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An example of life history antecedence in the European badger (Meles meles): rapid development of juvenile antioxidant capacity, from plasma vitamin E analogue

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An example of life history antecedence in the European badger (*Meles meles*): rapid development of juvenile antioxidant capacity, from plasma vitamin E analogue

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The way organisms cope with oxidative stress, to quench potentially toxic oxygen free radicals while maintaining oxygen species functionality, is crucial in shaping life-history traits. Antioxidant capacity plays an important role in this process. Here we use multi-model inference procedures to examine the age-class-dependent non-enzymatic antioxidant capacity of the European badger (*Meles meles*), testing non-enzymatic plasma antioxidant capacity and expressing the results as vitamin E analogue (VEA) equivalent units. Despite immaturity (ca 16 weeks old), cubs exhibited plasma antioxidant capacity equivalent to those of prime-age adults (1–5 years old). Compared to individuals aged 6 years and over, cubs exhibited significantly higher non-enzymatic plasma antioxidant capacity. There was no association between plasma antioxidant capacity and sex or other physiological variables, such as body condition or presence of wounding. We consider the adaptive significance of this life-history strategy with respect to pandemic endoparasitoses that affect badger cub survival, as well as a possible link to the free radical theory of ageing. Our finding on the interaction between age and antioxidant defences (linked to immune function) has implications for the controversy surrounding effective badger bovine tuberculosis management strategy in the UK.

KEY WORDS: antioxidant, innate immunity, ontogeny, oxidative stress, ROS, oxygen free radical.

INTRODUCTION

Organisms experience 'stress' due to exposure to physiological challenges, to which they have evolved a suite of endocrinological responses, for mitigating otherwise

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detrimental consequences (Cannon 1929; Selye 1936, 1956; de Kloet et al. 2005). One manifestation of physiological stress can arise from heightened production of reactive oxygen species (ROS) resulting from accelerated metabolic processes; termed "oxidative stress" (OS; Sies 1997) when ROS are not sufficiently quenched by protective mechanisms, where damage to DNA, proteins, lipids and polysaccharides can occur, leading to cell damage or death (Halliwell & Gutteridge 2007; Costantini et al. 2010). Fundamentally, OS results in a disruption of redox signalling (i.e. when electronically active molecules act as biological messengers) and control (Jones 2006). This phenomenon is thought to be responsible for the sequential deterioration in ROS mitigation that accompanies advancing age and the associated progressive increases in the chances of morbidity and mortality (Harman 1956; Beckman & Ames 1998; Packer & Cadenas 1999).

Antioxidants provide protective mechanisms in tissues and plasma that contribute to quenching, or inhibiting, ROS production (Packer 1999; Chen et al. 2004; Pamplona & Costantini 2011). Antioxidants comprise integrated systems of exogenous [e.g. tocophenols/tocotrienols (vitamin E; Packer 1991), ascorbic acid (vitamin C; Padayatty et al. 2003), retinol/retinal/carotenoids (vitamin A; Olson 1993; Galano et al. 2010)] and endogenous [e.g. uric acid (Becker et al. 1989) or enzymes Glutathione peroxidase, Catalase and Superoxide dismutase (Chaudiere & Ferrari-Iliou 1999)] molecules and enzymes.

While antioxidants are important for the termination of ROS production from immune reaction cascades, ROS are also essential for redox signalling (Thannickal & Fanburg 2000; Hancock et al. 2001; Powers & Jackson 2008) and immune function (Bogdan et al. 2000; Fang 2004). ROS therefore require subtle mediation to preserve the balance between vital cellular functions and toxicity (Halliwell & Gutteridge 2007); with antioxidant systems evolved to maintain optimal oxidant levels, rather than to resolve them completely (Rhee 2006). This is a particular challenge for free-living wild animals, where repeated acute and/or chronic stress has been seen to reduce reproductive fitness and survival rate (Moberg 1996; Bateson & Bradshaw 1997; Laugero & Moberg 2000; Montes et al. 2003, 2004).

While there have been a number of ecological studies on this subject focusing on bird populations (e.g. Costantini et al. 2007; Bize et al. 2008; Devevey et al. 2010), the investigation of mammals has been slower to develop (but see Nussey et al. 2009; Bergeron et al. 2011; Montes et al. 2011; Vazquez-Medina et al. 2011a; Costantini et al. 2012).

