter alia), as-well-as individual declines in body-condition and reproductive success (Newman et al. 2017; for broader discussion see Newman and Macdonald 2015). Specifically, badgers provide an informative model for studying responses to weather conditions because they preferentially forage for earthworms (*Lumbricus spp*.; see Newman et al. 2017), the availability of which is tied tightly to soil microclimate and prevailing weather (Curry 2004). However, badgers reside in communal burrows (termed setts) and, provided they have sufficient body-fat reserves, they can stay underground during periods of inclement weather and/or poor foraging conditions to mitigate net energy loss (Noonan et al. 2014; 2015). Consequently badgers undergo frequent short-term periodic swings in foraging activity, foraging success and body-condition, ultimately attempting to replenish depleted somatic reserves (Newman et al. 2011). This high metabolic turn-over, linked directly to variation in weather, is likely to generate ROS (Leeuwenburgh and Heinecke 2001). Under net-negative foraging conditions, when badgers do not meet their immediate energetic needs, they catabolize fat reserves (Domingo-Roura et al. 2001; Newman et al. 2011). Generally fat catabolism generates ROS (Morales et al. 2004) and causes redox imbalance, leading to increased levels of OD, despite upregulation of antioxidant enzymes (Vijayakumar et al. 2004). Once fat reserves are depleted, catabolizing muscle (protein) can further increase OS (Eisler et al. 2004; Finn and Dice 2006). Certainly, body-fat depletion adversely affects badger health (Domingo-Roura et al. 2001), over-winter survival (Macdonald and Newman 2002) and embryonic implantation (Woodroffe 1995; Macdonald et al. 2015).

Winter weather is especially critical for badgers, with frost making earthworms unavailable (Newman et al. 2017) and badgers use periods of torpor to mitigate food scarcity (Newman et al. 2011). Such scarcity has been linked with lower over-winter survival rates (Macdonald and Newman 2002), as-well-as lower cub recruitment into the adult population (Nouvellet et al. 2013). This leads us to predict that: (i) biomarkers of OS/OD and antioxidant capacity may be associated seasonal weather variation; (ii) individuals in poorer body-condition might exhibit higher OS/OD biomarker levels; and (iii) winter weather COE may affect individual OS/OD and antioxidant levels in the following spring. We consider, however, that OS/OD could occur even without apparent loss of body-condition over a season, due to the effective compensation of fat reserves.

Previous work in this same badger population by Macdonald and Newman (2002) identified that spring rainfall was critical to cub survival, with drought conditions leading to higher mortality rates (see also Macdonald et al. 2010). Building on this, Nouvellet et al. (2013) found distinct age-class specific responses, where cub survival probability was highest in years with intermediate annual rainfall (neither too wet nor too dry; a negative quadratic relationship) and intermediate temperature; whereas adult survival probability was greater in wetter years. These leads to a series of age-related predictions, positing that (iv) cubs and elderly badgers may be more vulnerable to OS/OD than prime-age adults, whereas (v) adults may benefit from more rainfall if weather interactions with OS/ OD biomarkers follow similar patterns to mortality effects. Furthermore, (vi) cub may experience more severe OS/OD effects in years with more extreme weather. Linked to this, we further predict that (vii) different OS phenotypes may be favoured under different weather conditions in different years (*sensu* Metcalfe and Alonso-Alvarez 2010).

Finally, we use emergent interactions between these biomarkers and weather conditions to parameterize simulations of how future weather conditions, according to the

IPCCs SRES climate change emissions scenarios (Murphy et al. 2009), might affect OS and OD. Herein we propose that this approach could provide a new tool for anticipating the effects of climate change on wildlife more broadly, better enabling appropriate and effective conservation action (Beaulieu et al. 2013).

105 **Methods**

106 *Trapping and sampling*

107 Badgers are highly tractable, enabling frequent recapture and large enough to yield sufficient
108 blood volumes, also for repeat sampling. For this study, a total of 220 unique individuals
109 were caught as part of ongoing socio-ecological population monitoring in Wytham Woods,
110 UK (see Macdonald et al. 2015). Briefly, marked (tattooed) individuals were captured
111 seasonally (spring (end of May – start of June) 2012-14, summer (end of August) 2012-14,
112 autumn (mid-November) 2012-13) sedated, measured and blood sampled, then given
113 sufficient time to recover from sedation prior to release back at their sett of capture; for full
114 handling protocol see Sun et al. (2015). Over the study period, these 220 individuals were
115 sampled between 1 and 11 times (median = 2), yielding a total of 564 unique capture records
116 and blood samples (Table 1). A body condition index (BCI) was calculated as:
117 log(weight)/log(length) (following Noonan et al. 2014). Age was known from year of birth
118 and divided into classes: Cubs <1 yr; prime adults 1-5 yr; old adults ≥ 6. Sex was also
119 recorded.

120

121 *Oxidative stress assays*

122 For details of assay methodologies, see Supporting on-line information, Appendix 1, but
123 briefly: Total antioxidant capacity (AOX) was measured as non-enzymatic plasma
124 antioxidant capacity (STA-360, Cell Biolabs, San Diego, USA) and enzymatic antioxidant
125 capacity via peroxidase (PER; STA-344, Cell Biolabs, San Diego). As a biomarker of OD,
126 lipid peroxidation (LP) was measured as malondialdehyde accumulation in plasma (STA-
127 330, Cell Biolabs, San Diego, USA) and red blood cell ½-life (RBC ½-life) was used as a

biomarker indicating resistance to OS, calculated as the time it took 50% of RBCs to lyze in the presence of an oxidant (Kirial, Courernon, France).

Weather data

Freely available weather records were obtained from the University of Oxford's Radcliffe Meteorological Station. Metrics of rainfall (total mm/month); minimum temperature (monthly mean daily minimum temperatures, in degrees Celsius); maximum temperature (monthly mean daily maximum temperatures, in degrees Celsius); and frost (number of days of frost/month) were extracted from this dataset. Mean weather conditions, used to analyze corresponding seasonal trapping sessions, were defined as: **Winter**: December – February (i.e. December 2011 to February 2012, inclusive, defines winter 2012); **Spring**: March – May; **Summer:** June-August; **Autumn**: September – November. Details of how weather in these seasons compared to 30 year average conditions are presented in Table 2.

Principal weather components

We used principal component analyses (PCA), conducted with scaling, to control for collinearity between weather metrics (for details see Appendix 2). This resulted in the retention of two principal components (PC) as predictive weather variables. For the PCA pertaining to seasonal analyses, factor loadings for temperature were the most influential contributors to the PC1 axis. Loadings were positive, thus higher values of PC1 correspond to higher temperatures. PC2 had a positive rainfall loading, where higher values correspond to wetter conditions. These components are henceforth referred to as PCtemp and PCrain.

For our COE analyses, PC1 factor loadings (PCtemp) included maximum temperature, minimum temperature and number of days of frost over the winter, such that

higher values of PC1 correspond to lower temperatures and more frost. PC2 (PCrain) had a negative rainfall loading where higher values correspond to drier weather.

Modeling seasonal weather effects on OS, OD and antioxidant defences

To identify predictors of variance in biomarkers, we built four global models that included these principal components, along with season, BCI, age-class and sex as fixed effects:

AOX = f(Age-class*PCrain*Season+Age-class*PCtemp*Season+Sex+BCI)

log(LP) = f(Age-class*PCrain*Season+Age-class*PCtemp*Season+Sex+BCI)

PER = f(Age-class*PCrain*Season+Age-class*PCtemp*Season+Sex+BCI)

RBC ½-life = f(Age-class*PCrain*Season+Age-class*PCtemp*Season+Sex+BCI)

To account for repeat sampling of individuals, badger ID was included as a random effect. We note that while our fixed effects were subject to temporal autocorrelation, the coarse scale at which these were measured did not result in any significant violation of the assumption of independence (see Appendix 2). LP values were log transformed to correct for heteroscedasticity. From these global models, we specified subsets of candidate models comprised of all possible combinations of fixed effects, both with and without interaction terms. We then used small sample size corrected Akaike's information criterion (AICc), to rank these candidates according to their statistical support (Burnham et al. 2011), additionally calculating the delta AICc (Δ_i), in relation to the highest-ranking model and the Akaike (or model) weight (w) for each model using the R package MuMIn (v. 1.15.6; Barton 2016).

Following Anderson (2008), rather than using Δ_i cut-off values, we applied weight-based averaging over all candidate models. From this, we derived averaged parameter estimates (θ), calculated by averaging their values over all candidate models that included the

parameter of interest, weighted by *w*, and their 95% confidence intervals (CI). We also calculated the 'relative influence' (RI) of each variable as the summation of *w* across all models that included the variable of interest (Burnham & Anderson, 2002).

