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## RESEARCH ARTICLE

# Dietary flavonoids advance timing of moult but do not affect redox status of juvenile blackbirds (*Turdus merula*)

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## ABSTRACT

Flavonoids are the most abundant plant polyphenols, widely occurring in fruits and berries, and show a strong antioxidant activity *in vitro*. Studies of avian species feeding on berries suggest that dietary flavonoids have health-promoting effects and may enhance the expression of melanin-based plumage traits. These effects are probably mediated by the antioxidant activity of flavonoids. However, the effect of dietary flavonoids on oxidative status has never been investigated in any bird species. We analysed the effects of dietary flavonoids on blood non-enzymatic antioxidants and protein oxidative damage of juvenile European blackbirds (*Turdus merula*). In addition, we analysed the effects of flavonoid-enriched diet on body condition and on the timing of moult from juvenile to adult plumage. Dietary flavonoids did not significantly affect redox status but significantly advanced the onset of moult, hastening plumage development. Moulting birds showed higher protein oxidative damage compared with those that had not yet started moulting. The probability of initiating moult after 40 days of dietary treatment was higher for birds with low circulating levels of oxidizing agents and high glutathione concentration. The metabolization of flavonoids could have altered their redox potential, resulting in no net effects on redox status. However, flavonoid consumption before and during moult may contribute to enhance plumage development. Moreover, our findings suggest that moulting feathers may result in redox imbalance. Given their effect on moult and growth of melanin-rich feathers, fruit flavonoids may have contributed to shape plant fruiting time in relation to fruit consumption preferences by birds.

KEY WORDS: Anthocyanins, Antioxidants, Berries, Feather replacement, Melanogenesis, Oxidative stress

## INTRODUCTION

Living organisms have evolved sophisticated mechanisms to cope with the pro-oxidant activity of reactive species in order to delay, prevent or reduce oxidative damage to biomolecules, cells and tissues. These mechanisms involve a large variety of enzymatic and non-enzymatic molecular antioxidants (Pamplona and Costantini, 2011). Dietary antioxidants, mainly acquired via plant food, crucially contribute to regulate the redox status of animals, as they can influence antioxidant defences and resistance to oxidative stress (Costantini, 2014). According to their chemical properties, dietary antioxidants are grouped into three broad classes: vitamin E, carotenoids and polyphenolic antioxidants (Costantini, 2014). Polyphenols constitute a large class of secondary plant metabolites that are abundant in fruits and vegetables. They include flavonoids (among which are anthocyanins, flavan-3-ols, flavones, flavanones and flavonols; Tsao, 2010), which collectively are the most abundant antioxidants in nature and show a strong antioxidant activity *in vitro* (Vinson et al., 1995). Coherently, studies of laboratory animals *in vivo* and of humans suggest that flavonoid consumption may positively affect organismal redox status by reducing oxidative damage, promoting endogenous antioxidant defences and upregulating antioxidant gene expression (e.g. Minato et al., 2003; Weisel et al., 2006). However, the actual antioxidant activity of flavonoids *in vivo* is debated as they are extensively metabolized, resulting in major alterations of their redox potential (Williams et al., 2004; Halliwell, 2008). Besides their possible effects on redox status, flavonoids accomplish several health-promoting actions. For example, experiments carried out on rats showed that flavonoids can have beneficial effects on cancer and cardiovascular disease (see García-Lafuente et al., 2009, and references therein), and retard the course of neuronal and behavioural ageing (Joseph et al., 1999; Galli et al., 2002).

Most wild fruits and berries contain high concentrations of flavonoids and are largely consumed by wild animals, including non-exclusively frugivorous birds that rely on these resources during specific phases of their life cycle (Snow and Snow, 1988). Non-exclusively frugivorous birds can be regarded as optimal candidates to study the effects of flavonoid diet because: (1) their diet can include large amounts of flavonoid-rich berries (*Ribes*, *Vaccinium* and *Sambucus* spp.; Määttä-Riihinen et al., 2004); and (2) they allow comparison of experimental groups fed with flavonoid-enriched diets compared with control diets without flavonoids (e.g. Catoni et al., 2008). Nonetheless, surprisingly few studies have used non-exclusively frugivorous birds as model species to investigate the health-promoting effects of dietary flavonoids. The most comprehensive studies of dietary flavonoid effects on wild birds have focused on blackcaps (*Sylvia atricapilla*) (Catoni et al., 2008, 2009; Schaefer et al., 2008). Blackcaps appear to actively select flavonoid-rich food and use fruit colour as a cue of food anthocyanin content (Schaefer et al., 2008). Moreover, anthocyanin-supplemented blackcaps showed a stronger humoral immune response after an immune challenge compared with birds fed a control diet (Catoni et al., 2008). Such positive effects of anthocyanins on immune function may depend on their antioxidant properties, which could allow the birds to cope with the increase in reactive oxygen and nitrogen species released by activated immune cells (Costantini and Dell'Omo, 2006; Hõrak et al., 2007). Finally,