Antioxidant systems and ROS production vary ontogenically from nascence to senescence (Packer & Cadenas 1999; Fontagne et al. 2008; Monaghan et al. 2009; Metcalfe & Alonso-Alvarez 2010), and are central to the understanding of life-history evolution (Costantini 2008; Monaghan et al. 2009). Repeated OS leads to cellular senescence, with severe consequences for the organism, characterised by the declining ability to respond to stress, increased homeostatic imbalance, and increased risk of gerontological diseases (Cadenas & Davies 2000; Martin & Grotewiel 2006; Monaghan et al. 2008; Ricklefs 2008). Older individuals of many taxa exhibit compromised antioxidant systems, or higher levels of OS (Martin & Grotewiel 2006; Richter & von Zglinicki 2007; Monaghan et al. 2008; Ricklefs 2008; Devevey et al. 2010; Isaksson et al. 2011b; reviewed in Metcalfe & Alonso-Alvarez 2010). To similar effect, antioxidant systems are often slow to develop during early maturation (e.g. Gitto et al. 2009; Vazquez-Medina et al. 2011b) – thought to be due to the high cost of investing resources and nutrients into such physiological traits in the face of high energy demands to achieve somatic growth (Stevens et al. 2000) – placing young

individuals at a greater risk of oxidative damage (reviewed in METCALFE & ALONSO-ALVAREZ 2010). As a consequence, antioxidant system development is subject to a life-history trade-off, particularly where food resources are limited. Indeed, different types of antioxidant defences mature at different rates in different tissues (see GAAL et al. 1995).

Here we investigate non-enzymatic plasma antioxidant capacity, as vitamin E analogue measurements, in the European badger (*Meles meles* L.), which provides an ideal wild mammal model. Badgers have an extensive geographical distribution (JOHNSON et al. 2002), are relatively tractable and trap compliant, and are sufficiently large to permit non-detrimental sampling protocols, even in immature individuals (DOMINGO-ROURA et al. 2001; MACDONALD et al. 2002).

Confinement and repeat sampling of wild animals is, however, in itself a source of stress, with the potential to confound observations, necessitating the measurement of OS in as naturalistic and immediate a way as possible. In this study, we therefore focus on a single parameter (CATONI et al. 2008; COHEN et al. 2009; MONTES et al. 2011), within a suite of OS components (ROS production, repair mechanisms and antioxidants; HALLIWELL & GUTTERIDGE 2007) by measuring circulating (non-enzymatic) antioxidant capacity at a single point in time (HORAK et al. 2007; CATONI et al. 2008). While this instantaneous approach is typical for wildlife studies (e.g. COSTANTINI et al. 2007; COSTANTINI 2008; BIZE et al. 2008; CATONI et al. 2008; ISAKSSON et al. 2011b), we acknowledge that single measurements at the time of capture do not account for antioxidant regeneration, or for any additional induced capacity (YAM et al. 1978; HALLIWELL 1999). This approach does, however, have the advantage of minimising restraint times, and avoids the need for repeat sampling and/or extended captivity. Antioxidants are only one side of the OS equation; therefore while this approach does not measure *actual* OS levels, it does provide a method to assess how well individuals could respond to an oxidative challenge (COSTANTINI & VERHULST 2009; COSTANTINI 2011). This is especially pertinent to the types of acute stressors animals often face in the wild (LEEUWENBURGH & HEINECKE 2001; CATONI et al. 2008).

This study investigates four main objectives:

(1) Trait development: We examine the interaction between badger ontogenescence (see LEVITIS 2011) and non-enzymatic plasma antioxidant capacity, focusing primarily on the physiological development of this trait in juveniles, where previous work has suggested antecedence for this trait in badgers (MONTES et al. 2011). As cubs, badgers suffer from pandemic endoparasitic infection (predominantly coccidia *Eimeria melis*), where severely infected individuals exhibit impaired growth (NEWMAN et al. 2001) and increased mortality rates (ANWAR et al. 2000). Without the adaptive (acquired) immune mechanisms necessary to fight coccidial challenges during early life, badger cubs rely primarily on their innate immunity (ANWAR et al. 2000; NEWMAN et al. 2001; DUGDALE et al. 2011). There is a major evolutionary cost within populations of death before reproductive age, compounded by the probability of death from external threats. "Antagonistic pleiotropy" (WILLIAMS 1957) proposes that natural selection will favour genes that have a positive effect on fitness (reproduction) early in life, rather than those that act on the preservation of non-germ cells, which are considered the "disposable soma" (ROSE 1991; ROSE & FINCH 1994; KIRKWOOD & AUSTAD 2000). Selective pressure to

compete effectively at an early age may guarantee a certain degree of OS and work against the conservation of the soma in the longer term.

We thus posit a particular selective advantage for badger cubs to be precocious in the development of their antioxidant physiology, in order to respond to an increase in ROS production resulting from the effects of infection, which also causes a failure to absorb dietary antioxidants effectively.

(2) Senescence: Badgers can live to more than 14 years of age, although only ca 10% live past 8 years old (MACDONALD & NEWMAN 2002; DUGDALE et al. 2011). Additionally, senescence is known to affect breeding status and reproductive capacity in badgers (BUESCHING et al. 2009; DUGDALE et al. 2011) with reproductive output (which we use to define 'prime-age' badgers) peaking at 5 years old for males and 3 years old for females, followed by a decrease with age (BUESCHING et al. 2009; DUGDALE et al. 2011). Informed by this evidence, as well as the free radical theory of ageing, i.e. the tendency for antioxidant levels to decrease, and ROS levels to increase, with senility (BECKMAN & AMES 1998), we also examine whether antioxidant levels are lower in older badgers (see MONTES et al. 2011).