Modeling winter weather COE on OS and antioxidant defences

Badgers were not trapped during winter due to a legal closed season to avoid stressing pregnant females (Protection of Badgers Act 1992). Instead we modeled the COE of winter weather on OS measurements in following the spring. Similar models were built to examine the COE of winter weather on spring OS measurements in adults (including cubs recruited from the previous year). Badger ID and age class were included as random effects to account for repeat sampling and for any differences between age classes (prime vs old), and AICc was derived for each model along with relative model weight (W).

AOX = f (PCrain+ PCtemp +Sex+BCI)

log(LP) = f (PCrain+ PCtemp +Sex+BCI)

PER = f (PCrain+ PCtemp +Sex+BCI)

RBC ½-life = f (PCrain+ PCtemp +Sex+BCI)

Model selection and averaging was then applied as described above. Note, cubs that survived the winter into the spring of the following were then included here as adults – age >1 year.

Climate change projections

We used these predictive models to parameterize simulations of how future climate change might affect OS, OD and AOX. Using the UK Climate Projections 2009 web interface (UKCP09; Murphy et al. 2009), we simulated 1000 projections of future seasonal weather

198 conditions for the 25 km^2 area around Wytham Woods into the years 2070-2099. UKCP09
199 projections were based on the IPCCs SRES low emissions (i.e., the B1 scenario, which
200 predicts 500 to 600 ppm CO_2, a 1.1 to 2.9 °C rise in mean temperature and no significant
201 trends in precipitation) and high emissions scenarios (i.e., the A1F1 scenario, which predicts
202 550 to 750 ppm CO_2, a 2.4 to 6.4 °C rise in mean temperature and no significant trends in
203 precipitation; Nakicenovic and Swart, 2005). We note that although more recent
204 'representative concentration pathway' (RCP) models have since replaced the SRES
205 emissions scenarios (Moss et al. 2010), recent analyses have demonstrated how the
206 UKCP09 projections still provide reliable projections (Sexton et al. 2016). Using each of
207 these weather projections and our predictive models, we estimated 1000 potential biomarker
208 responses using the predict() function in the R environment (v. 3.3.2; R Core Team 2016).
209 We acknowledge, however, that although trends in responses can be considered as robust,
210 both our parameter estimates and climate predictions are subject to modeling error and
211 therefore interpretations should be made cautiously.
212

Results

Short-term observational studies of natural weather effects are always hostage to fortune, because substantial variation may not occur within the study period, nevertheless our study years included sufficient weather deviation from long-term normative values to show meaningful effects on biomarkers (Table 2).

Analysis of seasonal weather effects on OS, OD and AOX

Yearly cub survival rate

Summary statistics for weather conditions are presented in Table 2 and for biomarkers in Table 3. The three study years included substantially different cub cohort sizes with different survival rates. In 2012, a total of 41 cubs were caught, of which 20 (49%) survived to adulthood. This was despite spring and summer weather both being considerably wetter than the long-term mean (1.45× and 2.15× greater rainfall respectively). Subsequently, in 2013, the winter was cold, with twice the normal number of frost days (50 vs the long-term mean of 26), followed by a cool spring (only 0.75× the long-term mean) and a drought summer that received only half of the normal rainfall. In this more challenging year, year only 28 cubs were caught, with 12 (43%) surviving to adulthood. In 2014, the winter was mild (just 5 frost days) and very wet, with twice the normal rainfall and although 60 cubs were caught, only 22 (37%) survived to adulthood, despite normative weather conditions throughout the rest of that year.

Antioxidant capacity – AOX

Of the modeled parameters, PCtemp, season, age-class*PCrain, PCrain*season, age-class*PCrain*season and age-class* PCtemp*Season all contributed significantly to variation

236 in AOX (Table 4). Sex had no distinct effect on AOX, or indeed on any other biomarker. For
237 cubs, AOX was lower when spring rainfall deviated from the long-term mean in either
238 direction and with cooler temperatures (Figure 1); conditions also associated with less inter-
239 individual variation. This occurred although we observed only modest spring rainfall
240 variation from the long-term mean (with 2012 wettest, at 1.45× the rainfall of long-term
241 mean), with no substantial variation in spring temperature. Cub AOX showed no significant
242 associations with summer weather (see top row of Figure 1), despite very wet summer
243 conditions in 2012 (2.15× the mean rainfall) and drought in 2013 (only 0.2× the mean
244 rainfall); albeit that the least inter-individual variability occurred when rainfall was abnormal.

245 Our dataset included minimal autumn weather variation, with only 2012 deviating
246 substantially from normative rainfall (1.41× the rainfall of long-term mean) and no
247 substantial temperature deviation; these conditions were associated with relatively low
248 autumnal cub AOX (see Table 3).

249 Cub AOX was lower than adult AOX in the spring, except in the dry, cold spring of
250 2013, when cub levels almost equaled adult levels. For prime adults, spring AOX was lowest
251 in 2013, which had normal rainfall and cooler temperatures; whereas for old adults AOX was
252 lower in spring 2012, with wetter conditions (1.45× the mean rainfall). Prime- and old- adults
253 exhibited similar AOX in summer, being lower with drier, warmer conditions. In autumn
254 AOX was lower with cooler, wetter conditions – although linked to greater inter-individual
255 variation.

256 <u>Antioxidant enzymes – peroxidase (PER)</u>

257 Of the modelled parameters, age-class, PCrain and PCtemp, age-class*season, PCrain*
258 season and the 3-way interaction PCtemp*age-class*season contributed significantly to
259 variation in peroxidase (Table 4). In spring, cub and adult PER tended to be lower with less

rainfall (Figure 1, second row). Inter-individual variability (Table 3) was greatest for cubs in the wettest year (2012); whereas inter-individual variability for both adult age-classes tended to be lower with drier conditions.

In summer, cubs exhibited similar mean PER levels between years, but with higher inter-individual variation with intermediate rainfall; whereas PER was highest for adults in the wettest summer (2012; 2.15× the mean rainfall). Low inter-individual variation was also associated with lower PER. In autumn, cubs and adults again showed similar responses, with lower PER occurring with slightly wetter, cooler conditions. For adults, high inter-individual variability was again associated with high PER.

Oxidative damage – lipid peroxidation (LP)

Of the modeled parameters, age-class, PCrain, PCtemp, season, PCrain*season, PCtemp*season contributed significantly to variation in LP (Table 4). Spring LP levels were higher for cubs than for adults. LP was highest in 2014, which had the warmest minimum temperature among study years (1.12× warmer than the long-term mean; 1.07× warmer than the long-term mean maximum temperature), but with typical rainfall (Figure 1, third row). Peak inter-individual variability also occurred with these conditions. In summer, cub LP was higher with intermediate rainfall. Similar cub LP levels continued into the autumn, although greater inter-individual variability was apparent in 2012 across a larger annual cohort, when rainfall was 1.41× greater than the long-term mean and temperatures were cool.

Prime- and old- adults exhibited similar LP associations with weather. Spring LP levels were higher with intermediate rainfall and higher temperature. In summer, LP was again higher with intermediate rainfall, which also associated with the greatest inter-

individual variability. In autumn, inter-individual variability was substantial under all conditions.

Resistance to oxidative stress – Red Blood Cell half-life (RBC ½-life)

Of the modeled parameters, age class, Age class*PCrain, Age class*PCtemp, Age class*Season and Age class*PCrain*season contributed significantly to variation in RBC ½-life (Table 4). There was little absolute difference between cub and adult RBC ½-life. For cubs, spring RBC ½-life was shortest in the coldest year (2012), whereas warmer temperatures were associated with greater inter-individual variability (Figure 1, fourth row). In summer, RBC ½-life was shortest for cubs in the driest year (2013), but with no clear effects in the other two years. In autumn, high RBC ½-life variation precluded any clear associations from being detected.

Adults showed no clear pattern with seasonal weather, although prime aged adults had higher RBC ½-life variability than old individuals.

Analysis of winter weather COE on OS and antioxidant defences in the following spring

There was substantial potential for COE during our study period: we observed variation in the number of winter frost days, from 50 in 2013 to just 5 in 2014 (2012 equaled the long-term average of 26) and winter rainfall in 2014 was twice the long-term average (other years had normal winter rainfall). All biomarker responses, below, refer to Figure 2 and Table 5.

Total antioxidant capacity - AOX

Winter PCrain and winter PCtemp had significant effects on badger AOX responses in the following spring, with 95% CIs not overlapping zero. The negative loadings on PCtemp and

PCrain indicate that warmer, frost free and drier weather conditions over the winter were associated with higher spring AOX and greater capacity to mitigate ROS.

Peroxidase – antioxidant enzymes

Only winter PCrain had a significant effect on PER, with 95% CIs not overlapping zero, with wetter winters associated with higher spring PER and thus greater capacity to cope with ROS.

Oxidative damage - lipid peroxidation

Only winter PCtemp had a significant explanatory relationship with LP, with 95% CIs not overlapping zero, with milder winters associated with higher oxidative damage.

