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Thrifty development: early-life diet restriction reduces oxidative damage during later growth

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Summary

1. Conditions during early stages of life may have an important effect on phenotype, by inducing programmed responses that may remain throughout the lifetime of an animal. One very important factor that can promote long-term changes in phenotype is restriction of food intake (dietary restriction, DR).

2. Recently, it has been shown that DR may induce an increase in antioxidant and repair mechanisms as a result of hormetic responses. Interestingly, the induction of antioxidant and repair mechanisms may be triggered by transitory increases in reactive oxygen species. Dietary-derived antioxidants, such as vitamin E, may be important to determine the compensatory effect of DR.

3. To investigate the effect of DR on attenuation of oxidative damage, we manipulated dietary intake (by restricting food ingestion) and antioxidant availability (by vitamin E supplementation) during the first days of life of yellow-legged gull (*Larus michahellis*) chicks. We then measured oxidative status and body mass during the early development of chicks.

4. We found that an early short event of food shortage strongly affected the oxidative status of the chicks and their growth patterns. We observed less oxidative damage to proteins and DNA in dietary restricted chicks, after the period of food restriction, than in non-restricted chicks. Unexpectedly, vitamin E supplementation did not suppress the hormetic effect of DR, but instead increased it.

5. These novel results support the idea that short events of DR during early development induce a reduction in oxidative damage in wild animals. The results suggest that DR promotes the induction of an early hormetic response in some antioxidant defence processes and/or repair mechanisms. These findings have important implications for our understanding of how early conditions may shape the phenotype of an organism, and also for the study of evolutionary trade-offs during early growth.

Key-words: caloric restriction, compensatory response, *Larus michahellis*, oxidative stress, phenotypic programming, vitamin E, yellow-legged gull

Introduction

Phenotypic development is the result of a complex interplay between the genetic architecture of an organism and environment (West-Eberhard 2003). Thus, environmental conditions can exert important effects on phenotype, and the strength of the effects appears to be particularly important in early stages of life (Monaghan 2008). The environmental conditions during early life can determine individual differences in a number of physiological traits such as metabolism and the immune response (Lindström 1999; Metcalfe & Monaghan 2001 and references therein). Importantly, they may also be major determinants of life-history trajectories (Alonso-Alvarez *et al.* 2006) and even sexual traits in adulthood (Ohlsson *et al.* 2002). It has recently been suggested that, during early development, organisms receive physiological cues that induce integrated phenotypic adjustments (Gluckman, Hanson & Beedle 2007; Mangel 2008). Such programming may promote survival, with the developing organism responding to environmental cues by following an appropriate path of development ('Thrifty phenotype hypothesis', reviewed by Wells 2007).

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Early development can be altered by many factors. Among these, food availability plays a crucial role because energy sources and specific nutrients are essential for correct maintenance of all physiological functions, with long-term consequences (Metcalfe & Monaghan 2001). Reduced food availability during early life may induce long-term changes in metabolic and endocrine functions, affecting the expression of enzymatic activities and genes (Burdge *et al*. 2007). Indeed, episodes of poor early nutrition have been linked to negative performance in adulthood in a number of taxa (Lummaa & Clutton-Brock 2002). Nevertheless, a short non-fatal food stress (i.e. dietary restriction, hereafter DR) during development may induce a programmed response that continues throughout the entire lifetime of an organism (Mangel 2008). DR is in fact the only well-known experimental manipulation that prolongs life span and delays the incidence of many age-related diseases in different taxa (Sohal & Weindruch 1996; Gredilla & Barja 2005).

The effects of DR have been found independently of whether the restriction applies to the total diet or only specific components (Merry 1995; Piper, Mair & Partridge 2005), although the underlying mechanisms are still unclear (Masoro 2005). Although many hypotheses have been proposed to explain the effects of DR, including growth retardation (McCay, Cromwell & Maynard 1935), reduction in body fat (Berg & Simms 1960) and reduction in metabolic rate (Sacher 1977), experimental evidence to support these remains scarce and controversial (Masoro 2005; Metcalfe & Alonso-Alvarez 2010). In this context, the most popular hypothesis posits that DR leads to a decrease in the cellular accumulation of oxidative damage ('oxidative damage attenuation hypothesis'; Sohal & Weindruch 1996; Finkel & Holbrook 2000; Masoro 2005). Oxidative damage is the result of oxidative stress, which is characterized by an imbalance between the production of reactive oxygen species (ROS) and antioxidant defences in favour of the former (Halliwell & Gutteridge 2007). Experimental studies in laboratory animals have shown that DR decreases oxidative damage and delays age-associated accumulation of such damage (Matsuo *et al*. 1993; Sohal *et al*. 1994; Dubey *et al*. 1996).

It has recently been hypothesized that the long-term effects of DR during early development are the consequence of a hormetic response (Minois 2000; Le Bourg 2003; Masoro 2006). Hormesis is a dose–response phenomenon characterized by unfavourable responses when organisms are exposed to high doses of stressors but favourable biological responses at low doses (Calabrese & Baldwin 2003). In evolutionary ecology, hormesis has been defined as an increase in fitness components as a response to a phase of exposure to mild levels of a stressor (Mangel 2008; Mattson 2008; Costantini, Metcalfe & Monaghan 2010). Thus, mild levels of stress imposed by DR would induce the release of physiological signals, thus inducing integrated responses, which would remain throughout life.