(3) Tissue repair: Antioxidants are important in healing mechanisms (SONEJA et al. 2005; SEN & ROY 2008); levels of most antioxidants decline following wounding in humans (SHUKLA et al. 1997). We therefore also consider whether badger antioxidant capacity is affected by wounding. Wild badgers often injure one another in agonistic interactions and consequently suffer from extensive wounds in varying degrees of severity and freshness (MACDONALD et al. 2004a).

(4) Dietary stress: Badgers undergo substantial changes in body-fat levels through the year (DOMINGO-ROURA et al. 2001), where emaciation is also known to compromise their antioxidant capacity (MONTES et al. 2011) with many circulating antioxidants being micronutrients (e.g. vitamins C and E), or requiring micronutrients to function (MATES et al. 1999; EVANS & HALLIWELL 2001). We therefore undertook this study during a particularly harsh spring season, with a limited food supply, which substantially alleviated the potential for confounding body condition effects on antioxidant capacity.

These findings on systems counteracting physiological stress in badgers are of particular relevance. Badger culling strategies, aimed at the control of the transmission of bovine tuberculosis (bTB) from badgers to cattle in the UK (DEFRA 2011), are known to lead to counterproductive outcomes, perhaps including immune-compromise, due to the 'perturbation effect' (e.g. MACDONALD et al. 2006; WOODROFFE et al. 2008, 2009; RIORDAN et al. 2011) with surviving individuals exhibiting sociological disruption.

METHODS

Study site

This study was conducted at Wytham Woods, Oxford, United Kingdom (GPS ref: 51:46:26N; 1:19:19W; mean annual temperature of 9.6 °C, mean annual precipitation 634.1 mm, Oxford Radcliffe Meteorological station, School of Geography), a semi-natural mixed woodland covering 424 ha. For full site details see (MACDONALD & NEWMAN 2002; MACDONALD et al. 2004b; SAVILL et al. 2010).

Trapping & sampling

Badgers were sampled as part of an ongoing study of their socio-ecology at Wytham Woods, Oxford, UK (see MACDONALD et al. 2009; BUESCHING et al. 2010), where this geographically discrete population has been subject to continuous study since the 1970s (KRUUK 1978). Since 1987, the protocol has been to trap this badger population 3 to 4 times per year (MACDONALD & NEWMAN 2002; MACDONALD et al. 2002, 2009) in steel mesh cage traps (85 × 37 × 38 cm), baited with peanuts. Upon capture, badgers are sedated with an intramuscular injection of ketamine hydrochloride at 20 mg kg^{-1} (MACKINTOSH et al. 1976; MCLAREN et al. 2005; THORNTON et al. 2005) in order to allow biometric parameters to be recorded, including sex, weight, body condition (subcutaneous fat score: 1 = thin; 5 = fat; SPEEDY & CLARK 1981), and wounds and scars (MACDONALD et al. 2004a) inter alia.Upon first capture (69% as cubs; MACDONALD et al. 2009), all individuals are given a unique and permanent tattoo in the inguinal region (CHEESEMAN & HARRIS 1982), to ascertain subsequent recapture history. This affords the opportunity of working with marked individuals that are trapped and handled frequently, and whose age is known. Furthermore, we benefit from complementary studies on this population, examining badger population dynamics (MACDONALD et al. 2002, 2009, 2010), parasitology (ANWAR et al. 2000; NEWMAN et al. 2001), endocrinology (DOMINGO-ROURA et al. 2001; BUESCHING et al. 2009), juvenile development and senescence (FELL et al. 2006; BUESCHING et al. 2009; DUGDALE et al. 2011), and stress (MONTES et al. 2003, 2004, 2011).

In order to assess non-enzymatic plasma antioxidant capacity, blood was taken by jugular venepuncture and collected in lithium heparin anticoagulant (BD Vacutainer® systems, Plymouth, UK). Within 15 min of collection, samples were centrifuged for 10 min at 1000 *g*, from which plasma was collected by pipette and stored at – 20 °C (COHEN et al. 2007) in 1-mL aliquots. All samples were taken between 08:30 and 11:00 in order to control for circadian variations (BENOT et al. 1998).

Eighty-four plasma samples were collected between 22 May and 4 June 2011, a period when both cubs and adults were lean and food-stressed, often bearing wounds, due to a paucity of natural food in the environment during that especially dry spring.

Antioxidant capacity measurement protocol

Non-enzymatic plasma antioxidant capacity (following COHEN et al. 2007) was established by comparison to water-soluble vitamin E analogue [VEA; Trolox™ (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Hoffman-LaRoche; DAVIES et al. 1988], as an antioxidant standard. A commercial kit (CS0790, Sigma-Aldrich, St. Louis, USA) was used where the formation of ferryl myoglobin radicals ($^{\bullet}$X-[Fe^{IV}=O]) – from metmyoglobin (XH-Fe^{III}) – and hydrogen peroxide (H_2O_2) oxidise ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)], triggers the production of an $ABTS^{\bullet+}$ cation, a soluble green chromogen which was measured spectrophotometrically at 405 nm (MILLER et al. 1993) using a 96-well plate spectrophotometer (FLUOstar OMEGA 415-0435, BMG LABTECH GmbH, Germany). Here, plasma antioxidants (mainly glutathione, uric acid and vitamins E, C and A) suppress radical cation production in a concentration-dependent manner: Higher antioxidants levels in a sample lead to greater suppression of $ABTS^{\bullet+}$ production, and thus a lesser intensity of green in the solution.