Resistance to oxidative stress – RBC ½-life

Only PCtemp had a significant effect on RBC resistance to OS, with 95% CIs not overlapping zero, with longer spring RBC ½-life following warmer winter weather.

Climate change projections

Although there were differences in predicted responses between the high and low emissions scenarios, projected climate change scenarios through the 21st Century appeared likely to drive changes in badgers' oxidative stress and antioxidant capacity. From our predictive models, we found that the generally warmer conditions predicted under both scenarios could promote substantial increases in lipid peroxidation in badgers (Figure 3d-f). Despite the potential increase in oxidative damage however, badger antioxidant coping capacity may also increase, as evidenced by the trends for greater AOX (Figure 3a-c) and longer RBC ½-life (Figure 3j-l), though with multi-directional responses in peroxidase concentrations (Figure 3a-c). Notably, apart from the summer RBC ½-life responses, all projected biomarker

responses fell within the range of values quantified in the present study, albeit with different distributions. This suggests that these responses are physiologically possible.

Discussion

In support of our primary prediction, we identified a range of associations between weather conditions likely to stress wild badger biology and biomarkers of OS, OD and AOX. Notably, the values for LP (indicating OD) that we observed (2.33 µM; SD 0.87 for males; 2.33 µM; SD 0.76 for females) were higher than typical values for domestic dogs (1.70µM for male; 1.5µM for females: Todorova et al. 2005), as a lab animal analogue. This is congruent with expectations that wild-living animals will experience higher oxidative stress than domestic animals. We also emphasize that we used metrics of both enzymatic and non-enzymatic antioxidant capacity here, where many previous studies have focused solely on antioxidant defences, erroneously assuming that this will indicate levels of OS. Absolute antioxidant levels are, in fact, only informative if the levels of ROS or OD are also known (see Monaghan et al. 2009).

Curiously, with regard to our second prediction, we found no evidence that individuals in good or bad body condition (BCI) showed different levels of OD or antioxidant defences (but see Montes et al. 2011). This suggests that any OS / OD arising was not due to weather-related effects of food supply, expenditure and starvation per se, but likely due to repeated short-term cycles of weight / loss gain, where cubs are known to have a lower tolerance for enduring and remediating periods of food scarcity (Newman et al. 2011; Macdonald and Johnson 2015). Sex also had no distinct effect on OS biomarkers, implying that the different life-history stressors affecting males and females caused similar levels of OS / OD.

In support of our third prediction, carry-over effects (COE) were apparent at the ensuing spring trapping, where our study coincided with critical variation in winter temperature, linked to substantial differences in number of frost days and double typical precipitation in the mild year (2014; Table 2). Drier and milder winter conditions were associated with higher LP, but also with longer RBC ½-life and higher AOX in the following spring. In the UK, badgers do not truly hibernate (i.e., conserve protein catabolism, see Newman et al. 2011), but undergo varying extents of torpor, dropping their activity levels and basal metabolic rate with colder winter conditions (Noonan et al. 2014; McClune et al. 2015). Exercise induces OS (Alessio 1993; Radak et al. 2008), conversely reduced activity and metabolic rate during torpor tends to lessen the risk of oxidative damage (Heldmaier and Ruf 1992). Reinforcing this proposition, wetter, less frosty winters (promoting earthworm availability) were associated with higher PER – plausibly linked to ROS generated by the metabolic cost of warmer conditions promoting higher activity rates (Noonan et al. 2014) at a time of year when thermoregulation (De Quiroga 1992) is expensive and food is scarce.

Our forth prediction, that badger cubs would be more susceptible to OS than adults, was largely supported. This was especially so when they were very young in the spring, when cubs were more prone to OD and suffered greater LP than adults; although both cubs and adults had similar RBC ½-life. Due to the scaling of metabolic rate to mass (McClune et al. 2015), cubs, initially in the 1.7 -3.0 kg range in early spring (vs adults ranging 7-9kg; Macdonald et al. 2015), would be expected to generate proportionately more ROS than adults. Badger cubs grow rapidly in the spring and faster juvenile growth-rate can confer an early survival advantage in badgers (Newman et al. 2001) and generally (Taborsky 2006; Dmitriew 2011). Growth rate and growth hormones are generally linked to higher ROS production, via metabolic activity (Holzenberger et al. 2003), potentially exacerbating OD, as seen in birds (e.g., Alonso-Alvarez et al. 2007; Kim et al. 2011).

374 Nevertheless, cubs concurrently also exhibited generally lower AOX than adults –
375 except in 2013, which was particularly cool and dry, when they matched adults. Potentially
376 badger cubs can only attempt to mitigate the ROS generated by growth when rainfall is not
377 too extreme – although they still fail to do so effectively because LP was consistently high
378 under these weather conditions. PER was similar between age-classes, except in the spring of
379 2012, when cubs had lower PER than adults. Cub RBC ½-life was shortest with the
380 abnormally dry conditions of 2013. Drought impacts badger foraging success (Macdonald
381 and Newman 2002) and exacerbates the morbidity caused by pandemic coccidiosis in badger
382 cubs (Newman et al. 2001) – potentially further exacerbating OS.

383 Higher cub LP actually arose with intermediate rainfall and warmer temperatures, but
384 higher rainfall resulted in more inter-individual variation. Similarly, wetter spring conditions
385 were associated with longer cub RBC ½-life, i.e., less OD; although longer adult RBC ½-life
386 was associated with warmer conditions and intermediate rainfall, more congruent with
387 weather effects on adult survival rate. High mean badger cub OS was also linked to greater
388 inter-individual variation, although variability decreased as seasons progressed within each
389 year, possibly due to selective mortality of mal-adapted individuals (Penteriani et al. 2009;
390 Gaillard and Yoccoz, 2003).

391 Interestingly, however, and contrary to our initial position, weather effects on OS /
392 OD biomarkers largely did not correspond with the negative quadratic weather effects on cub
393 mortality that Nouvellet et al. (2013) found in a more extensive and purely actuarial study of
394 this same population. Conforming with our fifth prediction, however, adult biomarkers were
395 more in accord with adult survival dynamics, with wetter (and slightly cooler) conditions
396 associated with higher AOX and PER, indicative of a greater ability to resist OS. This implies
397 that any mechanistic relationship between drivers of OS /OD and absolute mortality
398 outcomes in badgers is also influenced by other co-factors, at least for cubs.

In terms of cohort effects, our sixth prediction; minimum inter-individual variation was observed in the harshest year (2013), which supported that 'poor-quality' cubs may have died before the earliest opportunity to sample them in the spring (post-weaning). Conversely, in milder years, when higher numbers of cubs survived until the spring trapping (2012 and especially 2014), there was considerable inter-individual variation in OS measurements, but along a continuum rather than according to distinct phenotypes – refuting prediction seven. This suggests that individuals may follow trade-off strategies, investing differentially in mitigating OS /OD versus other developmental traits, which might have a selective advantage only under stressful weather conditions (Metcalfe and Alonso-Alvarez 2010; Bilham et al. 2013). For instance, in this same badger population Annavi et al. (2014) found advantages of paternal heterozygosity on cub survival rates only in years with benign weather; in harsh years all individuals were similarly prone to mortality, irrespective of subtle genetic advantages.

Conclusions

Identifying that distinct OS, OD and AOX biomarker responses were associated with prevailing and carry-over weather conditions, led us to consider how these biomarkers might be affected by climate change projections for the UK (Murphy et al. 2009). While our models suggest future conditions could lead to substantial increases in lipid peroxidation, badgers may well have the adaptability to cope with warmer conditions because simultaneously their antioxidant coping capacity was also predicted to increase. Indeed, this would be congruent with the European badgers' wide bioclimatic niche, spread from the Mediterranean to the Arctic (Johnson et al. 2002).

Schloss et al. (2012) predict that, for the western hemisphere, an average of 9.2% of mammals at any given location will be unable to respond to climate change adequately and in some regions up to 39% may be unable to keep pace. Berteaux et al. (2006) identify a lack of understanding on proximate causality as one of the main constraints when projecting the effects of climate change on mammals. Therefore, identifying mechanistic eco-physiological associations with climate change is broadly relevant (e.g., Helmuth et al. 2005), beyond badgers and may well provide an additional tool with which to assess climate change vulnerabilities.

Author Contributions Statement

KB and CN conceived the ideas; KB, CN, MN and CDB collected the samples, DWM directed the badger fieldwork; KB and AB analysed the samples, with ALS overseeing the lab work; KB and MN analysed the data; CN, KB, CDB and DWM led the writing of the manuscript. All authors contributed to the manuscript writing.

Data Accessibility

Data summaries included in the manuscript and appendices are comprehensive; however, all data will be archived in Dryad upon acceptance.

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614 **Table 1** Summary of the individuals sampled in the study. Age was known from year of

615 birth, and individuals were classified as Cub <1 yr; prime adult 1-5 yrs; old adult ≥ 6yrs.