Evidence for a hormetic effect is provided by data showing that DR may act as stressor (Han *et al*. 1995; Masoro 1998; Kitaysky *et al*. 1999), especially during early life (Honarmand, Goymann & Naguib 2010), although it may also up-regulate some cytosolic antioxidants (Yu 1994; Kaneko *et al*. 2011) and promote several cellular repair systems (Van Remmen *et al*. 1995; Cabelof *et al*. 2003; Stuart *et al*. 2004). Interestingly, short events of DR may enhance resistance to oxidative stress during the lifetime of an organism, an effect that may even be observed in the next generation (Kaneko *et al*. 2011). Moreover, DR may also increase the ability of an organism to cope with oxidative stressors such as toxins, high temperatures and inflammatory agents (reviewed in Masoro 1998). Thus, the oxidative damage hypothesis may in fact be based on a hormetic response. A recent study on the worm *Caenorhabditis elegans* has shown that under a DR regimen (reduction in glucose availability) ROS production increases, but so do antioxidant defences, thus extending the life span of the animals (Schulz *et al*. 2007). Interestingly, the effect was reversed when worms were supplemented with antioxidants (Schulz *et al*. 2007). This suggests that the DR may produce a hormetic response to oxidative (mild) stress-related cues, such as a transitory increase in ROS. Nevertheless, it is not known whether this mechanism is also present in vertebrates.

In the present study, we examined the effects of DR and antioxidant availability on oxidative stress during early development in a wild bird species. We manipulated dietary intake (by restricting food intake) and antioxidant availability (by vitamin E supplementation) during post-hatching development of first-hatched yellow-legged gull (*Larus michaellis*) chicks (Fig. 1). In gulls, the amounts of yolk antioxidants in the first two eggs to hatch are higher and less variable than in the third egg and they support less stressful conditions (Royle, Surai & Hartley 2001). Vitamin E is a fat-soluble antioxidant that cannot be synthesized *de novo* by animals (it is derived from the diet) and is stored in fat tissues (Surai 2002). In birds, dietary vitamin E is particularly important in preventing oxidative damage (Surai 2002) and also in improving growth (de Ayala, Martinelli & Saino 2006; Hall *et al*. 2009).

We measured the effect of DR and vitamin E supplementation on growth rates, plasma antioxidant capacity and ROS

Fig. 1. Yellow-legged gull chicks resting in the nest (photograph from Sin-Yeon Kim).

levels, as well as on the levels of oxidative damage in the main macromolecules (lipids, proteins and DNA) throughout early development of the gull chicks. As far as we know, this is the first experimental study on a wild species in which all of these oxidative stress markers have been analysed simultaneously. If DR induces an integrated response that attenuates oxidative damage (Sohal & Weindruch 1996; Masoro 2005), we predict that chicks exposed to early, short episodes of DR will subsequently develop lower levels of oxidative damage than non-restricted chicks. Moreover, if physiological cues for the effects of DR are related to a transitory mild increase in oxidative stress, antioxidant (vitamin E) supplementation should inhibit these compensatory responses.

Materials and methods

FIELD PROCEDURE AND EXPERIMENTAL DESIGN

The study was carried out between May and June 2008 in a large colony of yellow-legged gulls in the Parque Nacional das Illas Atlánticas, Sálvora Island, Galicia, Spain (42°28′N, 09°00′W). Yellow-legged gulls are monogamous colonial breeders that defend a small breeding territory. Clutches typically contain three eggs (modal clutch size) and eggs are laid at intervals of 1–3 days.

In the last week of May, the colony was examined to locate nests with a clutch of three eggs in which only one of the eggs was pipping (expected to hatch the next day). To recognize the chick after hatching (Fig. 1), the tip of the bill in the pipped egg ($n = 82$) was marked with a black permanent maker (toluene free). In this species, laying and hatching order are highly positively correlated (Rubolini *et al.* 2005), hatching is asynchronous, and the first-hatched chick has a strong competitive advantage (Boncoraglio *et al.* 2006). Small enclosures were installed around the nests (an area of 1·5 m^2 around the nest was surrounded with semitransparent mesh of height 30 cm) to prevent the chicks from moving to nearby nests. Before the experiment, we did not find any adverse effect on parental brooding and chick feeding in three pilot enclosures. The nests were checked daily, the first-hatched chicks (recognizable by dark markings remaining on the bill) were marked with a strip of coloured Velcro, and the tarsus length was measured (±0·01 mm). The first pipped egg from each nest was randomly assigned to one of four groups in a 2 × 2 factorial experiment: DR treatment (restricted or not) and vitamin E treatment (supplemented or not).

In the DR group, food intake was restricted only between the day 1 and day 2 of age by fastening a 5-mm-wide ring of tape around the chick's bill, just below the nostril, to prevent food intake. Prior to the experiment, we performed pilot trials ($n = 5$) to check the tape effect on chick behaviour. All chicks survived and showed normal behaviour. The tape limited bill opening but did not close the bill completely or constrain begging. The tape was put in place in the afternoon of day 1 (1 day after hatching) and was removed in the afternoon of the following day. Non-diet-restricted chicks were manipulated in the same way, but the tape was loosened to allow food intake. The duration of DR (mean ± SE: 22·61 ± 0·07 h) resembled the time that chicks can remain without being fed by their parents in the same colony under adverse natural conditions (J.C. Noguera, pers. obs).

