RESEARCH ARTICLE

Interactive effects of early and later nutritional conditions on the adult antioxidant defence system in zebra finches

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ABSTRACT

In vertebrates, antioxidant defences comprise a mixture of endogenously produced components and exogenously obtained antioxidants that are derived mostly from the diet. It has been suggested that early-life micronutritional conditions might influence the way in which the antioxidant defence system operates, which could enable individuals to adjust the activity of the endogenous and exogenous components in line with their expected intake of dietary antioxidants if the future environment resembles the past. We investigated this possibility by experimentally manipulating the micronutrient content of the diet during different periods of postnatal development in the zebra finch (*Taeniopygia guttata*). Birds that had a low micronutrient diet during the growth phase initially had a lower total antioxidant capacity (TAC) than those reared under a high micronutrient diet, but then showed a compensatory response, so that by the end of the growth phase, the TAC of the two groups was the same. Interestingly, we found an interactive effect of micronutrient intake early and late in development: only those birds that continued with the same dietary treatment (low or high) throughout development showed a significant increase in their TAC during the period of sexual maturation. A similar effect was also found in the level of enzymatic antioxidant defences (glutathione peroxidase; GPx). No significant effects were found in the level of oxidative damage in lipids [malondialdehyde (MDA) levels]. These findings demonstrate the importance of early and late developmental conditions in shaping multiple aspects of the antioxidant system. Furthermore, they suggest that young birds may adjust their antioxidant defences to enable them to 'thrive' on diets rich or poor in micronutrients later in life.

KEY WORDS: Environmental-matching model, Fitness, Oxidative stress, Programming

INTRODUCTION

During early life, organisms have to cope with a wide range of environmental factors that can influence their development and, in the longer term, affect their fitness (Burton and Metcalfe, 2014; Gluckman et al., 2007; Monaghan, 2008). Phenotypic plasticity during this early stage of life enables organisms to potentially tailor their phenotypes to the prevailing environmental conditions (Callahan et al., 1997; Monaghan, 2008; West-Eberhard, 2003). Phenotypic traits such as growth rate, stress responses, oxidative status and metabolism are very sensitive to variations in environmental conditions (Garland and Kelly, 2006; Metcalfe and Alonso-Alvarez, 2010; Surai, 2002). However, although the ability

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to cope with environmental variation is crucial in fitness terms, our knowledge of the potential costs of such early phenotypic changes is still in its infancy (Costantini, 2014; Saastamoinen et al., 2010).

An important component of the phenotype of an organism that can have substantial fitness consequences is its antioxidant defence system (Catoni et al., 2008; Cohen et al., 2008; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009). This includes a variety of endogenously produced antioxidants plus antioxidants obtained from the diet, and acts to detoxify reactive oxygen species (ROS) that otherwise can cause oxidative damage to a range of biomolecules (Halliwell and Gutteridge, 2007). Many physiological functions depend on the subtle balance between ROS and antioxidant defences (Finkel and Holbrook, 2000; Halliwell and Gutteridge, 2007; Monaghan et al., 2009); while ROS are used as signalling molecules within cells, overexposure to ROS can lead to cell and tissue oxidative damage and the eventual development of degenerative diseases, premature ageing and death (Finkel and Holbrook, 2000; Kirkwood and Austad, 2000); however, the relationship between ROS and ageing is still under debate (e.g. Speakman and Selman, 2011).

There is a large literature showing that the vertebrate antioxidant system shows a high degree of plasticity and can be influenced by a wide range of environmental factors, particularly the diet (Surai, 2002, 2006). Dietary micronutrients such as vitamin A, E or B that cannot be synthesised *de novo* are very important exogenous lipophilic and hydrophilic antioxidants in vertebrates (Costantini, 2014; Monaghan et al., 2009; Surai, 2002). Essential minerals such as selenium, copper or manganese are also involved in the absorption, recycling and formation of enzymatic and non-enzymatic antioxidants (e.g. glutathione peroxidase or vitamine E; Halliwell and Gutteridge, 2007; Surai, 2002, 2006). Furthermore, both vitamins and essential minerals play an important direct role in epigenetic regulation of gene expression (Hardy and Tollefsbol, 2011; Rao et al., 2012) and can influence key life-history traits such as growth, sexual development and reproduction in a wide range of taxa (reviewed by Catoni et al., 2008).

Most of the studies that have investigated the early-life influence of dietary micronutrients on antioxidant protection have focused on a single developmental period, usually during pre- or early postnatal development (de Ayala et al., 2006; Hall et al., 2010; Marri and Richner, 2014; Noguera et al., 2011b). However, the diet during later developmental periods is also relevant since there may be compensation for earlier periods of undernutrition if conditions subsequently improve (Metcalfe and Monaghan, 2001). Hence, not only nutritional conditions during early post-natal development (e.g. the growth period) but also later development (e.g. during sexual maturation and early adulthood) might alter the development of antioxidant defences and potentially, a wide range of phenotypic and life history traits (i.e. plumage colouration, growth, sexual maturation and reproduction; Catoni et al., 2008; Hõrak et al., 2000).

List of abbreviations

ABTS	2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)
GLMM	generalised linear mixed-effect model
GPx	glutathione peroxidase
LMM	linear mixed-effect model
M_b	body mass
MDA	malondialdehyde
NADPH	nicotinamide adenine dinucleotide phosphate
RBC	red blood cells
ROS	reactive oxygen species
TAC	total antioxidant capacity

Here, we use zebra finches (*Taeniopygia guttata*) to examine how the availability of micronutrients at two stages of development influences antioxidant defences and the level of oxidative damage individuals incur. We manipulated the availability of dietary micronutrients within the natural range during both the growth period (the first 40 days of life) and the period of sexual maturation and early adulthood (40–90 days of age). In order to see the extent to which the micronutrient intake during the growth phase influenced the antioxidant defence system during sexual maturation, half of the birds were switched from the high or low micronutrient treatment to the opposite treatment during sexual maturation in a full 2×2 design. Birds receiving the lower level of dietary micronutrients during the first period would be expected to show higher levels of oxidative damage unless they were able to make compensatory adjustments when dietary conditions improved.

RESULTS
Effect of dietary treatments on growth, antioxidant defences and oxidative damage

During the experiment, body mass (M_b) increased with age but differed between sexes (supplementary material Table S5); during the growth period both sexes showed a similar M_b (LSD *post hoc* tests, day 1: $t_{117}=-0.180$, $P=0.906$; day 20: $t_{117}=-1.116$, $P=0.262$; day 40: $t_{116}=-0.249$, $P=0.803$) but during the period of sexual maturation females were, on average, 10.7% heavier than males

(day 65: $t_{115}=3.549$, $P<0.001$; day 90: $t_{114}=4.396$, $P<0.001$). None of the other considered variables, including the micronutrient treatments, had any significant effect on M_b (supplementary material Table S5, Fig. S1).