Year	Season	Age class	*n*
2012	Spring	Cub	31
		Prime adult	31
		Old adult	25
	Summer	Cub	16
		Prime adult	37
		Old adult	30
	Autumn	Cub	14
		Prime adult	40
		Old adult	29
2013	Spring	Cub	23
		Prime adult	28
		Old adult	21
	Summer	Cub	9
		Prime adult	30
		Old adult	24
	Autumn	Cub	2
		Prime adult	25
		Old adult	19
2014	Spring	Cub	34
		Prime adult	17
		Old adult	19
	Summer	Cub	16
		Prime adult	24
		Old adult	20

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618 **Table 2** Mean weather conditions in Oxford, UK for the three study years. Averages for the
619 last 30 years are also presented.

Rainfall (mm/month)	**30 year mean**	**2012**	**2013**	**2014**
Winter	55.78	42.37	69.73	111.57
Spring	51.26	74.27	55.87	59.90
Summer	52.25	112.43	27.43	56.00
Autumn	62.88	88.36	60.77	-
Min temperature (°C)				
Winter	2.18	2.63	1.80	3.77
Spring	5.57	5.97	3.60	6.27
Summer	12.25	12.60	12.40	12.33
Autumn	7.75	6.83	7.87	
Max temperature (°C)				
Winter	7.85	8.8	7.07	9.67
Spring	13.94	14.77	11.70	14.97
Summer	21.72	20.40	22.63	22.40
Autumn	14.88	13.97	15.07	-
Frost (total days)				
Winter	26	26	50	5

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622 **Table 3** Summary statistics of badger (*Meles meles*) biomarkers in Wytham Woods, UK
623 throughout the study period. Values presented are means ± standard deviations. Total
624 antioxidant capacity (AOX) was measured as non-enzymatic plasma antioxidant capacity (in
625 µM) and enzymatic antioxidant capacity via peroxidase (PER; in mU/ml). As biomarkers of
626 OD, lipid peroxidation (LP) was measured as malondialdehyde accumulation in plasma (in
627 µM) and red blood cell ½-life (RBC ½-life) was calculated as the time it took 50% of RBCs
628 to lyse in the presence of an oxidant (in min). For sample sizes see Table 1.

Season	Biomarker	Age class	2012	2013	2014
Spring	AOX	Cub	160.73 ± 66.02	114.98 ± 57.60	206.71 ± 104.02
		Prime Adult	209.2 ± 69.16	121.83 ± 42.80	176.72 ± 82.68
		Old Adult	228.96 ± 52.05	127.95 ± 36.11	242.81 ± 105.12
	PER	Cub	0.45 ± 0.10	0.29 ± 0.05	0.22 ± 0.08
		Prime Adult	0.53 ± 0.15	0.34 ± 0.08	0.33 ± 0.13
		Old Adult	0.51 ± 0.07	0.33 ± 0.06	0.29 ± 0.12
	LP	Cub	18.65 ± 14.32	12.36 ± 5.40	59.54 ± 40.82
		Prime Adult	8.52 ± 3.55	5.59 ± 2.70	26.43 ± 23.30
		Old Adult	8.27 ± 3.88	5.53 ± 2.29	41.11 ± 26.59
	RBC ½-life	Cub	71.49 ± 12.62	61.74 ± 5.74	69.88 ± 11.40
		Prime Adult	71.24 ± 8.86	62.79 ± 8.89	88.18 ± 17.82
		Old Adult	66.3 ± 5.03	60.59 ± 4.72	72.25 ± 11.65
Summer	AOX	Cub	250.19 ± 61.41	220.02 ± 62.45	226.89 ± 122.70
		Prime Adult	255.26 ± 62.91	132.27 ± 84.11	234.58 ± 68.10
		Old Adult	266.98 ± 55.39	79.66 ± 55.06	246.92 ± 63.79
	PER	Cub	0.29 ± 0.07	0.48 ± 0.10	0.4 ± 0.15
		Prime Adult	0.46 ± 0.22	0.41 ± 0.07	0.46 ± 0.20
		Old Adult	0.71 ± 0.18	0.39 ± 0.06	0.35 ± 0.09
	LP	Cub	7.94 ± 4.00	9.63 ± 2.74	14.5 ± 4.82
		Prime Adult	6.31 ± 2.14	6.26 ± 1.35	12.22 ± 5.02
		Old Adult	4.76 ± 3.03	6.4 ± 2.03	11.15 ± 5.43
	RBC ½-life	Cub	65.17 ± 6.19	58.91 ± 2.79	64.85 ± 6.56
		Prime Adult	64.89 ± 4.41	60.37 ± 4.64	68.86 ± 4.97
		Old Adult	65.07 ± 5.16	62.42 ± 5.87	68.42 ± 4.85
Autumn	AOX	Cub	169.04 ± 20.43	495.74 ± 21.41	-
		Prime Adult	172.79 ± 23.06	452.9 ± 158.30	-
		Old Adult	173.01 ± 27.04	475.16 ± 183.21	-
	PER	Cub	0.23 ± 0.03	0.55 ± 0.14	-
		Prime Adult	0.24 ± 0.11	0.51 ± 0.09	-
		Old Adult	0.23 ± 0.04	0.48 ± 0.12	-
	LP	Cub	12.7 ± 5.97	17.09 ± 9.20	-
		Prime Adult	9.15 ± 8.77	30.36 ± 14.52	-
		Old Adult	8.35 ± 3.10	26.28 ± 12.88	-
	RBC ½-life	Cub	78.92 ± 10.14	78.21 ± 20.21	-
		Prime Adult	74.69 ± 5.42	68.87 ± 8.67	-
		Old Adult	73.6 ± 6.95	68.4 ± 9.40	-

Table 4 Model averaging for the variables predictive of variation in badger (*Meles meles*) biomarkers in Wytham Woods, UK. The model-averaged estimates (Θ), 95% confidence intervals (CI), and relative influence (RI) of each parameter are presented. Biomarkers include total antioxidant capacity (AOX), measured as non-enzymatic plasma antioxidant capacity (in µM); enzymatic antioxidant capacity, measured as peroxidase concentration (PER; in mU/ml); lipid peroxidation (LP), measured as malondialdehyde accumulation in plasma (in µM) and red blood cell ½-life (RBC ½-life), calculated as the time it took 50% of RBCs to lyze in the presence of an oxidant (in min). Asterisks denote coefficient estimates that differed significantly from zero (based on 95% confidence intervals).

		AOX				LP				PER				RBC ½ life			
	Category level	**RI**	***θ***	**Lower 95% CI**	**Upper 95% CI**	**RI**	***θ***	**Lower 95% CI**	**Upper 95% CI**	**RI**	***θ***	**Lower 95% CI**	**Upper 95% CI**	**RI**	***θ***	**Lower 95% CI**	**Upper 95% CI**
Intercept	-		266.80*	197.28	336.40		4.08	3.76	4.40		0.33	0.23	0.42		72.67*	65.42	79.92
Age class	Cub	0.93	-	-	-	1.00	-	-	-	1.00	-	-	-	1.00	-	-	-
	Prime		-14.94	-107.21	77.34		-0.44*	-0.71	-1.56		0.15*	0.03	0.27		21.25*	11.70	30.80
	Old		67.18	-25.83	160.20		-0.53*	-0.80	-2.72		0.10	-0.01	0.22		0.18	-9.43	9.78
BCI	-	1.00	-8.48	-2.55	237.97	0.43	0.10	-1.05	1.25	0.32	-0.09	-0.43	0.25	0.97	18.73	-7.36	44.81
PCrain	-	0.99	-72.45	-147.05	4.156	1.00	-1.39*	-1.69	-1.10	1.00	0.32*	0.23	0.40	1.00	6.78	-1.12	14.67
PCtemp	-	1.00	101.80*	4.89	154.69	1.00	1.46*	1.24	1.69	0.99	-0.10*	-0.17	-0.03	1.00	5.32	-0.12	10.77
Season	Spring	1.00	-	-	-	1.00	-	-	-	1.00	-	-	-	1.00	-	-	-
	Summer		-87.67	-1.59	1419.24		-18.79*	-23.11	-14.47		0.70	-0.49	1.88		-116.20	-271.92	39.05
	Autumn		190.70*	1.05	276.81		-0.04	-0.44	0.35		0.09	-0.04	0.22		3.54	-5.35	12.43
Sex	Female	1.00	-	-	-	0.09	-	-	-	0.01	-	-	-	0.46	-	-	-
	Male		-8.54	-21.32	6.15		-0.01	-0.05	0.04		> -0.01	> -0.01	< 0.01		0.282	-0.95	1.46
Age class*PCrain	Cub*PCrain	1.00	-	-	-	0.03	-	-	-	0.17	-	-	-	1.00	-	-	-
	Prime*PCrain		170.30*	54.31	286.36		< 0.01	-0.05	0.05		0.01	-0.04	0.06		-29.99*	-41.94	-18.04
	Old*PCrain		65.45	-49.53	180.43		> -0.01	-0.06	0.06		0.02	-0.09	0.13		-16.11*	-27.83	-4.29
Age class*PCtemp	Cub*PCtemp	1.00	-	-	-	0.07	-	-	-	0.85	-	-	-	1.00	-	-	-
	Prime*PCtemp		-71.04	-151.27	9.19		0.01	-0.10	0.12		0.04	-0.04	0.12		21.85*	13.60	30.11