For vitamin E supplementation, chicks received a daily dose of 7·1 mg of vitamin E (dl-α-tocopherol acetate, Chiesi España, Barcelona, Spain) during the first 2 days of life, on the day of hatching (day 0) and on the following day (day 1). The same dosage has previously been used and reported to increase vitamin E levels significantly within the natural range in the yellow-legged gull (Pérez, Lores & Velando 2008; Noguera *et al.* 2010a). The vitamin E was mixed with 0·5 mL of refined sunflower oil and administered orally. The control group (not supplemented) was administered with placebo (refined sunflower oil without vitamin E). Refined sunflower oil contained only traces of vitamin E (254 μg in the daily dose), probably negligible compared to dosage in vitamin E–supplemented chicks (approximately 30-fold lower) and well below the estimated daily vitamin E intake in natural conditions for this species (Pérez, Lores & Velando 2008).

To examine the effect of experimental treatments on chick's growth (body mass), the chicks were weighed (±0·1 g) at hatching and on days 1, 2, 5 and 8 of age. Body mass growth was calculated as final body mass minus initial body mass for three ages (1–2, 2–5 and 5–8 days). Chick survival was recorded until 8 days of age.

MEASURES OF OXIDATIVE STRESS AND OXIDATIVE DAMAGE

Blood was collected from each chick at hatching and 5 and 8 days of age for evaluation of oxidative stress. Blood samples were taken from the brachial vein by use of heparinized capillary tubes. The samples were kept cool until the plasma was separated from blood cells (within a few hours of collection) and then stored in liquid nitrogen. The plasma antioxidant capacity and ROS levels in plasma were analysed at all three ages, but the level of oxidative damage was only measured at age 5 and 8 days because of the small volume of each sample available. Repeatability was calculated in all analysis as described by Lessells & Boag (1987).

Plasma antioxidant capacity

The plasma antioxidant capacity was measured by the method described by Erel (2004). Briefly, the method consists of mixing plasma samples (5 μL) with 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate), which is decolourized by plasma antioxidants according to their concentration and antioxidant capacity. The change in colour was measured as the change in absorbance at 415 nm (Microplate reader 550; Bio-Rad Laboratories, S.A. Barcelona, Spain). Levels of plasma antioxidant capacity were expressed as millimoles of Trolox equivalent per litre. Samples were assayed individually, but previous assays in yellow-legged gull chicks were highly repeatable ($r = 0{\cdot}87$, $F_{113,114} = 14{\cdot}60$, $P < 0{\cdot}001$).

Reactive oxygen species

Reactive oxygen species levels in plasma were estimated in duplicate by the method described by Brambilla, Fiori & Archetti (2001). Note that we measured standing ROS levels, i.e. those levels affected by antioxidant defences and ROS production. Briefly, ROS in plasma samples (5 μL) are reacted with *N*,*N*-diethyl-p-phenylenediamine to produce a coloured complex that can be measured spectrophotometrically at 505 nm (Microplate reader 550; Bio-Rad Laboratories). ROS levels were expressed as millimoles of hydrogen peroxide (H_2O_2) equivalent per litre (repeatability: $r = 0{\cdot}78$, $F_{121,122} = 7{\cdot}74$, $P < 0{\cdot}001$).

Analysis of lipid peroxidation

Lipid peroxidation in plasma (levels of oxidative damage in lipids) was assessed in triplicate by quantifying malondialdehydes (MDA),

by high-performance liquid chromatography, according to Karatas, Karatepe & Baysar (2002), but modifying the volume of sample (10 μL) and reagents (Noguera *et al.* 2011). The absorbance of the eluent was monitored at 254 nm and quantified relative to external standards (calibration curves, $R^2 = 0{\cdot}999$; repeatability: $r = 0{\cdot}90$, $F_{104,210} = 28{\cdot}93$, $P < 0{\cdot}001$). Lipid peroxidation was expressed as microgram of MDA per millilitre of plasma.

Analysis of protein oxidation

Protein oxidation was assessed by the determination of levels of protein carbonyl groups in plasma (20 μL), which were quantified in duplicate by reaction with 2,4-dinitrophenylhydrazine (DNPH), as described by Levine *et al.* (1990). Briefly, plasmatic proteins were reacted with 0·2% DNPH in 2 M hydrochloric acid for 15 min at 25 °C, precipitated with 20% trichloroacetic acid and washed three times by resuspension in ethanol/ethyl acetate (1 : 1 v/v). Proteins were solubilized in 6 M guanidine hydrochloride and centrifuged to remove any trace of insoluble material. Carbonyl groups were measured spectrophotometrically at 370 nm (Biomate 3; Thermo Fisher Scientific Inc, Rochester NY, USA; repeatability: $r = 0{\cdot}68$, $F_{104,105} = 5{\cdot}21$, $P < 0{\cdot}001$). Carbonyl groups in plasma were expressed as nmol of carbonyl/mg of protein.