Pectoral muscle and body fat stores were significantly correlated with M_b throughout the experiment (pectoral muscle: estimate=0.030; body fat: estimate=0.153; supplementary material Table S5) but only fat stores significantly changed with age (supplementary material Table S5, Fig. S2). Body fat increased between 20 and 65 days (LSD *post hoc* tests, 20–40 days: $t_{238}=-25.243$, p<0.001: 20–65 days: $t_{233}=-3.178$, $P=0.002$) but significantly declined between 65 and 90 days of age ($t_{231}=-3.792$, $P<0.002$). On average, females also accumulated 7.35% more body fat than males during the experiment (estimate=0.199; supplementary material Table S5). No other variables, including the micronutrient treatments, had any significant effect on pectoral muscle and body fat stores (supplementary material Table S5).

The three-way interaction between the two dietary treatments and age had a highly significant effect on TAC levels (Table 1). Thus, at 20 days of age, the birds that were being reared on a low micronutrient diet ('L' group) had a lower TAC level than those reared on the high micronutrient diet ('H' group; LSD *post hoc* test: $t_{117}=-2.396$, $P=0.018$; Fig. 1). However, birds in the L group then showed an increase in TAC, so by the end of the growth period (40 days) the two dietary groups did not significantly differ in TAC ($t_{116}=0.664$, $P=0.664$; Fig. 1). Interestingly, the birds' TAC level during the period of sexual maturation (40–90 days) was influenced by the micronutrient treatment received during both the growth period and the period of sexual maturation (Table 1): only the birds that were on the same diet throughout development (L/L or H/H) increased their level of TAC during the period of sexual maturation (L/L: $t_{82}=-2.93$, $P=0.019$; H/H: $t_{84}=-2.99$, $P=0.014$). As a consequence, at 90 days, these birds had higher TAC levels than those that had experienced a switch to the opposite micronutrient diet at 40 days of age (L/L vs L/H: $t_{53}=2.465$, $P=0.017$; L/L vs H/L: $t_{57}=2.426$, $P=0.018$; H/H vs H/L: $t_{55}=2.351$, $P=0.022$; H/H vs L/H: $t_{51}=2.424$, $P=0.019$; Fig. 1). Our results also showed that females on

Table 1. Summary of linear mixed models for the effects of micronutrient treatments and covariates on antioxidant defences and oxidative damage levels

Source of variation	TAC			GPx			MDA		
	F or Wald Z	d.f.$_{n,d}$	P	F or Wald Z	d.f.$_{n,d}$	P	F or Wald Z	d.f.$_{n,d}$	P
Age	**20.317**	**2,227.46**	**<0.001**	**89.479**	**2,227.84**	**<0.001**	**82.436**	**2,230.75**	**<0.001**
Sex	**4.858**	**1,114.28**	**0.030**	0.475	1,108.91	0.492	0.005	1,114.74	0.944
M_b	**0.006**	**1,292.64**	**0.940**	3.888	1,331.95	0.071	1.389	1,343.96	0.239
T-growth	**0.665**	**1,110.04**	**0.417**	2.948	1,109.43	0.089	0.056	1,114.89	0.813
T-sexual	**1.321**	**1,113.40**	**0.253**	2.519	1,109.03	0.115	0.085	1,116.10	0.771
T-growth×T-maturation	**0.156**	**1,115.98**	**0.694**	0.162	1,110.29	0.688	1.181	1,112.84	0.279
T-growth×age	**3.582**	**2,227.44**	**0.029**	1.647	2,228.01	0.195	0.459	2,226.45	0.633
T-maturation×age	**0.434**	**2,227.44**	**0.648**	1.103	2,228.09	0.333	0.233	2,224.34	0.792
Sex×age	0.247	2,235.10	0.781	0.774	2,234.66	0.462	1.271	2,236.60	0.282
Sex×T-growth	1.231	1,114.08	0.269	0.103	1,108.28	0.749	0.105	1,112.37	0.747
Sex×T-maturation	1.484	1,116.64	0.226	0.479	1,111.67	0.490	0.000	1,112.07	0.989
Sex×T-growth×T-maturation	0.043	1,112.43	0.836	1.354	1,105.98	0.247	0.070	1,109.75	0.792
Sex×age×T-growth	0.364	2,221.74	0.695	0.067	2,221.66	0.935	1.598	2,222.21	0.205
Sex×age×T-maturation	1.328	2,223.11	0.267	2.892	2,223.35	0.058	1.052	2,219.82	0.351
T-growth×T-maturation×age	**6.363**	**2,227.46**	**0.002**	**5.224**	**2,228.05**	**0.006**	0.278	2,217.96	0.757
Sex×age×T-growth–T-maturation	2.656	2,219.33	0.072	0.635	2,219.09	0.531	0.306	2,215.77	0.736
Random factors									
Plate	**0.782**	–	**0.434**	1.806	–	0.071	-	-	-
Individual	**1.853**	–	**0.064**	**4.635**	–	**<0.001**	**5.266**	-	**<0.001**

'T-growth' and 'T-maturation' refer to the dietary treatment during the growth period and the period of sexual maturation, respectively. Variables retained in the final models are shown in bold. For removed terms, significance levels are those estimated when terms were dropped from the model.
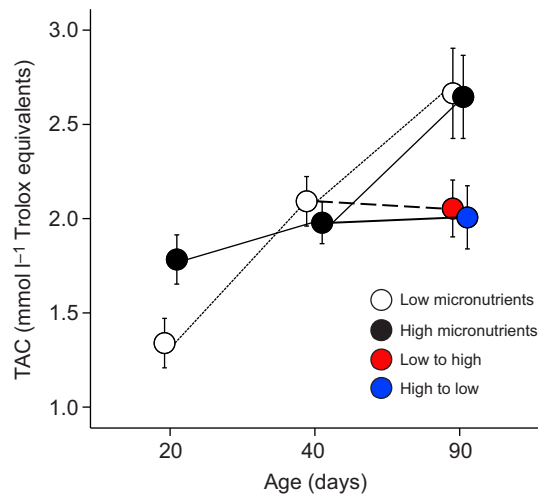
Fig. 1. Plasma non-enzymatic total antioxidant capacity in zebra finches during the growth period and at the end of sexual maturation. Growth period, 20–40 days; end of sexual maturation, 40–90 days. TAC values are means±s.e. Groups of birds (N=126) were fed a high (N=65) or low (N=61) micronutrient diet for 40 days, then either kept on the same diet or switched at 40 days from the low to the high diet or in the opposite direction (from high to low) for a further 50 days.

average had 15.4% higher TAC levels than males throughout development (estimate=0.255; Table 1). M_b had no significant effect on non-enzymatic antioxidant capacity, nor did the remaining two- and three-way interactions initially included in the model (Table 1).