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flavonoid consumption enhanced the visibility of the sexually dichromatic black head feathers of males (but not females) against the background, supporting the idea that melanin-based head coloration may signal immune function and suggesting that feather melanin biosynthesis can be affected by a flavonoid-enriched diet (Catoni et al., 2009).

The fitness benefits deriving from consumption of flavonoids may relate, either directly or indirectly, to their antioxidant properties. However, to our knowledge no previous study has tested the effect of dietary flavonoids on the redox status of any bird species. We aimed at filling this gap by experimentally assessing the effects of dietary flavonoids on the redox status [total non-enzymatic plasma antioxidant capacity, total amount of plasma oxidizing agents, protein oxidative damage and glutathione (GSH) concentration] and other condition indices (body mass, fat score) in juvenile European blackbirds (*Turdus merula*) during the post-fledging period. The blackbird is a non-exclusively frugivorous bird species that largely feeds on flavonoid-rich berries during the non-breeding season (Clement et al., 2000).

In addition, we investigated the effects of dietary flavonoids on feather replacement during the transition from the juvenile to the adult plumage. Adult blackbirds express a eumelanin-based black (males) or dark grey/brown (females) plumage (McGraw, 2006; Galván and Jorge, 2015), and previous evidence suggests significant links between dietary antioxidants and melanin biosynthesis (Catoni et al., 2009). By improving antioxidant defences, flavonoids may enhance melanocyte development (Bowers et al., 1994, 1999; Arck et al., 2006). Flavonoids may also increase the bioavailability of calcium, a crucial element favouring melanin biosynthesis (e.g. McGraw, 2007). Moreover, melanin deposition in tissues is critically related to low levels of circulating GSH (Galván and Alonso-Alvarez, 2008), the most abundant intracellular non-enzymatic endogenous antioxidant (Costantini, 2014). Thanks to their antioxidant properties, dietary flavonoids administered before or during moult may allow individuals to reduce circulating GSH and promote melanin biosynthesis without incurring oxidative stress.

We expected a generally improved redox status (higher antioxidant defences and reduced oxidative damage) and body condition (higher body mass and fat load) of flavonoid-supplemented blackbirds compared with controls. Moreover, we expected dietary antioxidants to affect eumelanic adult plumage development by, for example, promoting post-juvenile moult in terms of the timing of onset and/or advancement. In addition, we investigated the effects of redox status and body condition indices on moult status, while expecting individuals in better condition (irrespective of dietary flavonoid supplementation) to moult earlier and/or more extensively. Concomitantly, as moult is a highly energy demanding process that may induce oxidative stress (Raja-aho et al., 2012), we compared the redox status of actively moulting individuals versus those that have not yet started moult.

## MATERIALS AND METHODS
### Study species, maintenance and dietary flavonoid supplementation protocol
The European blackbird (*Turdus merula* Linnaeus 1758) is an omnivorous, medium-sized (80–100 g), widely distributed passerine bird that mainly feeds on invertebrates during the breeding period and consumes large amounts of berries and fruits from late summer to early winter (Clement et al., 2000). Blackbirds commonly breed in urban and suburban habitats throughout their range, where they are year-round residents (Cramp, 1998). Nestlings