Analysis of oxidative DNA damage

DNA from blood red cells was extracted by a chaotropic NaI-based method (Gedik & Collins 2005), which minimizes artefactual oxidation of DNA and is recommended by the European Standards Committee on Oxidative DNA Damage. Isolated DNA was quantified by a high-sensitivity fluorescent assay (Quant-iT™ High-Sensitivity DNA Assay kit, Invitrogen, LLobregat, Barcelona, Spain), and protein contamination was determined by the absorbance ratio A_{260}/A_{280} (>1·85 in all samples). DNA samples were diluted to 100 μg mL^{-1} in TE buffer, and two aliquots of 10 μL (hereafter 'subsamples') were stored at −80 °C. Oxidative DNA damage was assessed by measuring the total level of oxidative damage in red blood cell DNA, by means of the aldehyde-reactive probe (ARP) assay for apurinic/apyrimidinic (AP) sites coupled to *N*-glycosylases (Kow & Dare 2000). Briefly, one subsample was treated with endonuclease III (Endo III) and 8-oxoguanine *N*-glycosylase (hOGG1), which transform oxidative DNA damage in purine (Dizdaroglu, Laval & Boiteux 1993) and pyrimidine (Girard, Guibourt & Boiteux 1997) DNA bases into AP sites (AP-enzymatic), which can be measured by the ARP assay. The subsample not treated with enzymes (AP control) was used to quantify the number of 'AP site base lesions' in the DNA. Thus, oxidative DNA damage was calculated as the difference between subsamples and expressed as number of AP site equivalents/100 000 bp of DNA. The number of AP sites was determined by use of a commercial kit (ARP assay; Oxidative DNA Damage Quantitation kit-AP sites; Cell Biolabs, Inc. San Diego, California, USA), in accordance with the manufacturer's instructions. AP sites were labelled with ARP solution at 37 °C for 1 h. The ARP-labelled DNA was then precipitated with ethanol and the DNA pellet suspended in TE buffer. ARP-labelled DNA was fixed in a DNA high-binding plate and incubated with streptavidin–enzyme conjugate, which results in a coloured complex that can be measured spectrophotometrically at 450 nm (Microplate reader 550; Bio-Rad). Subsamples of DNA were assayed individually, although this assay showed was significantly repeatable in a previous sample of yellow-legged gull chicks ($r = 0{\cdot}72$, $F_{34,35} = 6{\cdot}21$, $P < 0{\cdot}001$).

STATISTICAL ANALYSES

The effects of DR and vitamin E supplementation on body mass, body mass growth, ROS, plasma antioxidant capacity and oxidative damage level (lipids, proteins and DNA) were analysed by repeated measure models (PROC MIXED in SAS software), with age as the repeated-measure factor (within chicks), individual birds as the subject term (REPEATED statement) and treatments as fixed factors. Age was also included as a between-groups fixed factor. Initial values (hatching day) of plasma antioxidant capacity or ROS, chick body mass, tarsus length, egg volume and hatching date were included as covariates. Two-way interactions between fixed factors and age, and fixed factors and hatching date were tested. Note that models differ with regard to age periods because not all variables were measured at all ages.

The effect of DR on oxidative damage may be related to an effect on growth rates (Metcalfe & Monaghan 2001). A greater increase in body mass appears to be related to increased susceptibility to oxidative stress (Alonso-Alvarez *et al.* 2007; Nussey *et al.* 2009; Kim *et al.* 2011). Thus, additionally, to test whether the effects of DR on oxidative damage remain after controlling for any possible effect of growth rates, we re-ran the full models including body mass growth (previous to sampling age, i.e. between 2–5 and 5–8 days of age) and the interactions of body mass growth with fixed factors as covariates. We did not include body mass growth in principal models because it is a response variable that would increase multicollinearity and hence type II errors (Quinn & Keough 2002). The effects of DR and treatments on chick survival until 8 days of age were analysed by a generalized linear model with binomial error and logit link.

All models were simplified by removing non-significant terms (in a backward deletion procedure), starting from two-way interactions; significance was estimated when terms were dropped from the model. The models were run by use of SAS software (SAS 9.1), with Satterthwaite's approximation for degrees of freedom, and the best covariance structure was selected in accordance with the likelihood ratio test (Littell *et al.* 2006). Prior to experimental manipulation, four pipped eggs were lost before hatching (probably predated) and three chicks died at hatching. Moreover, the data from five chicks in the DR group were excluded from the experiment because the bill tape was lost. Thus, 14 chicks were in the DR/vitamin E–supplemented group, 17 were in the DR/non-vitamin E–supplemented group, 19 were in the no DR/vitamin E–supplemented group and 20 were in the no DR/non-vitamin E–supplemented group. Differences in sample sizes in some analyses reflect missing values because of death or loss of chicks and/or insufficient volume of blood sample (i.e. ROS and oxidative DNA damage level). Data are presented as means ± standard error, and the significance level was set at 0·05.

Results

Egg volume, chick body mass, tarsus length at hatching and hatching date did not differ significantly between experimental groups ($P > 0{\cdot}17$ in all cases). After finishing the vitamin E supplementation and prior to starting the DR treatment (day 1 of age), gull chicks did not differ in body mass (DR: $F_{1,68} = 0{\cdot}34$, $P = 0{\cdot}55$; vitamin E: $F_{1,67} = 0{\cdot}193$, $P = 0{\cdot}66$; DR × vitamin E: $F_{1,66} = 1{\cdot}231$, $P = 0{\cdot}27$). Chick survival until 8 days of age was also similar among groups (DR: $\chi^2 = 1{\cdot}31$, d.f. = 1 $P = 0{\cdot}25$; vitamin E: $\chi^2 = 0{\cdot}07$, d.f. = 1, $P = 0{\cdot}79$; DR × vitamin E: $\chi^2 = 0{\cdot}31$, d.f. = 1, $P = 0{\cdot}57$).