GPx activity was also affected by the triple interaction among our dietary treatments and age (Table 1). During the growth period, GPx increased with age (Table 1; Fig. 2), but was not significantly affected by the micronutrient treatment received during that period (Table 1). However, as for TAC, there was an interaction between the two treatment periods (growth and sexual maturation) during the period of sexual maturation (Table 1; Fig. 2). In the group that received a low micronutrient diet during the growth period, only those birds that continued with the same micronutrient diet (L)
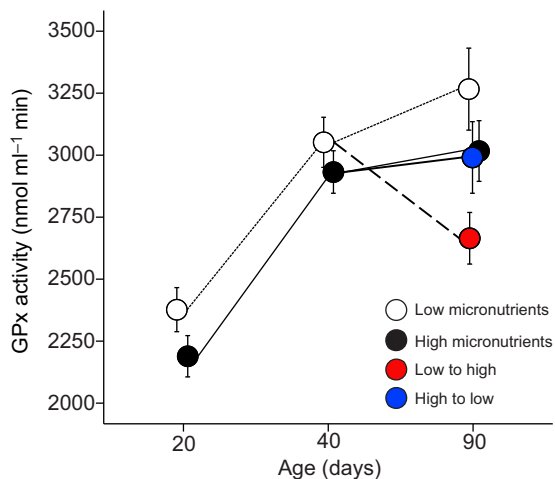

Fig. 2. Red blood cell GPx activity in in zebra finches during the growth period and at the end of sexual maturation. Growth period, 20–40 days; end of sexual maturation, 40–90 days. Values are means±s.e. Groups of birds (N=126) were fed a high- or low-micronutrient diet for 40 days, then either kept on the same diet or switched at 40 days from the low to the high diet or in the opposite direction (from high to low) for a further 50 days.

during sexual maturation did not experience a significant reduction in GPx activity levels (LSD *post hoc* test, L/L between 40–90 days: t_{84}=−1.034, P=0.304; L/L vs L/H at 90 days: t_{55}=2.919, P=0.005). In contrast, GPx activity in birds reared on a high micronutrient diet during the growth period was not significantly influenced by the dietary treatment received during the period of sexual maturation (H/H vs H/L at 90 days: t_{58}=0.135, P=0.893). There was no significant effect of sex or M_b, nor were any other two and three-way interactions significant (Table 1).

The level of oxidative damage to lipids (MDA) increased over the course of the experiment (Table 1), but levels were not significantly affected by our dietary treatments or their interactions with age and/or sex (Table 1). Neither M_b nor any interactions initially included in the model were significant (Table 1).

Within-individual consistency over time and covariation structure among measures
Both mixed-model and repeatability analyses indicated that GPx and MDA levels were significantly repeatable within individuals (GPx: $F_{109,220}$=2.743, P<0.001, r=0.37; MDA: $F_{109,220}$=2.078, P≤0.001 r=0.26; but see also estimates for random factors in Table 1, main text); the same was not true for TAC level ($F_{109,220}$=1.210, P=0.123, r=0.07). With regard to the covariance among measures, TAC, GPx and MDA levels were not significantly correlated with each other at 20 days of age (supplementary material Table S6). However, at the end of the growth period (40 days), TAC and MDA levels were weakly but significantly correlated (Pearson's r=0.28, P=0.002). In addition, at the end of sexual maturation, we also found a (weak) negative correlation between GPx and MDA level (Pearson's r=0.21, P=0.020).

Power analyses
The data sets had a high power (≥89.81% in all analyses) to detect medium to large effect sizes (r≥0.3; i.e. Cohen, 1992), although they provided lower power (18–20%) to detect small effects (r≤0.1).

DISCUSSION
The main aim of this study was to assess whether several components of the antioxidant system are influenced by variation in the micronutrient content of the diet at different developmental periods (i.e. during growth and sexual maturation). The dietary manipulations had no significant effect on rates of growth or final size attained, showing that the diets were different only in micronutrients and not the macronutrients that fuel growth. There were also no significant differences throughout the experiment in the level of oxidative damage sustained by the zebra finches, at least so far as we could tell from our measures. However, we did find effects of micronutrient intake on the activity of the non-enzymatic component of the antioxidant defences. During the middle of the growth period, the non-enzymatic antioxidant defence capacity was lower in those birds experiencing the low dietary micronutrient diet, but by the end of this period (at 40 days) these differences were no longer evident. Interestingly, we found that the diet during the growth period influenced antioxidant defences during sexual maturation. The only birds that showed a steady increase in their level of non-enzymatic antioxidant defences (TAC) throughout development were those that had experienced the same dietary treatment (low or high) throughout development; a switch in diet during development (whether from low to high or vice versa) resulted in a failure to increase this aspect of the antioxidant system. Similarly, the most sustained increase in levels of enzymatic antioxidant defences (GPx) was in the group that

experienced the 'low' diet throughout development, with the biggest drop in GPx occurring in those switched from the low to the high micronutrient diet. Together, these results suggest that the capacity of zebra finches to respond to variations in the intake of dietary micronutrients during later development may be determined by their nutritional experience early in life. Furthermore, the results raise the possibility that the balance between endogenous and exogenous antioxidant activity might be shaped by the micronutrient environment experienced in early life.

The reduced level of TAC shown at 20 days of age by zebra finches reared on the low micronutrient diet supports previous studies that have emphasised the important role that dietary micronutrients play in the body's antioxidant defences (Evans and Halliwell, 2001; Surai, 2002, 2006). The observed reduction in antioxidant capacity was probably the result of deficiencies in dietary antioxidant vitamins such as vitamin A or E, which have important and recognised antioxidant properties in birds (Surai, 2002) and were the most abundant in the micronutrient supplement. The lower availability of other micronutrients such as selenium (Se) could also have had an important role, since these trace elements are an integral part of selenoproteins such as glutathione peroxidase (Surai, 2006), although there was no evidence of a stimulatory or inhibitory effect on GPx activity in any of the groups of birds. While this may indicate that dietary micronutrients primarily influence the non-enzymatic antioxidant defence system, we cannot rule out the possibility that our dietary treatment had an effect on other (non-measured) antioxidant enzymes (e.g. catalase or gluthatione reductase; Shireen et al., 2008).