The assay was performed with all reagents at half of the manufacturer's specified volume, to fit well-plates. Plasma samples were diluted by half in order for readings to fall within the range of the standard curve, by mixing 2.5 μL plasma with 2.5 μL buffer. Means were calculated from sample duplicates. Blanks were also run for each sample, containing 135 μL ddH_20, 2.5 μL buffer and 2.5 μL experimental sample. Plasma samples with high turbidity (probably due to lipid content DOMINGO-ROURA et al. 2001), for which the absorbance reading for the blank was > 0.2 (n = 18; range = 0.209–0.497), were excluded from the analysis, as these samples gave inconsistent replicates of antioxidant measurements. For the remaining samples (n = 66), the absorbance value of the blank at 405 nm (mean = 0.104, SD = 0.036) was subtracted from the antioxidant

experimental absorbance value at 405 nm, thus correcting for individual sample turbidity. Results were calculated using a Trolox standard curve and the antioxidant capacity was expressed as mM VEA equivalent (Trolox).

Statistical analysis

All statistical analyses were performed in open source statistical program R 2.13.1 (R Development Core Team 2011) using the 'MuMIn' (Bartòn 2011), 'graphics', and 'stats' packages.

Using a multi-model inference procedure (to avoid multiple testing on the same dataset; Burnham & Anderson 2002, 2004; Whittingham et al. 2006; Anderson 2008), each model's capacity to predict non-enzymatic plasma antioxidant capacity was compared.

- Age, as a categorical variable; where age-classified models have been applied successfully to various other aspects of badger population dynamics and endocrinology (see Buesching et al. 2009; Macdonald et al. 2009, 2010): Age class – *Ac*: cubs ($n = 12$), young adults (1–5 years old, $n = 39$; prime reproductive age; Buesching et al. 2009; Macdonald et al. 2009; Dugdale et al. 2011) and older adults (6 years +, $n = 15$);
- Body condition [*Bc*: 1 = thin; 5 = fat, *Bc* of 1 ($n = 18$), *Bc* of 2 ($n = 35$), *Bc* of 3 ($n = 13$); Speedy & Clark 1981];
- Sex [*Sx*: females ($n = 35$), males ($n = 31$)];
- Weight-age residuals [W_r ($n = 66$): substituting for weight, correcting for the correlation between weight and age to avoid artificially inflating confidence intervals for the correlated parameters; Freckleton 2011)];
- Fresh wounds [Fr_w: present ($n = 5$), absent ($n = 61$)] and healed wounds [Hld_w: present ($n = 9$), absent ($n = 57$)].

Forty-two general linear models were constructed with non-enzymatic plasma antioxidant capacity as the response variable, with a maximum of three explanatory variables [i.e. one model with no predictor (intercept only), six models with one, 15 models with two and 20 models with three explanatory predictors], which defined a balanced set of candidate models that represented all predictors equally. Small sample size prevented the investigation of interactions between variables.

For each model the *AICc* value was derived (*AIC* corrected for small sample size in relation to the number of parameters; Hurvich & Tsai 1989), and used to rank models, where lower values indicate better support (Anderson 2008), along with the Akaike weight (*Wi*) for each model (Buckland et al. 1997; Anderson 2008). Different estimation of the parameter (θ) was obtained from each model, linking predictor variables (or each level of a predictor, in the case of categorical variables) to non-enzymatic plasma antioxidant capacity. This enabled the derivation of relative influences for every predictor variable as the sum of the Akaike weight of models containing each particular variable (Burnham & Anderson 2004; Anderson 2008).

Model averaging was then applied to determine a weighted average of the predictions ($\hat{\theta}$) for each of the R models, and their associated 95% confidence intervals, accounting for uncertainty in model selection (Buckland et al. 1997; Burnham & Anderson 2002, 2004; Anderson 2008). The predicted values (θ_i) from each model are dependent both on the magnitude of parameter estimates and on the relative weight (W_i) of each model including that parameter (adapted from Anderson 2008):

$$\bar{\hat{\theta}} = \sum_{\hat{I}=1}^{R} W_i \hat{\theta}_i \tag{1}$$

Significant predictors for non-enzymatic plasma antioxidant capacity were those for which 95% confidence intervals for the weighted average parameter estimates did not include 0.

In order to retain influential information, occurring due to animals showing extreme responses, statistical outliers were included. This is a particularly pertinent approach for studies on wild animals, where factors such as disease status, health, nutrition, and body condition can vary over a wide range (MONTES et al. 2011).