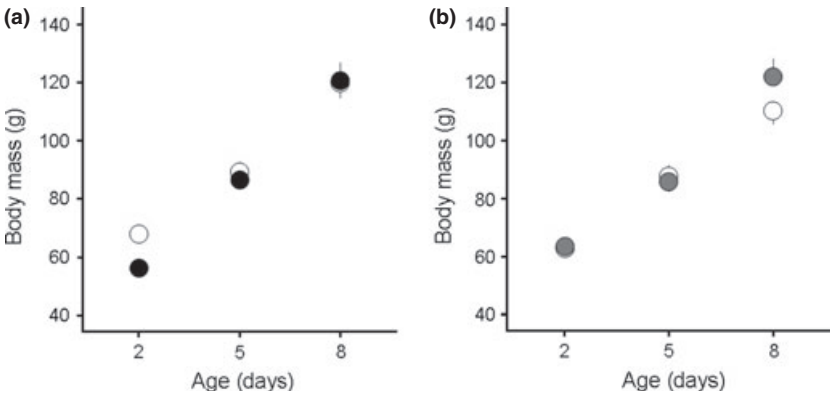

Fig. 2. Body mass (estimated marginal mean ± SE) in (a) dietary restricted (filled circles) and non-restricted (open circles) chicks and in (b) vitamin E–supplemented (filled circles) and non-vitamin E–supplemented (open circles) chicks.

EXPERIMENTAL EFFECTS ON CHICK GROWTH

Chick body mass increased with age ($F_{2,50·6} = 112·91$, $P < 0·001$) and was positively related to chick body mass at hatching ($F_{1,66·3} = 42·28$, $P < 0·001$). Overall, the body mass of chicks in the diet-restricted group was significantly lower than in non-diet-restricted chicks during post-DR development ($F_{1,66} = 62·25$, $P < 0·001$) (Fig. 2a), but the interaction between DR and age was not significant ($F_{2,47·7} = 1·47$, $P = 0·23$). The interaction between vitamin E supplementation and age had a significant effect on body mass (age × vitamin E: $F_{2,50·6} = 3·44$, $P = 0·039$). Chick body mass was similar between groups until day 5, but vitamin E–supplemented chicks grew more and attained a greater body mass than non-supplemented chicks at 8 days of age (Fig. 2b).

Body mass growth was affected by DR treatment and the effect varied with age (age: $F_{2,51·3} = 98·48$, $P < 0·001$; DR: $F_{1,51·6} = 0·19$, $P = 0·664$; age × DR: $F_{2,51,3} = 12·49$, $P < 0·001$). Body mass growth was lower in chicks under the DR regime than in control chicks during DR treatment (between 1 and 2 days of age). The DR chicks then grew faster than control chicks (between 2 and 5 days of age), although the differences disappeared after 5 days of age. Vitamin E supplementation ($F_{1,66·9} = 0·81$, $P = 0·372$), its interaction with DR treatment ($F_{1,65·8} = 0·14$, $P = 0·713$) or age ($F_{2,50·2} = 1·44$, $P = 0·246$) did not have a significant effect on body mass growth.

EXPERIMENTAL EFFECTS ON PLASMA ANTIOXIDANT CAPACITY AND ROS LEVELS

At hatching, neither plasma antioxidant capacity nor ROS levels differed between experimental groups (ROS; vitamin E: $F_{1,38} = 0·505$, $P = 0·482$; DR: $F_{1,39} = 1·575$, $P = 0·217$; vitamin E × DR $F_{1,37} = 0·06$, $P = 0·807$; plasma antioxidant capacity; vitamin E: $F_{1,66} = 0·056$, $P = 0·814$; DR: $F_{1,64} = 0·064$, $P = 0·938$; vitamin E × DR $F_{1,63} = 1·821$, $P = 0·182$). Plasma antioxidant capacity was positively related to egg size and hatching date (Table 1), but was not affected by experimental treatments (vitamin E: $F_{1,97} = 0·94$, $P = 0·335$; DR: $F_{1,90} = 0·51$, $P = 0·479$; vitamin E × DR: $F_{1,87} = 0·51$, $P = 0·477$) or chick age (age: $F_{1,91} = 1·05$, $P = 0·40$). ROS levels decreased with age of the chicks (Table 1), but did not differ between experimental treatments (vitamin E: $F_{1,33·6} = 0·43$, $P = 0·516$; DR: $F_{1,41·4} = 0·66$, $P = 0·422$; vitamin E × DR: $F_{1,30·1} = 1·68$, $P = 0·205$).

EXPERIMENTAL EFFECTS ON THE LEVEL OF OXIDATIVE DAMAGE

Lipid peroxidation level was not significantly affected by experimental treatments (vitamin E: $F_{1,90} = 0·08$, $P = 0·771$; DR: $F_{1,99} = 1·11$, $P = 0·295$; vitamin E × DR: $F_{1,89} = 1·73$, $P = 0·192$) but increased with age (Table 1). In the model including previous body mass growth as a covariate (see Methods), the relationship between oxidative damage to lipids and body mass growth was significant (Table 2, Fig. 3), especially at 8 days of age, although the interaction between age and body mass growth was not significant ($F_{1,90} = 2·12$, $P = 0·148$).