Interestingly, birds on the low micronutrient diet initially had a reduced TAC but this capacity then increased significantly over time. This compensatory response occurred in the absence of any increase in the intake of dietary micronutrients, but could have occurred through at least three mechanisms. Firstly, low levels of circulating antioxidants could have triggered the up-regulation of endogenous non-enzymatic antioxidants such as vitamin C, which can occur via ROS-sensitive signalling transduction pathways when oxidative stress levels increase (Chatterjee et al., 1975; Surai, 2002). Secondly, previously stored antioxidants may have been mobilised; powerful antioxidants such as vitamin E or A are often stored in the adipose tissue or internal organs such as the liver, and may be mobilised when the body antioxidant requirement increases (Surai, 2002). However, it seems unlikely that animals on the low micronutrient diet could have built up stores of surplus antioxidants, and fat reserves remained similar in both experimental groups, both of which suggest that any antioxidant mobilisation would have been on a small scale. Lastly, given the highly plastic nature of the digestive system of birds (McWilliams and Karasov, 2001), it is possible that the birds in the low micronutrient group increased their absorption and assimilation efficiency of dietary antioxidants over time. However, the assimilation of dietary micronutrients in zebra finches has previously been thought to be promoted by a high rather than a poor micronutrient diet during this growth period (Blount et al., 2003). Therefore, the results seems to better support the idea that environmentally induced oxidative stress may have triggered the up-regulation of endogenous nonenzymatic antioxidant defences (Dimova et al., 2008), which, in turn, would explain why birds in the low diet did not suffer higher levels of oxidative damage than those in the high diet.

In contrast to our predictions, we did not find evidence of improved antioxidant defences when birds were switched from a low to a high micronutrient diet; indeed, birds that started life on a particular diet (whether low or high) actually had better antioxidant defences if they continued with that same diet throughout sexual maturation. This suggests that early micronutrient conditions might have shaped the birds' antioxidant phenotype in such a way that prepared them for a similar (micronutrient) environment in future, an idea that fits with the 'environment-matching' hypothesis of developmental plasticity (often also called the 'predictive adaptive response' hypothesis; Gluckman et al., 2005). The evolution of environment-matching responses relies on the ability of developing organisms to capture accurate information about the quality of the existing environment, and on early environmental conditions being accurate predictors of adult environmental conditions (Monaghan, 2008; Nettle et al., 2014). This is more likely to be the case in fast-developing species in which development through to adulthood may occur before the environmental conditions have radically altered (e.g. less than 2 months from birth to sexual maturity in zebra finches). This might explain why studies that support the environment-matching hypothesis tend to be of fast-developing species (e.g. Oksanen et al., 2012; Saastamoinen et al., 2010) rather than those that develop slowly (Douhard et al., 2014; Hayward et al., 2013). However, given the small number of studies carried out so far, more work is needed to examine this further.

In contrast to expectations, the pattern of oxidative damage (as assessed by levels of MDA) did not vary significantly between diet treatments despite differences in levels of antioxidant protection. These results, however, do not necessarily indicate that there were no differences in oxidative stress between groups. On one hand, supplementation studies often show that a higher availability of micronutrients does not always reduce the level of all biomarkers of oxidative damage (Kim et al., 2013; Lucas et al., 2014; Noguera et al., 2011b), given that they may vary between tissues and organs (Veskoukis et al., 2009). Such variability can therefore make it difficult to reach conclusions relating to oxidative status when measurements are of damage to only a single tissue or type of macromolecule (Monaghan et al., 2009; Selman et al., 2012). By extension, the same problem can be applied to the use of one single biomarker of antioxidant defences. However, this was not a problem in this study since we have shown that diet micronutrients induced a similar effect in both non-enzymatic and enzymatic antioxidant defences. On the other hand, the lack of a significant effect of our dietary treatments on MDA levels might reflect a limitation of statistical power, if for instance, our dietary treatment had a very small effect on the MDA level (Cohen, 1992). Hence, more work is needed to determine how early and later environmental conditions interact to affect different biomarkers of oxidative stress.

In conclusion, this study found that a low availability of dietary micronutrients during the growth period of zebra finches initially compromised their non-enzymatic antioxidant defences, but the birds later responded by up-regulating their endogenous antioxidants defences. Most importantly, those individuals that experienced similar nutritional conditions (regardless of the micronutritional content) during both postnatal growth period and the period of sexual maturation had better enzymatic and non-enzymatic antioxidant defences than those that experienced a change (either upward or downward) in their micronutrient intake. These data support the hypothesis that nutritional conditions experienced in early life act as predictors of the later-life environment, and affect the later regulation of antioxidant defences. Future studies should investigate whether environmental stressors other than nutrition (e.g. population density, predation risk, competition) may induce similar responses in the antioxidant defence system and to what extent such physiological responses affect different components of fitness.

MATERIALS AND METHODS

General procedures and experimental design

In September 2012, 50 pairs of adult zebra finches (*Taeniopygia guttata* Reichenbach 1862) from our outbred stock population (∼1500 birds) at the University of Glasgow were allowed to breed under standardised conditions, each pair in a separate breeding cage (60×50 cm and 50 cm high). Paired birds were all experienced breeders and of different pedigree. During the experiment, the birds were housed in standard conditions, in a room maintained at 20.5±2°C under full spectrum, artificial light (16 h:8 h light: dark cycle). The birds' diet prior to the hatching of the chicks comprised an *ad libitum* supply of mixed seeds (common millet, yellow millet and canary seed in an approximate ratio of 3:1:1; Johnson & Jeff, U.K.), oyster shell grit, cuttlefish and water. Once a week the birds also received Calcivet calcium supplement (Vetafarm, Wagga Wagga, NSW, Australia), a protein-conditioning supplement (J. E. Haith, Cleethorpes, UK) and fresh vegetables. One day after the experimental chicks hatched, all pairs continued receiving an *ad libitum* supply of mixed seeds (see below), oyster shell grit, cuttlefish and green vegetables but without any calcium or protein supplement. At hatching, all chicks (N=126 from 36 families) were weighed using an electronic balance (±0.01 g) and marked with a unique nest colour and individual colour combination by dyeing their toes with permanent marker pens. Each brood of chicks was coupled with another brood with a similar hatching date (±1 day) and clutch size; one of the two pairs was then assigned to follow a 'high' dietary micro-nutritional treatment and the other a 'low' treatment ('H' or 'L', respectively; details below). One day after the chicks hatched, half of the chicks within each brood were cross-fostered between the two nests to account for family (genetic) and environmental (rearing) effects (see statistical section). Consequently, half of each genetic brood was reared under H and half under L micronutrient treatment but all chicks in a rearing nest received the same treatment. Chick M_b did not significantly differ between experimental groups either at hatching (day=0; $F_{1,124}=0.573$, $P=0.451$) or after being cross-fostered (day=1; $F_{1,122}=0.141$, $P=0.708$).