RESULTS

Age effects

Badger non-enzymatic plasma antioxidant capacity differed between age classes (*Ac*; Fig. 1), with cubs having the highest plasma antioxidant capacity (mean = 164.37, SD = 19.99), prime-age adults (1–5 years) having intermediate capacity (mean = 152.12, SD = 24.95), and older individuals (6 + years) having the lowest non-enzymatic plasma antioxidant capacity (mean = 133.08, SD = 30.97).

Of 42 models compared and ranked according to their statistical support (see Table 1 for the top seven models), the model including only age class (*Ac*) was the most supported, followed by a second rank of similarly supported models containing the other variables (*Bc*, W_r, Fr_w, *Sx*, Hld_w) in addition to *Ac*. Age class was thus the most influential variable predicting non-enzymatic plasma antioxidant capacity (Table 2). Based on 95% confidence intervals for the parameter estimates, cubs exhibited antioxidant capacities equivalent to, indeed marginally in excess of, those measured in 'prime-age adults' (but non-significantly, i.e. confidence interval overlapping 0 marginally, Table 2). Cubs' levels exceeded those of older individuals with statistical significance (i.e. confidence intervals did not include 0; Table 2).

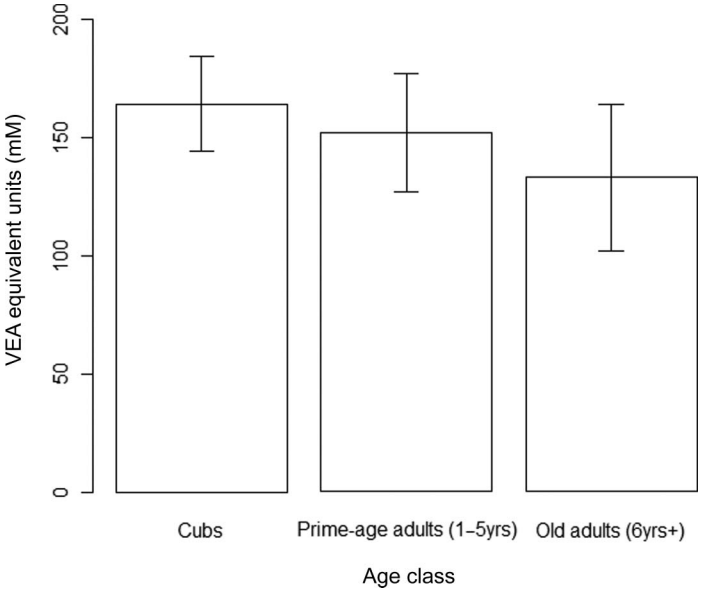

Fig. 1. — Non-enzymatic plasma antioxidant capacity in the European badger expressed as vitamin E analogue (VEA) equivalent units (mM) for cubs ($n = 12$), young adults ($n = 39$) and old adults ($n = 15$). Plots show the mean and standard deviation for each category.

Table 1.

Statistical summary of the seven most supported models linking plasma antioxidant capacity to predictor variables. Based on Akaike weight (W_i) the most supported model (lowest AICc) ranks higher, followed by several similarly ranked models. θ values represent the coefficient linking non-enzymatic plasma antioxidant capacity to each predictor index (or level of predictor in the case of categorical variables) with standard errors (SE) in parenthesis. *Ac*= age class (cubs, 1–5 years, 6 years +), *Bc*= body condition (1 = thin; 5 = fat), W_r = weight-age residuals, Fr_w = Fresh wounds (present ☑/absent ☒), *Sx*= Sex, Hld_w = healed wounds (present ☑/absent ☒).

Model	AIC_c	ΔAIC_c	W_i	Intercept (SE)	$_{Ac}$ (SE)	$_{Bc}$ (SE)	$_{W_r}$ (SE)	$_{Fr_w}$ (SE)	$_{Sx}$ (SE)	$_{Hld_w}$ (SE)
Ac	621.6	0	0.218	164.37 (7.41)	Cub 1–5 y –12.25 (8.47) 6 y + –31.29 (9.94)					
Ac,Bc	622.9	1.7	0.093	154.35 (9.88)	Cub 1–5 y –9.53 (8.60) 6 y + –29.28 (9.96)	1 2 12.65 (7.54) 3 6.36 (9.40)				
Ac,W_r	623.0	1.8	0.091	164.37 (7.43)	Cub 1–5 y –12.25 (8.50) 6 y + –31.29 (9.97)		–2.26 (3.01)			
Ac,Fr_w	623.3	2.1	0.078	164.37 (7.45)	Cub 1–5 y –12.91 (8.61) 6 y + –3172 (10.03)			☒ ☑ 6.42 (12.13)		
Ac,Sx	623.5	2.3	0.069	164.92 (7.95)	Cub 1–5 y –12.05 (8.59) 6 y + –31.48 (10.06)				♀ ♂ –1.32 (6.58)	
Ac,Hld_w	623.5	2.3	0.068	164.37 (7.47)	Cub 1–5 y –12.34 (8.66) 6 y + –31.41 (10.19)					☒ ☑ 0.60 (9.46)
Ac,Bc,W_r	623.7	2.5	0.064	150.53 (10.28)	Cub 1–5 y –8.48 (8.60) 6 y + –28.52 (9.92)	1 2 15.64 (7.86) 3 13.63 (10.95)	–4.43 (3.47)			

Table 2.