Protein oxidation level decreased with age (Table 1), and protein oxidation was lower in DR chicks than in non-diet-restricted chicks (Table 1, Fig. 4). In contrast, vitamin E supplementation did not have any effect on protein oxidation ($F_{1,90} = 0·09$, $P = 0·765$). Protein oxidation level was not related to the previous growth rate (body mass growth: $F_{1,89} = 1·05$, $P = 0·30$; age × body mass growth: $F_{1,85} = 1·39$, $P = 0·24$).

Oxidative damage was significantly affected by the interaction between DR and vitamin E supplementation (Table 1; Fig. 5). In the non-diet-restricted group, chicks supplemented with vitamin E had higher oxidative DNA damage than non-vitamin E–supplemented chicks (Tukey's test; $P = 0·025$), but no differences were found in the diet-restricted group (Tukey's *post hoc* test; $P = 0·243$; Fig. 5). Oxidative DNA damage was lower in diet-restricted chicks than non-diet-restricted chicks only in the vitamin E group (Tukey's *post hoc* test; vitamin-E group: $P < 0·001$ non-vitamin E group: $P = 0·948$). When previous body mass growth was included in the model (see Methods), it was positively related to oxidative DNA damage (Table 2). Although this relationship was particularly pronounced at 8 days of age, the interaction between age and body mass growth was not significant (age × body mass growth: $F_{1,33·3} = 0·25$, $P = 0·62$; Fig. 6). Importantly, the effect of the interaction between DR and vitamin E on oxidative DNA damage remained significant

Table 1. Mixed models analyses of the effect of vitamin E supplementation and DR treatments on plasma antioxidant capacity, ROS and oxidative damage level. Non-significant terms were excluded from the final analysis

Dependent variable	Source of variation	Estimate	*F*	d.f.	*P*
ROS†	Intercept	29·667			
	Age*	−13·416	36·70	1, 48·8	< 0·001
Plasma antioxidant capacity†	Intercept	−23·225			
	Hatching date	0·138	7·59	1, 99	0·007
	Egg volume	0·000	4·49	1, 99	0·036
Lipid peroxidation†	Intercept	0·867			
	Age*	0·233	5·10	1, 103	0·026
Protein oxidation†	Intercept	0·531			
	Age*	−0·120	12·28	1, 102	< 0·001
	DR	−0·078	4·28	1, 102	0·041
Oxidative DNA damage†	Intercept	−37·574			
	Hatching date	0·725	9·28	1, 20·8	0·006
	Tarsus length at hatching	−2·536	42·8	1, 23·7	< 0·001
	Age*	2·989	4·84	1, 43·9	0·033
	DR	−6·003	16·50	1, 21·1	< 0·001
	Vitamin E	−3·284	0·51	1, 19·1	0·482
	DR × Vitamin E	5·483	12·40	1, 18·9	0·002

*Age: Two-level fixed factor (5, 8 days of age).
†ROS, plasma antioxidant capacity, lipid peroxidation, protein oxidation and oxidative DNA damage measured as mmol H_2O_2 equivalent per L, mmol TROLOX equivalent per L, μg MDA mL^{-1}, nmol carbonyls per mg and AP sites equivalent per 10^5bp, respectively.
‡Full model: dependent variable = age + vitamin E + DR + plasma antioxidant capacity/ROS (day 0) + body mass (day 0) + egg volume + tarsus (day 0) + hatching date + hatching date × vitamin E + hatching date × DR + age × vitamin E + age × DR + vitamin E × DR.
DR, dietary restriction; ROS, reactive oxygen species.

Table 2. Mixed models analyses of the effect of vitamin-E supplementation and DR treatments on oxidative damage level after include body mass growth and the interaction with fixed effects as covariates. Non-significant terms were excluded from the final analysis

Dependent variable	Source of variation	Estimate	*F*	d.f.	*P*
Lipid peroxidation	Intercept	0·716			
	Age*	0·210	4·17	1, 101	0·043
	Body mass growth†	0·006	4·85	1, 101	0·030
Protein oxidation	Intercept	0·531			
	Age*	−0·120	12·28	1, 102	< 0·001
	DR	−0·070	4·28	1, 102	0·041
Oxidative DNA damage	Intercept	−19·047			
	Hatching date	0·589	5·92	1, 25·6	0·022
	Tarsus length at hatching	−2·536	41·28	1, 29·2	< 0·001
	Age*	3·132	5·86	1, 45·2	0·019
	DR	−5·254	14·60	1, 24·6	0·001
	Vitamin E	−2·657	0·46	1, 22·9	0·505
	Body mass growth†	0·065	6·90	1, 44	0·011
	DR × Vitamin E	4·267	7·11	1, 23·9	0·013

*Age: Two-level fixed factor (5, 8 days of age).
†Body mass growth corresponds with body mass growth previous to sampling age (i.e. between 2–5 and 5–8 days of age).
DR, dietary restriction.

when previous body mass growth was included in the model (Table 2).

Discussion

In this study, we found that a short episode of food scarcity early in life strongly affected the oxidative status of yellow-legged gull chicks. Oxidative damage in proteins and DNA was lower in diet-restricted chicks after the period of diet restriction than in non-diet-restricted chicks. Contrary to our expectations, vitamin E supplementation did not suppress the hormetic effect of DR, but instead increased it. In turn, there was an increase in oxidative DNA damage in non-diet-restricted chicks supplemented with vitamin E.

The present results are consistent with those of previous studies in laboratory models (Sohal *et al*. 1994; Forster, Sohal & Sohal 2000; Zainal *et al*. 2000), confirming the 'oxidative damage attenuation hypothesis' (Sohal & Weindruch 1996).