From hatching, all chicks were fed with a low micronutrient diet (common and red millet in a 1:1 ratio) specially formulated to reduce the daily intake of the main dietary micronutrients (i.e. Vitamin A, B, C and E) within the natural range of variation in captive conditions (see below). Chicks allocated to the H group received individually an extra dose of diet micronutrients every 3 days, diluted in water and via oral administration from hatching to 40 days old (hereafter 'growth period'). The main components of the commercial micronutrient supplement were vitamins A, B, C, D, E and K, and essential minerals such as selenium, magnesium or copper (see Noguera et al., 2015 and below for further details). The micronutrient supplement was adjusted to double the natural daily intake of main micronutrients that birds would obtain from the low antioxidant diet. Chicks in the 'H' group received an increasing dose of micronutrient supplement in order to match their increasing size (see below for a detailed description of dosage). Nestlings in the L group were manipulated in the same way as chicks in the H group, but they received only the vehicle (water). Chick survival (119 of 126; 94.4%) at the end of the dietary treatment (40 days old) was not significantly different between experimental groups and sexes [generalised linear mixed-effects model (GLMM); dietary treatment: $F_{1,121}=0.00$, $P=0.93$; sex: $F_{1,120}=0.00$, $P=0.96$; dietary treatment×sex: $F_{1,119}=0.00$, $P=0.96$].

When the broods reached 40 days of age, the parents were removed from the cages and half of each brood was assigned to follow with the same micronutrient treatment as during the growth period (low to low or high to high), and half to change to the opposite treatment (low to high or high to low) until they were 90 days of age. This second treatment corresponded with the period of sexual maturation and early adulthood in this species (Zann and Bamford, 1996) (hereafter 'sexual maturation period'). This experimental design resulted in four treatment groups that differed in the micronutrient availability the birds received during their growth period and the period of sexual maturation (i.e. L/L, L/H, H/H or H/L). Survival of the birds during the period of sexual maturation (117 of 119; 98.3%) was not significantly different among experimental groups and sexes (GLMM; growth dietary treatment: $F_{1,116}=0.298$, $P=0.586$; sexual maturation dietary treatment: $F_{1,117}=0.311$, $P=0.578$; growth dietary treatment×sexual maturation dietary treatment: $F_{1,115}=0.233$, $P=0.630$).

We used blood samples to examine the oxidative status of the birds to avoid terminal sampling and to enable effects on later life performance to be assessed in future studies. During the experiment, all birds were blood sampled at 20, 40 (the end of the growth period) and 90 days of age (the end of sexual maturation). Blood samples were maintained on ice and then centrifuged to separate plasma from red blood cells (RBCs). Several aliquots were made for both plasma and RBCs samples and were stored at −80°C. Birds were weighed (±0.01 g) at 1, 20, 40, 65 and 90 days old, and their fat reserves and the size of pectoral muscle assessed at 20, 40, 65 and 90 days old. Fat reserves were assessed by visual inspection using an 8-point scale (Kaiser, 1993) and pectoral muscle profiles were scored on a 4-point scale following Bairlein (1995).

Dietary manipulation and treatment

Many seeds present in commercial seed mixtures, such as pearl or yellow millet, are especially rich in micronutrients such as vitamin E (supplementary material Table S1; Royle et al., 2013). Because zebra finch parents feed their chicks (Zann and Bamford, 1996), so preventing us from independently controlling the amount of micronutrients each chick ingested, all broods were fed with a low-micronutrient seed mix specially formulated to reduce the daily intake of several antioxidants and vitamins to what we presumed would be the lower end of the natural range in captive conditions (supplementary material Table S2), but every 3 days those chicks in the H treatment also received an oral dose of a micronutrient supplement. This supplement was made from 1 g of a commercial micronutrient complex (Magic Antistress Mix/Performax, Feed-Food Ltd, UK), specially formulated for bird requirements, mixed with 17.6 mg of water-soluble vitamin E (50% w/w) and diluted in water (5 ml); the H chicks received an increasing dose of this suspension (6.9 µl between 1 and 5 days of age, 17.1 µl between 6–10 days, 25.28 µl between 11 and 15 days, and 28.1 µl between 16 and 40 days) in order to match their increasing size. The main components of the commercial micronutrient supplement were vitamins A, D_3, C, E, B and K and essential minerals Se, $MgSO_4$, $MnSO_4$ and $ZnSO_4$ (supplementary material Table S3). Given that vitamin E and C were qualitatively and quantitatively the most important micronutrients present in the micronutrient complex, the dosage was adjusted to be approximately double the daily amount of vitamin E and C estimated to be consumed by chicks on the low-micronutrient diet (supplementary material Table S4). These calculations assumed that birds consumed the two types of seeds present in the low micronutrient seed mix (common and red millet, 1:1) in direct proportion to their availability (McGraw et al., 2002) and that chicks had a daily seed consumption of 0.74 g when aged 1 to 5 days, 1.83 g when aged 6–10 days, 2.7 g when aged 11–15 days and 3 g from day 16 onward (Zann and Bamford, 1996). Similar manipulations of the micronutrient intake have been successfully used to quantify the effect of diet quality on development, sexual maturation and fitness in this species (i.e. daily increase in micronutrients of between 4 and 71% of the base level for birds receiving the dietary supplement; calculation based on our own data and the data provided in Birkhead et al. (1999), Blount et al. (2003), Royle et al. (2003). Broods in the L group were handled every 3 days in the same way as the H birds and received an oral dose of the same amount of water but lacking the micronutrient complex.

Quantification of oxidative status

Non-enzymatic total antioxidant capacity (TAC)

The total non-enzymatic antioxidant defence capacity was measured in plasma using the method described by Erel (2004). The main antioxidants contributing to this assay are hydrophilic antioxidants such as the –SH group of proteins, uric acid and vitamin C, and to a lesser extent, vitamin C and others hydrophobic antioxidants. In brief, plasma samples (5 µl) were diluted 1:2 in ultra-pure water and allowed to react with a coloured 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS) in a 96-well microplate. The ABTS is decolourised by antioxidants according to their concentration and antioxidant capacity (Erel, 2004). This change in colour was measured as a change in absorbance at 660 nm (Thermo Scientific Multiskan Spectrum spectrometer, Fisher Scientific UK Ltd, Loughborough, UK) and the assays were calibrated with Trolox. Levels of non-enzymatic plasma antioxidant capacity were expressed as millimoles of Trolox equivalent per

litre. Analyses were carried out in duplicate and were repeatable at each age (20 days: $r=0.75$, $F_{119,120}=4.506$, $P<0.001$; 40 days: $r=0.75$, $F_{116,117}=5.565$, $P<0.001$; 90 days: $r=0.76$, $F_{111,112}=6.228$, $P<0.001$; inter-assay CV=5.73%). Repeatability estimates were calculated for all analyses as described in Lessells and Boag (1987). During the TAC and GPx assays (see below), samples from the same individual were run on the same plate and the plate included the same number of individuals from each one of our four experimental groups in order to distribute the measurement error equally among treatments and ages.