Model averaging for the parameters linking non-enzymatic plasma antioxidant capacity to the predictor parameters. Akaike weights were used to give relative importance of each parameter. Additionally present are the model averaged estimated values of each parameter with standard error (SE), and associated 95% confidence intervals (95 CI). *Ac*= age class (cubs, 1–5 years, 6 years +), *Bc*= body condition (1 = thin; 5 = fat), W_r = weight-age residuals, Fr_w = Fresh wounds (present ☑/absent ☒), *Sx*= Sex, Hld_w = healed wounds (present ☑ /absent ☒).

	Importance	Category level	θ (SE)	Lower 95 CI	Upper 95 CI
Intercept	–	–	160.00 (10.50)	139.00	181.00
Ac	0.92	Cub			
		1–5 y	−10.70 (8.91)	−28.40	7.11
		6 y +	−28.40 (12.70)	−53.50	−3.31
Bc	0.28	*Bc* 1			
		Bc 2	3.86 (7.47)	−10.90	18.60
		Bc 3	2.47 (6.86)	−11.10	16.10
W_r	0.27	–	−0.80 (2.19)	−5.14	3.55
Fr_w	0.21	☒			
		☑	1.43 (6.31)	−11.10	14.00
Sx	0.19	♀			
		♂	−0.09 (2.97)	−6.03	5.85
Hld_w	0.18	☒			
		☑	0.12 (4.15)	−8.18	8.42

Effects of wounds

The presence of fresh or healed wounds was not a significant predictor of non-enzymatic plasma antioxidant capacity (Table 2): VEA equivalent units were not significantly different whether wounds were present (Fresh: mean = 154.12, SD = 36.42;

healed: mean = 146.27, SD = 22.45) or absent (Fresh: mean = 149.68, SD = 26.78; healed: mean = 150.61, SD = 28.11).

Effects of body condition

As our primary focus was to look at the effects of maturation on antioxidant capacity, we were fortunate to be able to time our work to coincide with not only a season when animals exhibit a limited spread of body-condition, but also with a year in which all individuals were especially thin (i.e. BC $\leq$ 3; SPEEDY & CLARK 1981), and thus we observed no significant body condition differences between individuals (Table 2). Indicative trends were apparent; as per MONTES et al. (2011) emaciated badgers with body condition 1 had the lowest antioxidant capacity (mean = 140.96, SD = 22.47).

Weight-age residuals did not predict antioxidant capacity significantly (Table 2).

Effects of sex

Sex was not a significant predictor of antioxidant capacity, with females (mean = 149.17, SD = 28.12) and males (mean = 150.98, SD = 26.76) having similar antioxidant capacity (Table 2).

Effects of weight-age residuals

Weight-age residuals, calculated to take into account differences in age class, did not significantly predict antioxidant capacity (Table 2).

DISCUSSION

The effect of age class on plasma antioxidant response proved both significant and insightful: Somewhat counter-intuitively, badger cubs exhibited the *highest* non-enzymatic plasma antioxidant capacity. We observed that cubs at ca 16 weeks of age exhibited non-enzymatic plasma antioxidant activity that at least equalled that of prime-age adults (1–5 years), with an indicative, albeit marginally non-significant, trend for cub levels to exceed that of these adults (see MONTES et al. 2011).

Typically in this Wytham Woods badger population, annual cub cohort sizes have a mean of 44.55 $\pm$ 5.37 (range = 16 to 86, years 2000–2010; see MACDONALD & NEWMAN 2002; MACDONALD et al. 2009). In this focal study year, only 12 cubs were trapped, as a consequence of especially dry spring conditions exacerbating early cub mortality (MACDONALD & NEWMAN 2002; MACDONALD et al. 2010), restricting our statistical sample size. That we were still able to observe age-dependent antioxidant interactions – despite pre-trapping mortality of the most vulnerable cubs – underscores the strength of antioxidant development in shaping juvenile badger life-history dynamics.

This finding is contrary to the limited range of observations available from other studies, where the gradual, conservative, development of antioxidant systems is more typical, and similar to the process through which acquired immune function is established. For example, spotted snow skinks (*Niveoscincus ocellatus*; ISAKSSON

et al. 2011b) and greater flamingos (*Phoenicopterus ruber roseus*; DEVEVEY et al. 2010) exhibit *higher* OS among immature individuals (RILEY 1998; BAKER et al. 1999; PALACIOS et al. 2009; SINKORA & BUTLER 2009).

Antioxidant systems are costly to implement, where energy is more typically invested in growth and development (MANGEL & STAMPS 2001; MCDADE 2003), underpinning an evolutionary trade-off (METCALFE & ALONSO-ALVAREZ 2010). That badger cubs do not fit the typical ontogenic pattern indicates intense selection – through differential survival (i.e. higher mortality rate among cubs with low antioxidant capacity) – for the rapid primary development of adult (or higher) levels of plasma antioxidants as a life-history imperative (see also LEVITIS 2011).