	Old*PCtemp		6.56	-74.69	87.80		0.02	-0.14	0.17		0.03	-0.05	0.11		7.23	-1.12	15.56
Age class*Season	Cub/Spring	1.00	-	-	-	0.17	-	-	-	0.97	-	-	-	1.00	-	-	-
	Prime*summer		-1520.00	-3388.72	348.10		0.06	-0.49	0.60		0.43	-0.13	0.99		-109.40	-301.73	83.023
	Old*Summer		-2749.00*		-838.51		0.01	-0.63	0.65		1.49*	0.20	2.79		-37.50	-233.82	158.83
	Prime*Autumn		-52.78	-4658.70	41.47		0.10	-0.45	0.66		-0.23	-0.57	0.10		-18.11*	-27.92	-8.30
	Old*Autumn		-80.53	-147.02	14.98		0.09	-0.40	0.57		-0.26	-0.56	0.11		-5.68	-15.58	4.19
				-176.04													
PCrain*Season	Spring/PCrain	1.00	-	-	-	1.00	-	-	-	1.00	-	-	-	1.00	-	-	-
	Summer*PCrain		85.33	-79.30	249.96		2.89*	2.39	3.40		-0.43*	-0.56	-0.30		4.46	-12.52	21.45
	Autumn*PCrain		-186.4*	-328.62	-44.08		1.04*	0.61	1.47		-0.67*	-0.84	-0.50		-2.36	-17.00	12.26
PCtemp*Season	Spring/PCtemp	1.00	-	-	-	1.00	-	-	-	0.97	-	-	-	1.00	-	-	-
	Summer*PCtemp		-67.28	-942.82	808.25		8.44*	5.93	10.95		-0.26	-0.96	0.44		54.05	-37.09	144.17
	Autumn*PCtemp		0.00	-0.036	0.04		< 0.01	> -0.01	< 0.01		< 0.01	> -0.01	< 0.01		<0.01	> -0.01	< 0.01
Age class* PCrain*Season	Cub/Spring/PCrain	1.00	-	-	-	< 0.01	-	-	-	<0.01	-	-	-	1.00	-	-	-
	Prime*Summer*PCrain		2.86	-210.27	215.98		< 0.01	-0.03	0.03		<0.01	-0.01	0.01		37.40*	15.42	59.38
	Old*Summer*PCrain		227.00*	10.51	443.59		< 0.01	-0.05	0.05		<0.01	-0.01	0.01		18.45	-3.83	40.74
	Prime*Autumn*PCrain		-161.5	-345.22	22.21		< 0.01	-0.03	0.03		<0.01	-0.01	0.01		42.66*	23.69	61.63
	Old*Autumn*PCrain		-33.86	-217.53	149.80		< 0.01	-0.03	0.03		<0.01	-0.01	0.01		22.27*	3.40	41.14
Age class* PCtemp*Season	Cub/Spring/PCtemp	1.00	-	-	-	0.02	-	-	-	0.85	-	-	-	1.00	-	-	-
	Prime*Summer*PCtemp		951.80	-135.63	2039.27		-0.01	-0.29	0.27		-0.35	-0.73	0.03		29.63	-82.26	141.52
	Old*Summer*PCtemp		1536.00*	423.64	2648.38		> -0.01	-0.34	0.33		-0.89*	-1.67	-0.11		15.00	-99.26	129.27
	Prime*Autumn*PCtemp		0.01	-4.31	4.33		0.02	-0.37	0.41		-0.15	-0.54	0.23		-0.02	-2.26	2.22
	Old*Autumn*PCtemp		0.01	-3.88	3.89		0.02	-0.34	0.38		-0.16	-0.56	0.23		-0.01	-1.50	1.48

635

Table 5 Model averaging for the variables predictive of carry-over-effects in badger (*Meles meles*) biomarkers in Wytham Woods, UK. The model-averaged estimates (Θ), 95% confidence intervals (CI), and relative influence (RI) of each parameter are presented. Biomarkers include total antioxidant capacity (AOX), measured as non-enzymatic plasma antioxidant capacity (in µM); enzymatic antioxidant capacity, measured as peroxidase concentration (PER; in mU/ml); lipid peroxidation (LP), measured as malondialdehyde accumulation in plasma (in µM) and red blood cell ½-life (RBC ½-life), calculated as the time it took 50% of RBCs to lyze in the presence of an oxidant (in min). Asterisks denote coefficient estimates that differed significantly from zero (based on 95% confidence intervals)

	AOX				LP				PER				BC ½ life			
	RI	***Θ***	**Lower 95% CI**	**Upper 95% CI**	**RI**	***Θ***	**Lower 95% CI**	**Upper 95% CI**	**RI**	***Θ***	**Lower 95% CI**	**Upper 95% CI**	**RI**	***Θ***	**Lower 95% CI**	**Upper 95% CI**
Intercept		214.68	41.36	387.99		0.61	1.21	3.61		0.08	0.26	0.59		63.88*	35.42	92.34
BCI	1.00	-68.62	-639.50	502.26	0.68	1.99	-4.53	3.35	0.30	0.26	-0.60	0.44	0.98	13.40	-78.38	105.17
PCrain	1.00	38.38	25.44	51.31	0.77	0.08	-0.27	0.03	1.00	0.01	0.09	0.13	0.44	-0.14	-1.40	1.12
PCtemp	1.00	-20.00	-26.67	-13.34	1.00	0.03	-0.42	-0.33	0.01	<0.01	0.01	<0.01	1.00	-3.77*	-4.83	-2.71
Sex – Male	0.95	-13.49	-38.43	11.45	0.27	0.01	-0.25	0.15	0.07	0.01	-0.02	0.02	0.66	0.80	-2.77	4.37

645 **Figure Legends**

646 **Figure 1. Scatter-plots depicting the relationships between biomarkers measured in badgers (*Meles meles*) in Wytham Woods, UK and**
647 **weather variables across all age categories**. The left-hand panels depict the biomarkers as a function of seasonal mean weather metrics in the
648 reduced dimension space of PCrain; in the right-hand panels weather metrics are reduced according to the dimension space of PCtemp. Cubs <1
649 yr; prime adults 1-5 yr; old adults ≥ 6.

650

651 **Figure 2. Scatter-plots depicting the carry-over effect (COE) relationships between biomarkers measured in badgers (*Meles meles*) in**
652 **Wytham Woods, UK in the spring and the previous winter's weather variables**. X-axis depicts extent PCrain and PCtemp axis loadings. All
653 animals here are classed as adult.

654

655 **Figure 3 Density estimates of projected responses of badger (*Meles meles*) biomarkers in Wytham Woods, UK to future climate projections**
656 **under a low (IPCC SRES B1), and high emissions scenario (IPCC SRES A1F1) in relation to the present distributions.** The top row (panels a; d; g; and
657 j) depicts spring responses, the middle row (panels b; e; h; and k) summer responses; and the bottom row (panels c; f; I; and l) autumn responses. We note that
658 although the negative Red blood cell (RBC) half-lives in panel k) are clearly impossible, these were included to depict the substantial negative trend in this
659 biomarker predicted under the high emissions scenario.