Enzymatic antioxidant defences

Enzymatic antioxidant defences were assessed by analysing RBC activities of GPx, the most important and widespread intracellular antioxidant enzyme (Halliwell and Gutteridge, 2007). GPx catalyses the reduction of hydroxyperoxides and its main function is to protect against the damaging effect of endogenously formed hydroxyperoxides. GPx activity was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI, US; Catalog Number 703102), and following the manufacturer's instructions. Briefly, this assay measures GPx activity by way of a coupled reaction with glutathione reductase. Glutathione is oxidised during reduction of an organic hydroperoxide (cumene) by GPx, and is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP$^+$ decreases absorbance at 340 nm which is directly proportional to the GPx activity of the sample. During the assays, all samples (RBC lysate) were diluted 1:25 with dilution buffer prior to analysis, and reactions were carried out in a 96-well plate and the absorbance monitored at 340 nm (Thermo Scientific Multiskan Spectrum spectrometer, Fisher Scientific UK Ltd, Loughborough, UK) at five time points spanning 5 min. All samples were run in duplicate and activity measures were expressed as nmol min^{-1} mg^{-1} protein. GPx concentrations were highly repeatable at each age (20 days: $r=0.90$, $F_{118,119}=19.019$, $P<0.001$; 40 days: $r=0.89$, $F_{116,117}=17.750$, $P<0.001$; 90 days: $r=0.91$, $F_{116,117}=21.630$, $P<0.001$; inter-assay CV=5.79%).

Oxidative damage

The level of oxidative damage to lipids was assessed by quantifying in duplicate the concentration of plasma malondialdehyde (MDA) by high-performance liquid chromatography, following the method described by Karatas et al. (2002) but modifying the volume of sample (10 µl) and reagents as describe previously (Noguera et al., 2011a). MDA is one of the major secondary oxidation products of polyunsaturated fatty acids (Gutteridge and Halliwell, 1999). The absorbance of the eluent was monitored at 254 nm and quantified relative to external standards (calibration curves, $R^2=0.999$). Lipid peroxidation was expressed as micrograms of MDA per millilitre of plasma and showed a high repeatability at each age (20 days: $r=0.99$, $F_{117,118}=329.80$, $P<0.001$; 40 days: $r=0.99$, $F_{117,118}=838.521$, $P<0.001$; 90 days: $r=0.99$, $F_{114,115}=1621.415$, $P<0.001$).

Statistical analyses

Body mass growth, fat stores and development of pectoral muscle

All statistical analyses were carried out using IBM SPSS 22 for Windows. The effect that the micronutrient treatments had on M_b, fat stores and pectoral muscle was analysed using linear mixed effect models (LMM) or generalised lineal mixed-effect model (GLMM) as appropriate. The models included age, sex, micronutrient treatment during the growth period (H or L) and micronutrient treatment during sexual maturation (H or L) as categorical fixed factors as well as all their possible interactions. For the models analysing pectoral muscle and fat stores, M_b was also included as a covariate. Bird identity was included as a random factor in all models.

Antioxidant defences and oxidative damage

The effect of our micronutrient treatments on antioxidant defences (TAC and GPx activity in RBCs) and oxidative damage level (plasma MDA) were analysed by using LMM. The models included age, sex, micronutrient treatment during growth period (H or L) and micronutrient treatment during sexual maturation (H or L) as categorical fix factors as well as all their possible interactions. Bird M_b was included as covariate in all models. In all models, bird identity was included as a random factor to account for the non-independence of measures from the same individual. For the models analysing TAC and GPx, the identity of the microplate where the samples were assayed was also included as a random factor.

The identity of the two nests between which chicks were switched ('cross-foster dyad') and the identities of biological and rearing nest (nested within the cross-foster dyad) were initially also included as random effects in the growth (M_b, pectoral muscle and fat stores) and oxidative stress (TAC, GPx and MDA) models to control for non-independence of chicks from the same genetic or rearing family. However, these random factors had no significant effect and hence, were removed in subsequent analyses. All models were simplified by removing non-significant terms (in a backward deletion procedure), starting from four-way interactions; reported significance levels are those estimated when non-significant terms were dropped from the model. The Satterthwaite approximation was used for the estimation of denominator degrees of freedom, and *post hoc* comparisons were carried out using Fisher's least significant difference (LSD) test. Sample sizes in some analyses can slightly differ because a small number of chicks died over the course of the experiment (9 out of 126) and because in a few cases there was insufficient blood in a sample to perform all the lab analyses. Data are presented as means±s.e. and significant level was set at 0.05. The statistical power of the tests was analysed by using GPOWER v3.1 freeware (Faul et al., 2009), following Cohen's (1992) conventions.

Within-individual consistency over time and covariation structure of measures

The within-individual consistency over time was assessed by calculating repeatability estimates (r) for TAC, GPx and MDA following the method described in Lessells and Boag (1987). These analyses only included birds for which TAC, GPx and MDA levels were available at all ages ($N=110$). In addition, we also assessed the covariance among these oxidative stress markers by calculating the Pearson's correlation coefficients separately for each age (20, 40 and 90 days old). Results of these analyses together with power estimates (see above) are reported in the text.

Author contributions
The experiment was conceived and designed by J.C.N., P.M., and N.B.M. Data were acquired and analysed by J.C.N and the interpretation of the data and writing were done by J.C.N. with the support of P.M., and N.B.M.

Supplementary material
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Table S1. Micronutrient content (µg/g) in different types of seeds commonly present in commercial seed mixtures for tropical birds. Only the values of those micronutrients for which there were published data are shown.

	Pearl millet	Red millet	Common millet	Yellow millet	Canary seed
Vitamin A	0.2	0.1	0	1.9	-
Vitamin E	64.8	21.6	18.9	31	31.6
Vitamin B1	3.1	2.4	7.3	4.8	8.5
Vitamin B2	2.9	1.1	3.8	1.2	1.6
Vitamin C	20.0	10	35	25	-

Sources: Abdel-Aal et al., 2011; Blount et al., 2003; Li and Beta, 2012; Nambiar et al., 2011; Royle et al., 2003; Ruskin, 1999; Shahidi, 1997; Simwemba et al., 1984 and The USDA Nutrient Database for Standard Reference (SR27)

Table S2. Mean, standard deviation (SD) and range of variation (µg/day) of micronutrients intake in normal aviary conditions, and the experimental treatments for an adult-size bird and assuming a daily intake of 3g of seeds. For aviary conditions, values are based in the estimated micronutrient content of 4 different seed mix [common millet-yellow millet-canary seed (3:1:1); pearl-common millet (1:1); yellow-common millet (1:1) and pearl-yellow (1:1)].