Surviving adolescent challenges has been noted to be an especial vulnerability in badger life-history, with implications for their population processes (MACDONALD & NEWMAN 2002; MACDONALD et al. 2009). Due to badger cubs suffering from pandemic and severe juvenile coccidiosis (*E. melis*), we speculate that high-capacity antioxidant defences may help individuals cope with the ROS produced by their innate immune system, as well as contributing to healing tissue damage caused by parasitosis (ANWAR et al. 2000). Moreover, high levels of antioxidants could also provide protection against additional ROS production resulting from increased metabolic rate accompanying juvenile growth (ROLLO 2002).

Variation in the speed of development of innate and acquired immunity is thought to reside in the ontogenic cost of each component (APANIUS 1998). Acquired immunity requires more time to develop than innate immunity and has higher nutritional costs, principally due to the requirement to generate sufficient lymphocyte diversity to recognize a comprehensive scope of prospective threats without them posing a risk to the body itself (KLASING & LESHCHINSKY 1999; KLASING 2004; PALACIOS et al. 2009).

In contrast to their precocious development of antioxidant capacity, cubs commence production of other energetically costly adult traits much later. For example, badger subcaudal gland secretion production, used for scent marking and individual fitness advertisement (BUESCHING et al. 2002b, 2002c) does not begin until at least 16 weeks of age, with cubs producing significantly less secretion volume than adults until they are approximately one year of age (BUESCHING et al. 2002a). Despite the sociological importance of scent marking (BUESCHING et al. 2002b, 2003; FELL et al. 2006), the ontogenic imperative to do so is clearly subject to less intense life-history trait selection than is the priority of antioxidant systems. Cubs deficient in subcaudal secretion (which also contains varying amounts of vitamin E, depending on individual-specific parameters; C.D. BUESCHING, H.V. TINNESAND et al. unpubl. data), can mitigate this deficiency in part through scent-theft (FELL et al. 2006). By contrast, cubs with an immune/antioxidant deficit may simply die of the consequences of parasitic infection – a very intense selection criterion.

Badgers aged 6 years or over exhibited significantly lower plasma antioxidant capacity than did cubs. This is consistent with senescence (WILLIAMS 1957; ZERA & BOTTSFORD 2001) and concordant with the free radical theory of ageing (see HARMAN 1956; BECKMAN & AMES 1998; FINKEL & HOLBROOK 2000). Again, from the limited availability of comparable wildlife studies, resistance to OS has been reported to lower in older individuals in both spotted snow skinks (ISAKSSON et al. 2011b) and greater flamingos (DEVEVEY et al. 2010). In cross-sectional studies, however, it is not possible to deduce definitely whether the variations are simply due to inter-individual heterogeneity rather than decreasing antioxidant capacity over an animal's life span (HOFER & SLIWINSKI 2001; HOFER et al. 2002; see also NUSSEY et al. 2009). The type of degenerative impairments of antioxidant responses that can result from ageing cause

a progressive inability to quench ROS production sufficiently, leading to OS. As ROS were not measured in this study, however, we are unable to infer actual exposure to OS (MONAGHAN et al. 2009). As mitochondria accumulate damage, mitochondrial function is modified, increasing free radical leakage during oxidative respiration, and thus further contributing to oxidative damage (SHIGENAGA et al. 1994; WEI et al. 1998). Germ-line cells and mitochondrial DNA are especially vulnerable to OS (MONAGHAN et al. 2008; METCALFE & ALONSO-ALVAREZ 2010), leading to damage of germ-line cells and age-related reduction in fecundity (MARTIN & GROTEWIEL 2006; ANGELOPOULOU et al. 2009), as well as trans-generational effects on offspring viability (BLOUNT et al. 2001; VELANDO et al. 2008). This consequently has implications for life-history traits that contribute to the understanding of badger population dynamics (MACDONALD & NEWMAN 2002; BUESCHING et al. 2009; MACDONALD et al. 2009; DUGDALE et al. 2011). More research is needed to establish the full effects of OS ecology and age-effects, especially in wild populations, and ideally using several different OS markers to establish the causes and consequences of ROS production and antioxidant degeneration.

In many species, OS levels differ between sexes (VIÑA et al. 2003; ISAKSSON et al. 2011b), due to higher mitochondrial H_2O_2 production in males (VIÑA et al. 2005). It has also been found that injecting zebra finch eggs with testosterone leads to decreased antioxidant capacity in the hatched individuals at age 10 days (TOBLER & SANDELL 2009). Here, we observed similar non-enzymatic plasma antioxidant activity in male and female badgers. This does not, however, equate necessarily with equivalence in OS exposure; both sexes simply exhibit a similar capacity to mount an antioxidant response, where they may be mitigating different levels of ROS production. The senescence rate is higher for male badgers than for females (DUGDALE et al. 2011), and male germ-lines are generally more susceptible to oxidative damage than female germ-lines due to spermatozoid characteristics (VELANDO et al. 2008; DOWLING & SIMMONS 2009). Consequently, differential ability to resolve OS could impact on male fertility.