Figure 1

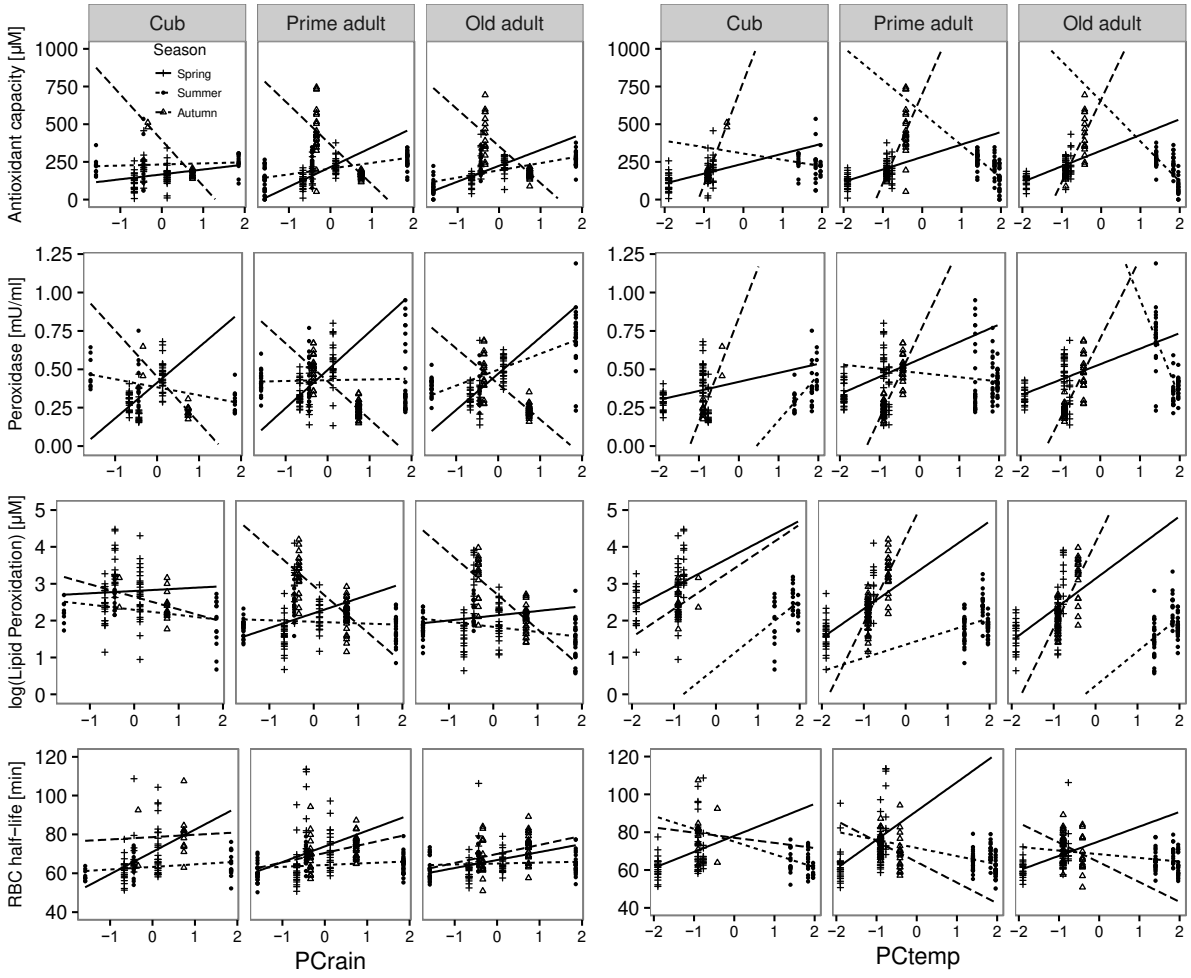

Cub
Prime adult
Old adult
Season
Spring
Summer
Autumn
Antioxidant capacity [µM]
Peroxidase [mU/ml]
log(Lipid Peroxidation) [µM]
RBC half−life [min]
PCrain
PCtemp

Figure 2

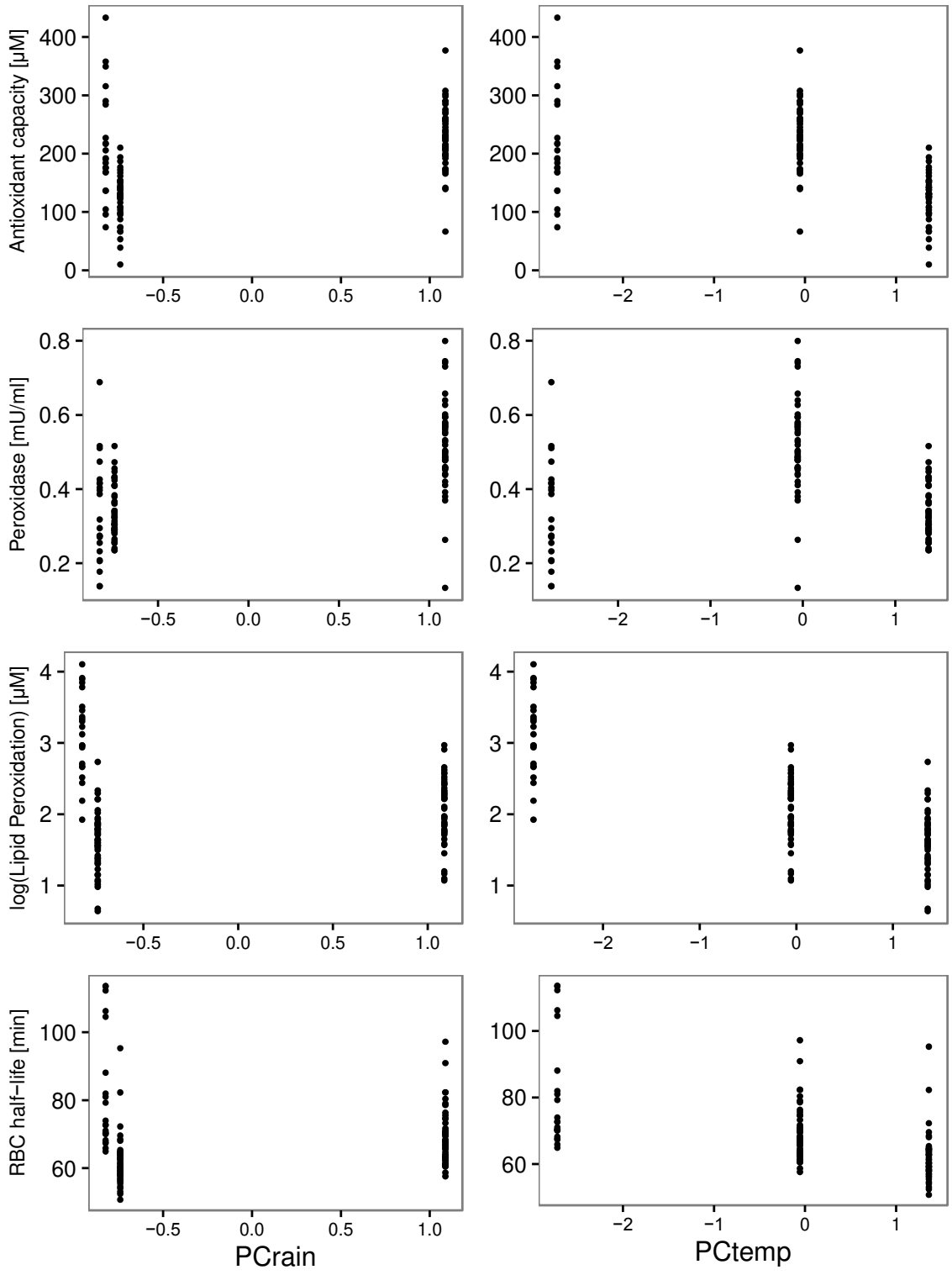
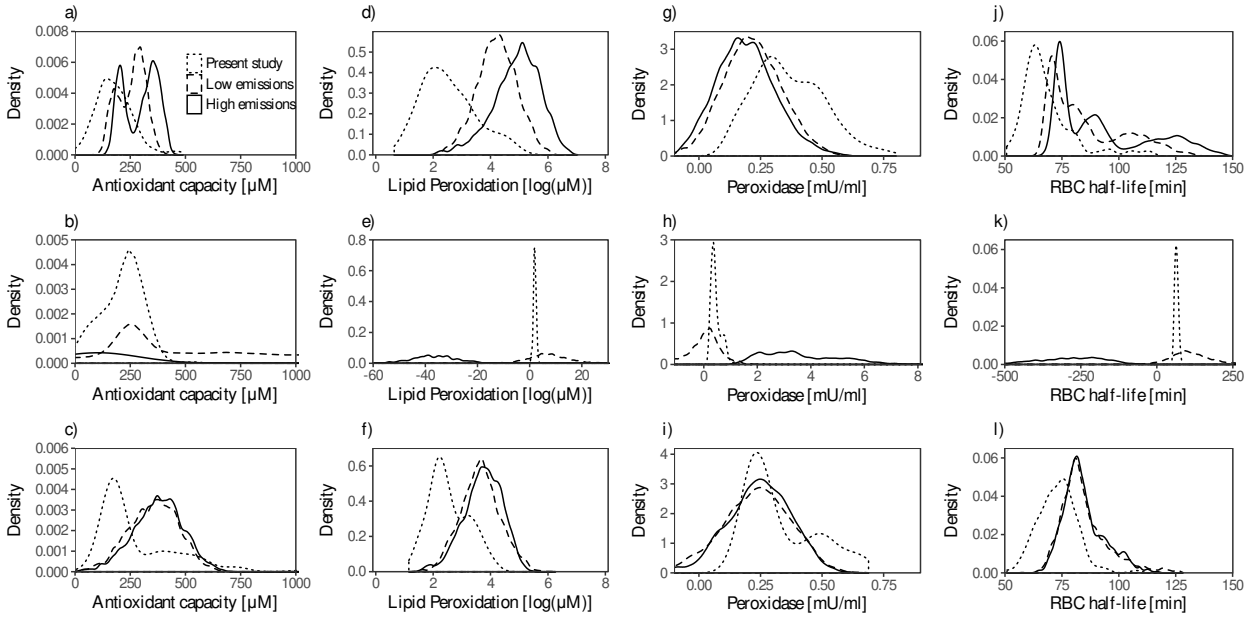

Antioxidant capacity [µM]
Peroxidase [mU/ml]
log(Lipid Peroxidation) [µM]
RBC half−life [min]
PCrain
PCtemp

Figure 3


a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
k)
l)
Present study
Low emissions
High emissions
Density
Antioxidant capacity [µM]
Lipid Peroxidation [log(µM)]
Peroxidase [mU/ml]
RBC half-life [min]

This Supplementary Information:

The effects of weather conditions on oxidative stress, oxidative damage and antioxidant capacity in a wild-living mammal, the European badger (*Meles meles*)

Blood sampling

Blood samples were collected from spring 2012 until August 2014, between 8.30 and 11 am, to minimise circadian variations. Individuals were marked with a temporary livestock marker dye to identify recaptures within trapping sessions and thus avoid unnecessary re-sampling.