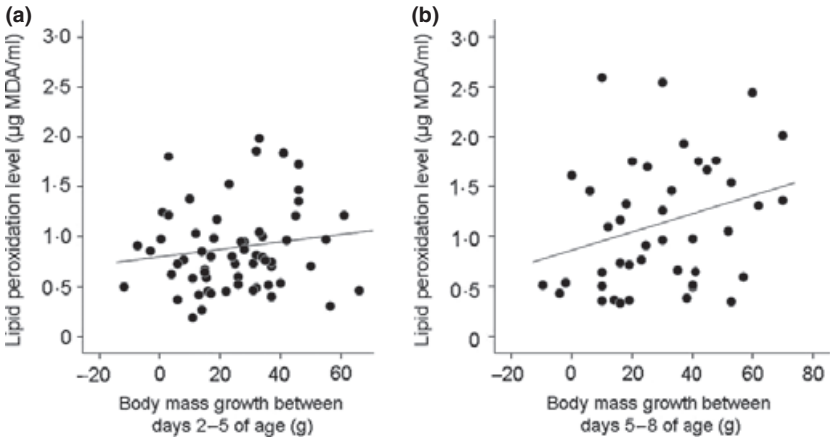

Fig. 3. Relationship between lipid peroxidation in plasma, estimated as MDA concentration, and previous body mass growth at (a) day 5 of age and (b) day 8 of age. Lines show the adjusted linear regression.

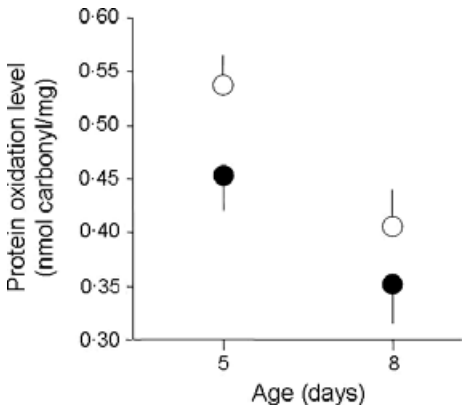

Fig. 4. Protein oxidation level in plasma, estimated as carbonyl groups (estimated marginal mean ± SE; Table 1) in dietary restricted (filled circles) and non-restricted chicks (open circles) during the first 8 days after hatching.

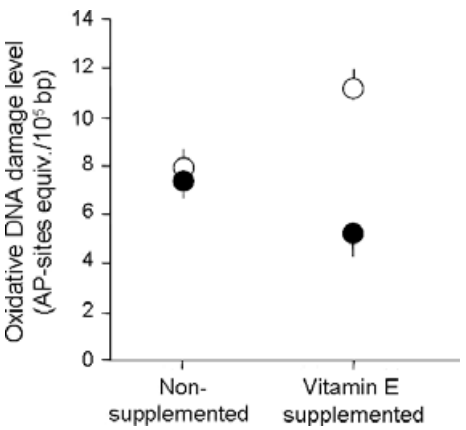

Fig. 5. Oxidative DNA damage in red blood cells, measured as the number of apurinic/apyrimidinic sites (estimated marginal mean ± SE), in dietary restricted (filled circles) and non-restricted (open circles) chicks in relation to vitamin E supplementation.

In gull chicks, DR attenuated oxidative damage on proteins and DNA, but not in lipids. This may indicate that different oxidation or reparation processes occur among biomolecules. Particularly, the maintenance of genetic integrity is needed for the correct expression of many enzyme-dependent mechanisms and to prevent a number of diseases (Jackson & Bartek 2009). The decrease in oxidative damage observed in chicks subjected to an early episode of diet restriction may be a direct result of decreased ROS production (Gredilla *et al.* 2001; López-Torres *et al.* 2002). Nevertheless, standing ROS levels were similar in DR chicks and non-diet-restricted chicks. The results therefore appear to support the hypothesis that DR, as an early mild stressor, promotes a compensatory (i.e. hormetic) response, thus promoting mechanisms that attenuate oxidative damage during subsequent development (Dimova, Bryant & Chankova 2008). However, note that we did not measure the effects of DR later than 8 days of age. In terms of hormetic mechanisms, chicks may have responded to DR by enhancing antioxidant defences or repair mechanisms, thereby reducing oxidative damage. Nonetheless, we did not find any differences in plasma antioxidant capacity between experimental groups. However, note that we did not measure other (e.g. enzymatic) antioxidants that may have been activated (Kaneko *et al.* 2011). On the other hand, DR may have affected repair mechanisms. DR has been shown to promote genomic stability in laboratory mammals, by induction of DNA base scission repair systems such as uracil (UDG) and 8-oxoguanine (hOGG1) DNA glycosidase, which are involved in oxidative DNA protection and repair (Cabelof *et al.* 2003; Stuart *et al.* 2004). Moreover, food restriction induces the expression of several genes related to protein synthesis and turnover rates (Tavernarakis & Driscoll 2002 and references therein), the up-regulation of cytosolic antioxidants (Yu 1994; Kaneko *et al.* 2011) and proteolytic removal of damaged proteins (Van Remmen *et al.* 1995). The mechanism underlying the hormetic response leading to the decrease in oxidative damage remains to be explored in future studies.