	Aviary condition (µg/day)			Experimental treatments	
	Mean	SD	range	Low	High
Vitamin A	1.85	1.37	0.3-3.15	0.09	1.22
Vitamin E	103.92	36.23	71.6-143.7	60.8	81.06
Vitamin B1	16.67	3.92	11.8-21.1	14.6	16.47
Vitamin B2	8.05	1.64	6.1-10.1	7.4	11.15
Vitamin C	79.5	9.40	67.5-90	67.5	90.02

Table S3. Micronutrient composition of 'Magic Antistress Mix'/'Performax' (Feed-Food Ltd, UK).

Fat-soluble vitamins	Concentration (units)
Vitamin A	2,000.000 (IU/kg)
Vitamin D3	10 (mg/kg)
Vitamin E	2 (g/kg)
Vitamin K	1 (g/kg)
Water-soluble vitamins	
Vitamin B1	1 (g/kg)
Vitamin B2	2 (g/kg)
Vitamin B5	6 (g/kg)
Vitamin B6	2.3 (g/kg)
Vitamin B12	11 (mg/kg)
Vitamin C	12 (g/kg)
Amino acids	
Dl-methionine	7.5 (g/kg)
L-Lysine	15 (g/kg)
Betaine	2.5 (g/kg)
L-Carnitine	2.5(g/kg)

The Mix also contained trace amounts of $MgSO_4$, $MnSO_4$, $ZnSO_4$, $NaHCO_3$, KCl, NaCl, Se, citric acid, formic acid, propionic acid, sorbic acid.

Table S4. Estimated daily consumption (µg every third day; see text) of different diet micronutrients by zebra finches fed with a low, standard or high micronutrient diet during different age periods. The low and high diets were the ones used in the experimental manipulations; the standard one is the commercial diet recommended for cage finches (Johnson & Jeff Ltd, UK) that all birds received before and after the experiment. All calculations assume that birds consumed the different types of seeds present in the seed mix (see text) in direct proportion to the degree to which they were available, and a daily consumption of seeds of 0.74 g on days 1 to 5. 1.83 g on days 6 to 10. 2.7g on days 10 to 15 and 3 g from day 16 onward.

Age	0-5 days			6-10 days			11-15 days			16 days onward		
	Low	Standard	High	Low	Standard	High	Low	Standard	High	Low	Standard	High
Vitamin A	0.02	0.30	0.77	0.05	0.70	2.09	0.08	1.00	3.13	0.09	1.10	3.48
Vitamin E	15.00	17.70	30.00	37.10	46.70	74.20	54.70	64.40	109.40	60.80	71.60	121.60
Vitamin B1	3.60	5.20	4.99	8.90	12.90	12.33	13.10	19.00	18.15	14.60	21.10	20.23
Vitamin B2	1.80	2.10	4.54	4.50	5.20	11.35	6.60	7.70	16.64	7.40	8.50	18.66
Vitamin C	16.70	19.20	33.41	41.20	47.60	82.43	60.80	70.20	121.65	67.50	78.00	135.06

Table S5. Summary of mixed models (LMM and GLMM) for the effects of micronutrient treatments and covariates on body mass growth, pectoral muscle and body fat stores. 'T-growth' and 'T-maturation' refer to the dietary treatments during the growth period and the period of sexual maturation, respectively. Variables retained in the final models are shown in bold. For removed terms, significance levels are those estimated when terms were dropped from the model.

	Body mass			Pectoral muscle			Fat stores		
Source of variation	F or Wald Z	$df_{n,d}$	P	F or Wald Z	$df_{n,d}$	P	F or Wald Z	$df_{n,d}$	P
Age	**3223.167**	**4,462.97**	**<0.001**	0.685	3,464	0.562	**60.934**	**3,394.60**	**<0.001**
Sex	**5.893**	**1,116.90**	**0.017**	0.121	1,463	0.729	**6.496**	**1,119.26**	**0.012**
Body mass	-	-	-	**7.538**	**1,471**	**0.006**	**55.823**	**1,434.68**	**<0.001**
T-growth	0.619	1,115.09	0.433	0.090	1,462	0.764	0.614	1,115.53	0.435
T-maturation	1.113	1,115.94	0.294	0.668	1,467	0.414	0.281	1,114.51	0.597
T-growth x T-maturation	0.077	1,111.80	0.782	0.100	1,460	0.752	0.588	1,112.13	0.445
T-grow x age	0.757	4,458.95	0.786	0.115	3,456	0.952	1.158	3,342.96	0.326
T-maturation x age	0.432	4,455.00	0.786	0.332	1,461	0.565	1.911	3,345.90	0.128
Sex x age	**12.145**	**4,462.97**	**<0.001**	0.108	3,453	0.955	0.663	3,347.43	0.575
Sex x T-growth	0.704	1,112.76	0.403	0.005	1,459	0.944	0.008	1,111.99	0.928
Sex x T-maturation	2.535	1,113.74	0.114	0.022	3,450	0.996	3.529	1,114.35	0.630
Sex x T-growth x T-maturation	0.609	1,110.90	0.437	0.011	1,449	0.916	0.341	1,110.48	0.560
Sex x age x T-growth	2.210	4,450.98	0.067	0.036	3,440	0.991	1.020	3,333.74	0.384
Sex x age x T-maturation	1.027	4,447.03	0.393	0.057	3,443	0.982	0.768	3,330.67	0.513
T-growth x T-maturation x age	0.801	4,442.95	0.525	0.063	3,446	0.979	1.562	3,336.96	0.199
Sex x age x T-growth-T-maturation	1.727	4,391.01	0.143	0.104	3,437	0.958	1.672	3,328.16	0.173
Random factors									
Individual	**5.289**	-	**<0.001**	0[a]	-	1	**4.216**	-	**<0.001**

[a]Parameter estimate bound to zero, therefore no Z and SE are estimated.

Table S6. Pearson's correlation coeficients among oxidative stress measures. Significant correlations are shown in bold.