For oxidative stress assays, approximately 12 ml of blood (never more than 5% estimated badger blood volume by weight) were collected from the jugular vein in heparinised vacutainers (BD Vacutainer® systems, Plymouth, UK) using 21'G x 1½" needles.

Blood was used in 2 different ways depending on assay:

1) *Plasma*: Full blood was centrifuged for 10 min at 1500 g (4ºC). The plasma was aliquoted and frozen on site immediately at -20ºC, before being transferred to -80ºC at the end of each week of trapping and stored until further analysis.
2) *Red blood cells*: After removal of the plasma, 10 µl of red blood cells (obtained by centrifugation) were diluted in 740 µl of 'KRL mammal assay buffer' and stored under the same conditions as (1) until further analysis.

Oxidative stress assays

For all assays, samples were run in duplicate, with appropriate negative and positive controls, and measurements performed in a 96 well plate spectrophotometer (FLUOstar OMEGA 415-0435, BMG LABTECH GmbH, Germany). For all absorbance assays, Grainer flat bottomed 96 well plates were used (Greiner Bio-One Ltd., UK).

AOX: Total antioxidant capacity Assay

Following Costantini et al. (2007) and Isaksson et al. (2011), we measured total antioxidant capacity (AOX) as total, non-enzymatic, circulating antioxidants. We used a commercial kit (STA-360, Cell Biolabs, San Diego, USA) where antioxidants reduce Cu^{2+} to Cu^{+}, which reacts with neocuproine to form an orange chromogen measurable at 490 nm. 20 µl of plasma was added to 180 µl of reaction buffer in a 96 well plate. Blank absorbance was measured at 490 nm and 50 µl of copper ion reagent was added. The plate was incubated on an orbital shaker for 5 min, before the addition of 50 µl of stop solution. Final absorbance was read at 490 nm. To calculate AOX as uric acid equivalent units, blank values were subtracted from the final reading and values were compared to a uric acid standard curve.

Peroxidase (PER) Assay

We measured PER using a fluorometric assay kit (STA-344, Cell Biolabs, San Diego), where hydrogen peroxide reacts with ADHP in the presence of horseradish peroxidase (HRP) to produce resorufin, which was measured fluorometrically. 50 µl of plasma was added to 50 µl of reaction mix containing 100 µM of ADHP and H_2O_2 (2 mM; for peroxidase assay) in the wells of a black 96 well plate (Nunc, Sigma-Aldrich, Dorset, UK). The plate was then incubated in the dark for 30 min before reading the fluorescence (excitation 530 nm, emission 590 nm). PER content was then calculated by comparison to a standard curve.

Lipid Peroxidation (LP): Malonaldehyde (MDA) Assay

We measured LP as the amount of MDA present in the sample using a commercial thiobarbituic acid reactive species (TBARS) assay kit (STA-330, Cell Biolabs, San Diego, USA). The principle of the assay is that two molecules of thiobarbituric acid react with one molecule of MDA (from the sample) to produce a pink molecule with a peak absorbance at 532 nm. Butylated hydroxytoluene (BHT) was then added to samples in a final concentration of 0.05 % to avoid further lipid peroxidation during the assay (Pikul, Leszczynski and Kummerow 1983). Following the manufacturer's protocol for hydrophilic samples, 100 µl of plasma, or standard, was incubated

with 100 µl of sodium dodecyl sulphate (SDS) lysis solution for 5 minutes. 250 µl of TBA reagent (pH adjusted to 3.5) was then added and incubated at 95ºC for 60 min. Samples were centrifuged for 15 min at 3000 g, and the supernatant (300 µl) was re-suspended in 300 µl of N-butanol. This was vortexed for 2 min, followed by centrifugation at 30,000 g for 5 min. The butanol fractions were transferred to a 96 well plate and absorbance was measured at 532 nm. Concentrations of MDA were then calculated by comparisons to a standard curve.

RBC ½ -life: Red blood cell killing assay

We used the a red blood cell (RBC) killing assay, to assess the capacity of RBCs to resist lysis in the presence of a strong in vitro oxidant (see Bize et al. 2008). 135 µl of 150 mM AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride (Sigma-Aldrich, Dorset, UK) was added to 90 µl of diluted RBC. Absorbance was measured spectrophotometerically at 450 nm, every 2.5 min for 3 h. The plate was maintained at 37 ºC for the entirety of the reaction, and the machine was programmed to shake the plate before every measurement to avoid RBC sedimentation. RBC ½ -life was calculated by plotting absorbance values against time, and these data were smoothed using a quadratic curve (Fox and Weisberg 2010). Half-life was calculated as the time for the initial absorbance to halve. Mean values were calculated from sample duplicates. Assays were undertaken within 48 h of blood sample collection.

Table S1 Summary of the first two principal components for the seasonal weather analysis.

Variable	PC1	PC2
Eigenvalue	1.972	1.016
% of variance explained	65.730	33.870
Cumulative % of variance explained	65.730	99.600
Minimum temperature	0.703	0.135
Maximum temperature	0.709	-0.059
Rainfall	-0.054	0.989

87 Table S2 Summary of the first two principal components of winter weather data for carry-over-
88 effect analyses.

Variable	PC1	PC2
Eigenvalue	3.25	0.75
% of variance explained	81.24	18.76
Cumulative % of variance explained	81.24	100
Days of frost	0.549	-0.184
Minimum temperature	-0.527	0.364
Maximum temperature	-0.556	0.025
Rainfall	-0.335	-0.913

89

90

Appendix 2

Details on the Principal Component Analysis (PCA) of weather data, and assessment of autocorrelation

In this appendix we first provide details on the PCA used to generate the predictive weather variables used in our models as a means of accounting for the collinearity in these data. We then provide details on the assessment of autocorrelation in the fixed effects in these models.

Principal Component Analysis (PCA) of weather data

The mean seasonal weather data used in our analyses were subject to collinearity. To account for this we used principal component analyses (PCA), conducted with scaling, to transform these data into linearly uncorrelated variables. To do this we applied the `prcomp()` function from the `R` environment to our data on minimum temperature; maximum temperature; and rainfall, with the argument `scale = TRUE`. The resulting factor loadings for temperature were the most influential contributors to the PC1 axis (see Table A2.1). Loadings for temperature variables were positive, thus higher values of PC1 correspond to higher temperatures. PC2 was dominated by the rainfall data, where this had a positive loading, such that higher values correspond to wetter conditions. These components are henceforth referred to as PCtemp and PCrain in the main text. These two components were retained as the linearly uncorrelated predictive weather variables in our models.

Table A2.1: Summary of the first two principal components of a PCA on seasonal weather data in Wytham Woods, UK, over the study period.

Variable	**PC1**	**PC2**
Eigenvalue	1.972	1.016
% of variance explained	65.73	33.87
Cumulative % of variance explained	65.73	99.6
Minimum temperature	0.703	0.135
Maximum temperature	0.709	-0.059
Rainfall	-0.054	0.989

For our analyses on cary over effects (COE) of winter weather on spring biomarkers of oxidative stress/damage, we conducted a similar PCA, but restricted this to winter weather data. Here, these data also included the variable 'days of frost'. PC1 factor loadings (PCtemp) included maximum temperature, minimum temperature and number of days of frost over the winter, such that higher values of PC1 correspond to lower temperatures and more frost (Table A2.2). PC2 (PCrain) had a negative rainfall loading where higher values correspond to drier weather.

Table A2.2: Summary of the first two principal components of a PCA on winter weather data in Wytham Woods, UK, over the study period.