No interaction between antioxidant capacity and either fresh (MACDONALD et al. 2004a; DELAHAY et al. 2006) or healed wounds from intra-specific aggression was observed (SHUKLA et al. 1997). Extensive wounds can cause badgers chronic health issues, such as secondary infections, sepsis, and even maggot infestation (MACDONALD et al. 2004a). Generally, immune-challenged animals are known to be able to divert resources to maintain antioxidant status, rather than invest them in other functions (COTE et al. 2010). Consequently, the lack of any effect in our analyses may arise because wounded individuals are obligated to invest more resources into maintaining antioxidant levels, at the expense of other functions. In our sample, individuals with the worst wounds, which would be expected to require high levels of antioxidants to compensate for the ROS produced by this immune response, were also severely emaciated (see also DELAHAY et al. 2006). It is the balance between ROS and antioxidant defences, however, which is important in the mediation of oxidative damage (MONAGHAN et al. 2009). Thus, despite wounded badgers not differing in antioxidant responses, they may still be producing more ROS due to wound healing (SONEJA et al. 2005; SEN & ROY 2008), exposing them to an increased risk of oxidative damage (MONAGHAN et al. 2009; METCALFE & ALONSO-ALVAREZ 2010).

Among the limited range of variation in this study's sample, weight-age residuals showed no interaction with non-enzymatic plasma antioxidant capacity, indicating no difference in antioxidant levels between lighter and heavier individuals within each age class. Weight varies considerably between seasons in badgers (autumn: mean = 9.9 kg, SD = 1.9; summer: mean = 7.5 kg, SD = 1.6; MONTES et al. 2011), as does antioxidant capacity (MONTES et al. 2011). Examination for within-season variation using body condition, based on an index of subcutaneous fat, also revealed no correlation with

non-enzymatic plasma antioxidant capacity. This is similar to findings in the Seychelle Warbler (VAN DE CROMMENACKER et al. 2011) but unlike MONTES et al. (2011), sampling badgers in a previous year. This is because we timed our sampling to coincide with all individuals being at the thin end of the condition scale, due to drought-induced food (earthworm, *Lumbricus terrestris*) paucity; of the 84 animals we captured during May–June 2011 none had a body condition exceeding 3, limiting the range of body-condition extremes in our sample. The majority of individuals captured were thus in a state of modest-severe malnutrition, where their capacity to generate sufficient circulating antioxidant protection was compromised (PODMORE et al. 1998; TRITES & DONNELLY 2003). Malnutrition not only has the potential to impact on individuals' capacity to deal with OS immediately (MONTES et al. 2011), but may also have more long-term ontogenetic effects on cubs, as low food quality in early life lowers adult antioxidant levels (BLOUNT et al. 2003). Furthermore, malnutrition intrinsically impairs the development of the immune system over the long-term (HUANG & FWU 1993; PARK et al. 2003).

CONCLUSION

Despite the profound importance of OS and antioxidants in immuno-physiology and to the evolution of life-history traits (MONAGHAN et al. 2008, 2009; METCALFE & ALONSO-ALVAREZ 2010), until recently studies have been applied predominantly to captive or domesticated animals (MCGRAW et al. 2010) as well as to the biomedical sciences (e.g. SHELTON-RAYNER et al. 2010). There is, therefore, a need for more work to be applied to ecological field research (MONAGHAN et al. 2009; METCALFE & ALONSO-ALVAREZ 2010; ISAKSSON et al. 2011a).

Our conclusions are limited to estimating individuals' capacity to deal with an oxidative challenge, rather than assessing their actual oxidative status, which will require further combinations of oxidative markers as well as longitudinal studies, informed by these findings. Nevertheless, this study highlights the intricate interactions and trade-offs that occur during ontogenescence, often overlooked in studies on the evolution of life-history traits (LEVITIS 2011). We provide a basis for further investigation into cellular senescence in badgers (BUESCHING et al. 2009; DUGDALE et al. 2011) to expand on interpretation of oxidative senescence in other species (DEVEVEY et al. 2010; ISAKSSON et al. 2011b). Badger OS responses have particular relevance to the ongoing debate surrounding the implications of badger management in the transmission of bTB (GALLAGHER & CLIFTON-HADLEY 2000; WOODROFFE et al. 2006a). MACDONALD et al. (2006), MATHEWS et al. (2006) and RIORDAN et al. (2011) all proposed that a major contributor to the counterproductive perturbation effect observed to result from badger culling (WOODROFFE et al. 2006a, 2006b, 2008, 2009; DONNELLY & WOODROFFE 2012) was the resultant behavioural changes in members of the residual population under social stress. If individuals of different age classes vary in their capacities to mount an antioxidant response and adapt to acute stressful situations, it would appear that age classes should not be treated with equivalent emphasis (MAHMOOD et al. 1987; GORMLEY et al. 2008; CORNER et al. 2011) when developing bTB control policy.

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