In the present study, vitamin E supplementation promoted an increase in body mass growth. Similar results have been observed in response to antioxidant supplementation in nestlings of barn swallow (*Hirundo rustica*) and red-winged blackbirds (*Agelaius phoeniceus*) (de Ayala, Martinelli & Saino 2006; Hall *et al.* 2009). Because growth rate appears to be related to an increase in oxidative stress (Alonso-Alvarez

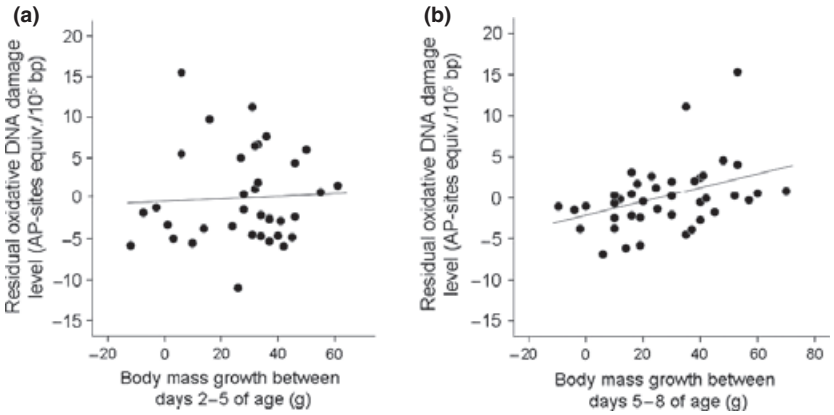

Fig. 6. Relationships between oxidative DNA damage in red blood cells and previous body mass growth at (a) day 5 of age and at (b) day 8 of age. The presented values for oxidative damages are residuals from the final model (Table 2), without age and body mass growth. Lines show the adjusted linear regression.

et al. 2007; Nussey *et al*. 2009; Kim *et al*. 2011), the present results suggest that dietary antioxidants may be used to mitigate growth-related oxidative costs, particularly when growth rates, and therefore ROS production, are high (Rollo 2002; Kim *et al*. 2011). An intriguing finding of the present study was that vitamin E affected DNA damage (but not lipid and protein damage), although in the opposite direction to that predicted, i.e. it increased the hormetic effect of DR and promoted oxidative damage in control birds. This strongly contrasts with the findings of a study in the nematode *C. elegans*, in which antioxidant supplementation reversed the enhancement of oxidative stress resistance after DR, probably because the cue leading to the hormetic response (the transient oxidative mild stress) was eliminated (Schulz *et al*. 2007). Vitamin E may function as an antioxidant under conditions of low oxidative stress, such as in diet-restricted chicks, but may, in turn, function as a pro-oxidant, especially under conditions of high oxidative stress (Rietjens *et al*. 2002). Birds may endure high oxidative stress during accelerated growth, as suggested by the correlation between growth and oxidative damage (Figs 3 and 6), which may explain why vitamin E supplementation intensified (but did not reverse) the hormetic response to oxidative stress in DR birds, but note that this result could differ with other (unmeasured) vitamin E dosage. This may also explain why in other bird species a lower level of oxidative damage is observed in marginal offspring (which usually have more limited access to parental feeding) given antioxidant supplements, but that the opposite pattern is observed in their core broodmates (Hall *et al*. 2009).

The inclusion of growth rates in the models used to test the effects on oxidative damage showed that body mass growth was related to oxidative damage, suggesting that growth entails oxidative costs. The oxidative cost of growth would be the result of trade-off allocations (Metcalfe & Monaghan 2001). Thus, greater investment in growth will result in a lower investment in prevention or repair of molecular damage (Cichon 1997). Previous studies in birds have revealed a negative relation between resistance to oxidative stress and body mass growth (Alonso-Alvarez *et al*. 2007; Kim *et al*. 2011). The results of the present study provide evidence that oxidative damage in lipids and DNA is positively related to body mass growth (see also Nussey *et al*. 2009 for evidence in mammals). On the other hand, DR attenuated oxidative damage of proteins but in turn also affected body mass growth, leading to a period of rapid growth. These results indicate that physiological trade-offs among different process or functions may be especially complex (Zera & Harshman 2001); trade-offs could differentially affect different macromolecules. Complex trade-offs may explain why different individuals recover from the DR at different rates. Additionally, the hormetic effects of DR could involve unmeasured costs not only for chicks, but also for parents, that could be paid at different times (i.e. short vs. long term). For instance, the need of a constant supply of resources for antioxidant system and repair mechanisms during growth could trade reproduction at sexual maturity; parents could experience high predation rates during chick provisioning as a result of altering their feeding strategies.

In conclusion, the present study shows that short events of DR during early development may induce a reduction in oxidative damage, at least in short-term, in wild animals confirming previous results in laboratory animals. In contrast to prolonged DR, our results suggest that a short event of DR may promote the induction of early hormetic responses to some antioxidant defence mechanisms and/or repair processes (not evaluated here). Moreover, the effects of DR on antioxidant systems appear to be mediated by the availability of specific dietary antioxidants (i.e. vitamin E), which in turn may affect growth patterns. The results have important implications for our understanding of how early conditions may shape the phenotype of an organism, and for understanding evolutionary trade-offs during early growth. Future studies should explore how DR may improve oxidative resistance phenotypes in the long term, which would result in best adapted individuals to stressful conditions experienced through their lifetime. Moreover, the costs that this metabolic programming may entail in different life-history contexts remain to be explored.

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