Variable	**PC1**	**PC2**
Eigenvalue	3.25	0.75
% of variance explained	81.24	18.76
Cumulative % of variance explained	81.24	100
Days of frost	0.549	-0.184
Minimum temperature	-0.527	0.364
Maximum temperature	-0.556	0.025
Rainfall	-0.335	-0.913

Assessment of autocorrelation

In addition to issues of collinearity, the fixed effects used in this study (i.e., age class; body condition; and minimum/maximum temperature; and rainfall) are variables that are subject to temporal autocorrelation. If sampled finely enough, any significant autocorrelation in these data would violate the assumption of independence of the linear mixed-effects models used in our analyses. To test for this we quantified autocorrelation functions (ACFs) for time series of the means and variances of these data and assessed whether there was any significant autocorrelation. We did this by first quantifying the means and variances of each of these parameters at each time step using the `mean()` and `var()` functions in the `R` environment. We note that because we used mean seasonal weather metrics, there was no variance in these data, precluding analysis. Using the `ts()` function we then converted each of these datasets into a time series and then used the `acf()` function to quantify the ACF of each time series (see Figures A2.1 and A2.2). Finally, we assessed each ACF for significant autocorrelation. Significance was determined by autocorrelation that exceeded $\pm 2/\sqrt{(T)}$ where T is the length of the time series (here 8 seasons long).

Notably, because of the coarse temporal scale at which these data were measured, there was no significant autocorrelation in any of the fixed effects used in our analyses.

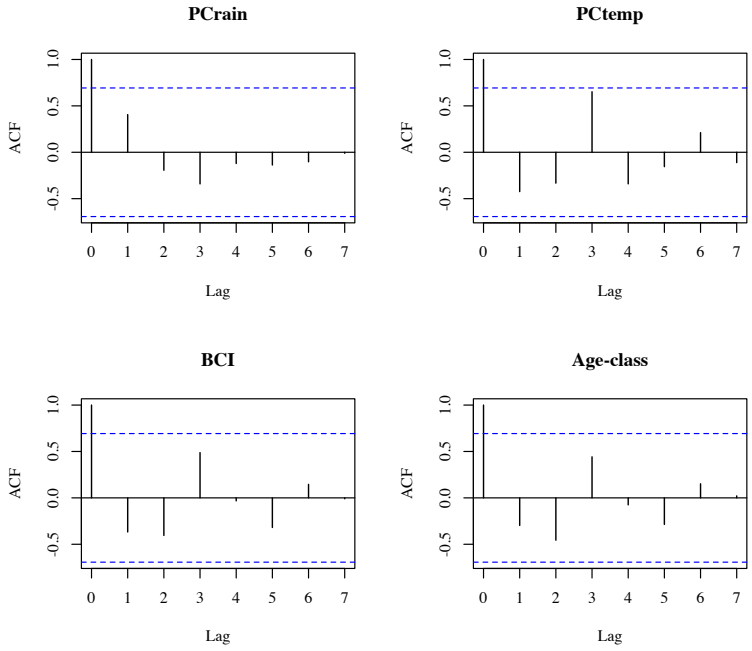


Figure A2.1: Time lagged autocorrelation in the means of the fixed effects used in our linear models. In all panels the blue dashed line depicts the significance threshold. Notably, there was no significant autocorrelation in any of these data.

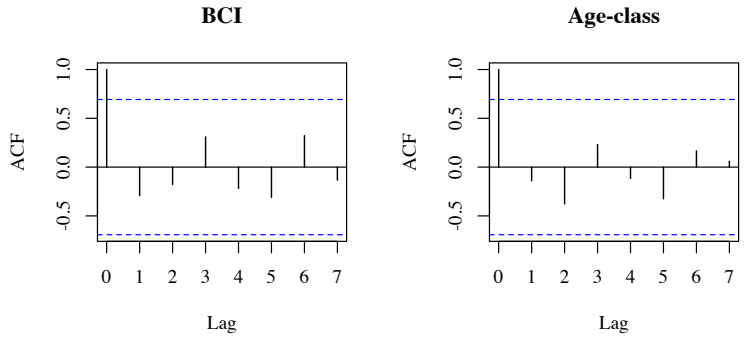


Figure A2.2: Time lagged autocorrelation in the variance of the fixed effects used in our linear models. In all panels the blue dashed line depicts the significance threshold. Notably, there was no significant autocorrelation in any of these data.

Re: PBZ-17099R1

Reviewer 1 Comments

You have done a great work with the revision. Some sections in the results need further attention and I hope that my comments below will be helpful in the revised MS.

Thanks once more for your careful attention to our manuscript, and especially for noticing that our column headers in Tables 4 and 5 had become changed to white, and were thus invisible. Throughout we have implemented your recommendation that we are dealing with predictions, rather than hypotheses.

We feel our manuscript is much improved, thanks to your input.

Abstract

The take home message is improved compared to the first submission but it is still too general.

Unfortunately, to summarise one biomarker more specifically rather imposes that we should do so for all, and this approach instantly takes us over word limits. There is rather a lot to squeeze into the Abstract (age, sex, weather, biomarker effect, COE, etc) and so it does inevitable have to skim the discoveries made rather superficially, given that we also have to provide a conceptual framework for our work. We hope you think the Abstract is adequate.

L68 suggest to change to energetics needs instead of caloric needs

Done

L80 -96, I think that these are all predictions and not hypotheses because you indicate a direction for the response.

OK changed to 'we predict'.

L182 should be "in following the spring" in the following spring?

I think there is perhaps a misunderstanding here. We do indeed mean in the following (ie subsequent) spring that follows after the winter – ie the carry over effect of the winter is observed in the 'following spring'. Nothing here 'follows the spring'. I hope this is clear.

L 186 Is the model weight is the same as the Akaike weight (w) for you calculated using the R package MuMIn (v. 1.15.6; Barton 2016)?

Correct. For complete clarity we have added: "…Akaike (or model) weight (w) for each model…"

L 249-255: The information is descriptive. Where it is shown (no reference in the text to table or figure). Where these changes significant?
Antioxidant enzymes - peroxidase (PER) - no reference in the text to table or figure and indicate if the trend in PER (L259) was significant or not.

The model was significant, as described under the sub-heading 'Antioxidant Capacity – AOX', with statistical values given in Table 4, as stated in the text.

Apologies for oversight on PER, we have added (Table 4) and reference to (Figure 1) for each biomarker [all stats outputs are shown in Table 4].

L262- 267: Where it is shown (Table 4?), please refer to the stat. Where the high and low inter-individual variation is shown and how do you define high and low?

The Stat is given in Table 4. Inter-individual variation are depicted by the ± standard deviations provided in Table 3 (now specified in the text).

Note, we do not define inter-individual variation in absolute terms, and therefore not as either 'high' or 'low'. Rather we examine inter-individual variation in relative terms, talking about circumstances where it is 'higher' or 'lower'.

L278-282: Where it is shown (Table 4?), please refer to the stat.

Yes – we were remiss in not repeating that each model statistic is presented in Table 4. We have added this throughout, as necessary.

L284-290: Where it is shown?

Table 4 – added.

L291-292 Delete

Respectfully, we would like to retain the RBC ½-life results for adults, even though there were no clear patterns, for consistency of reporting relative to other biomarker sections.

L305-313, Pool the sections to one section and refer to the relevant table or figure.

Respectfully, we would like to retain this sub-heading format for consistency and easy comparison to the preceding section.

We had mentioned that Figure 2 and Table 5 pertained to these COE results under first subheading (AOX), and hoped that it would be clear that the same Figure and Table depicted results from other biomarkers (so as not to burden the text). Acknowledging your concern about short segments here, rather than add (Figure 2; Table 5) to each biomarker, instead we have specified that all biomarker statistics refer to Figure 2 and Table 5 at the start of this section (end of "Analysis of COE…" section).

L348 In support of our third hypothesis, carry-over effects: add (COE)

Actually, there seems little point in specifying an acronym and then not using it, and so we considered just using COE here; however, because this is the first mention in the Discussion we thought it best to write it out in full, but that repeating the already-defined acronym could be redundant. Nevertheless, for total clarity, we have added it as you suggest.

ALSO- with respect to your recommendation in the Into that we should phrase our 'hypotheses' as 'predictions', we have also changed phrasing to 'predictions' in the Discussion, for consistency.

L383 inter-individual variation, see my previous comment.

See accompanying response. Depicted by the Standard Deviation in Table 3, but we think this is now clear from revision to the Results section, without adding table cross-references to the Discussion.

L398, this is your sixth hypothesis: "cub may experience more severe OS/OD effects in years with more extreme weather"

Correct, our 6th 'prediction', as stated.

Notice that you may mix between hypothesis and predictions in the discussion. In line 368 you mention hypothesis "third hypothesis" and in line 363 you mention prediction. See also my earlier comment.

Noted, and we accept that indeed strictly we do phrase these as predictions, and we have amended our phrasing accordingly.

Table 4 and 5: All measured biomarkers should appear in the top line (first line of the table). Right now, they are missing from both tables.

Well spotted – thank you. For reasons I don't understanding, these headings had changed to white font on a white background – now restored to default black.

Reviewer 3 Comments

My major concerns have been carefully addressed by the authors. The manuscript has been greatly improved.

We are sincerely grateful for your input and for your approval of our revision, which has benefited enormously from your advice.