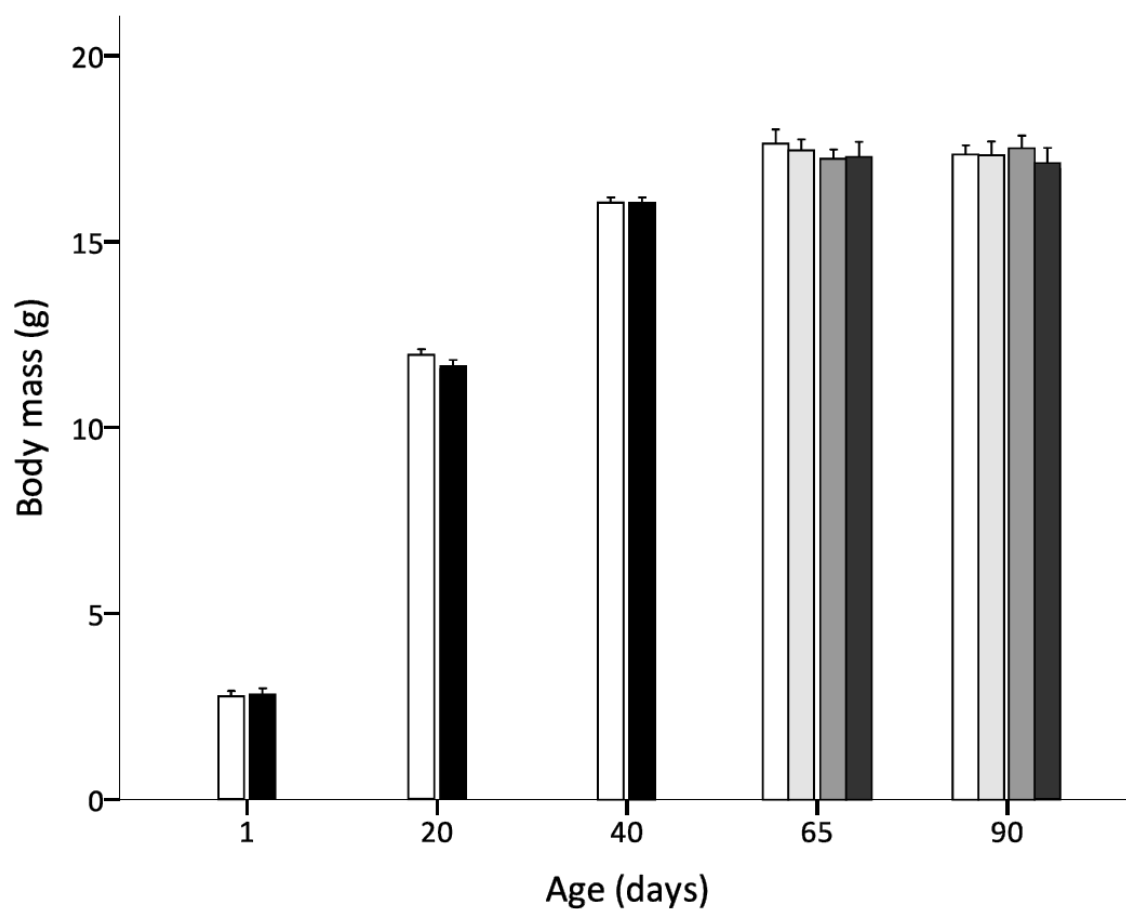|	20 days			40 days			90 days		
	TAC	GPx	MDA	TAC	GPx	MDA	TAC	GPx	MDA
TAC	-			-			-		
GPx	r=0.041 p=0.657	-		r=0.084 p=0.365	-		r=0.143 p=0.132	-	
MDA	r=0.155 p=0.093	r=-0.58 p=0.528	-	**r=0.283 p=0.002**	r=-0.133 p=0.154	-	r=0.165 p=0.084	**r=-0.216 p=0.020**	-

Fig. S1. Body mass (mean + SE) during the growth period (0-40 days) and the period of sexual maturation (40-90 days). Birds on the Low micronutrient diet are represented by white bars and those on the High diet by black bars. Birds that were switched at 40 days from the Low to the High diet are represented by light grey bars while those switched in the opposite direction (from High to Low) are shown as dark grey bars.
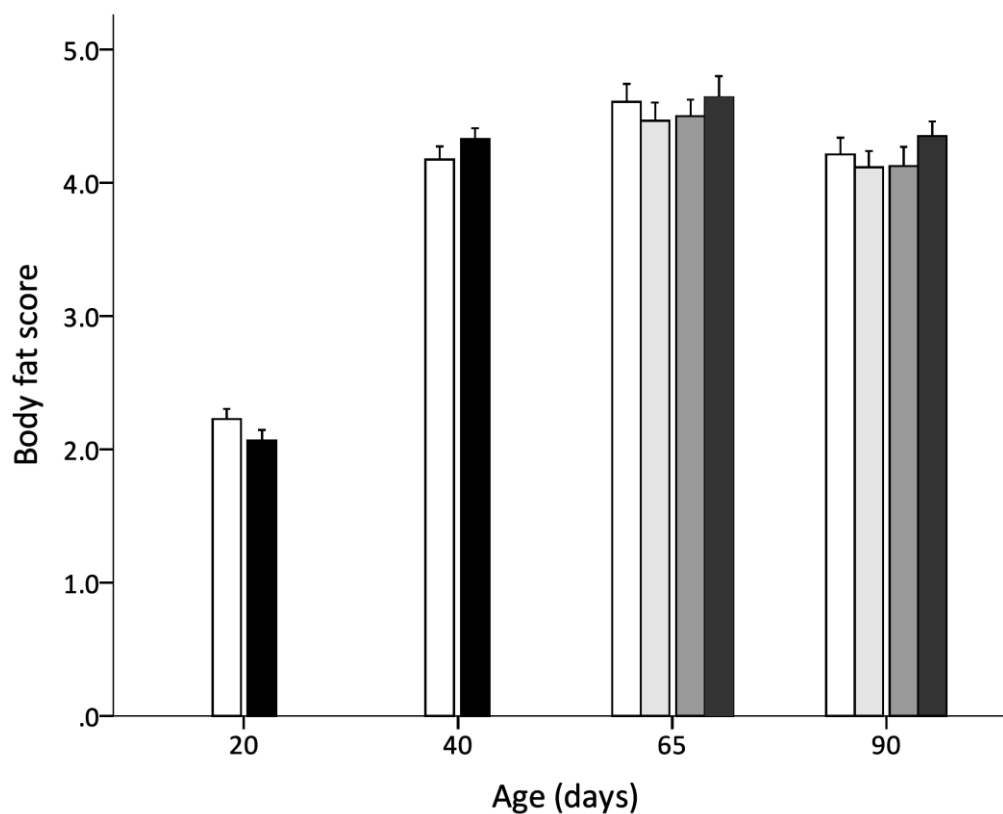
Fig. S2. Body fat scores (mean + SE) during the growth period (0-40 days) and the period of sexual maturation (40-90 days). Birds on the Low micronutrient diet are represented by white bars and those on the High diet by black bars. Birds that were switched at 40 days from the low to the high diet are represented by light grey bars while those switched in the opposite direction (from High to Low) are shown as dark grey bars.