are often found in gardens and picked up by people who, in most cases, erroneously believe they are orphaned and take them to wildlife rehabilitation centres. Juvenile blackbirds recruited for this study were obtained from two wildlife rehabilitation centres located in central-northern Italy. They were admitted to these centres at an approximate age of 10 days and were hand-raised until ca. 30 days of age (approximate time of fledging). While at the rehabilitation centres, blackbirds were fed only mealworm larvae (*Tenebrio molitor*). When birds became able to feed on their own, they were taken to the Institute for Environmental Protection and Research (ISPRA, Ozzano Emilia, Bologna, Italy) aviaries and individually housed in sheltered cages (60×25×35 cm) under natural temperature and photoperiod until the end of the experiment (55 days). Temporary housing and food supplementation of blackbirds were carried out under the prescriptions of Article 7 (5) of the Italian law 157/1992, which regulates wildlife life management and research activities on mammals and birds. A total of 71 juveniles arrived at ISPRA in three different batches of 18 (7 May 2014), 27 (8 June 2014) and 26 birds (30 June 2014). Within each batch, individuals were assigned alternately to two different dietary treatments: flavonoid-supplemented diet (35 birds) and control diet (36 birds). All experimental individuals were fed with a commercial semi-synthetic food optimized for small omnivorous birds (Delikat Insect pâté, Kiezebrink Ltd, UK) for 55 days, after which they were all released back into the wild. No mortality occurred during the experiment. Water was available *ad libitum* and replaced daily. The feeder was replenished every morning with ca. 13 g of food (i.e. the amount of food we preliminarily estimated was sufficient to satiate most individuals; details not shown). We therefore provided each individual with this amount of food and verified that no appreciable amount of food was left in the feeder every day. The commercial food content was 16% crude protein, 11% crude fat, 4.5% crude ash, 2.5% crude fibre, added vitamins and chemical elements (per kg of food: 15,000 IU vitamin A, 3000 IU vitamin D3, 20 mg vitamin E, 3 mg vitamin B1, 10 mg vitamin B2, 10 mg vitamin B6, 0.02 mg vitamin B12, 5 mg copper, 0.5 mg cobalt, 30 mg iron, 70 mg magnesium). This food was carefully chosen as it contained no berry or fruit derivatives, and thus contained no flavonoids. Each individual of the flavonoid-supplemented group received a daily amount of 32 mg of bilberry (*Vaccinium myrtillus*) ethanol extract (Linnea SA, Riazzino, Switzerland), corresponding to 12 mg flavonoids, mixed in the food. The amount of flavonoids per bird body mass administered in this study (12 mg per 73.6±0.86 g, mean±s.e.m.) was similar to that used by Catoni et al. (2008) in their studies on blackcaps (2.8 mg per 16.8±0.1 g), which provided evidence of positive effects of flavonoids on fitness-related traits.

The daily food intake (12.9±0.3 g per individual) was measured four times for each batch and did not significantly differ between the two groups (mixed model with batch as random effect, effect of treatment=0.31±0.44, $t$=0.70, $P$=0.49). After 40 days of dietary treatment, a blood sample (ca. 200 µl) was taken from the brachial vein of each bird into microhaematocrit capillary tubes. Plasma was separated by centrifugation and stored at −80°C for the assessment of redox status (see below). All birds were sexed molecularly by typing of the *CHD* gene (Griffiths et al., 1998).

### Body condition indices and moult assessment
At 40 and 55 days, we recorded body mass (accuracy 0.1 g), tarsus length (0.1 mm), fat score (visible amount of subcutaneous fat deposition inside the furcula and on the abdomen; Kaiser, 1993) and moult status. Moult status was scored as moult onset (0=no visible

sign of new feathers, 1=presence of at least one new feather; juveniles perform a partial summer moult which involves body feathers, wing coverts and a few flight feathers; Jenni and Winkler, 1994) and moult advancement (0=0%, 1=0.1–10%, 2=11–60%, 3=61–99%, 4=100% of the body feathers and wing coverts moulted). Both variables were visually estimated by J.G.C.

### Assessment of redox status

#### Plasma non-enzymatic antioxidant capacity and total oxidant status
The total non-enzymatic antioxidant capacity (TAC) of plasma was measured by using the OXY-Adsorbent test (Diacron, Grosseto, Italy). This test quantifies the ability of plasma non-enzymatic antioxidants to cope with the oxidant action of hypochlorous acid (HClO). Plasma (5 μl) was diluted 1:100 with ultrapure water; 5 μl of the diluted plasma was added to 200 μl of a titred HClO solution. The solution was incubated for 10 min at 37°C and, subsequently, 5 μl of an alkyl-substituted aromatic amine solubilized in a chromogenic mixture was added. The amine is oxidized by the residual HClO, forming a pink-coloured complex, whose intensity can be read at 492 nm using a spectrophotometer. The colour intensity is directly proportional to the HClO excess, while it is inversely related to the plasma TAC. A standard sample of known TAC and a blank sample (5 μl of ultrapure water) were processed in the same plates of blackbird plasma and used as a reference. TAC was expressed as μmol neutralized HClO ml$^{-1}$. The mean (±s.d.) TAC intra-assay coefficient of variation (CV) was 2.8±0.5% (N=3 replicates), while the mean inter-assay CV was 6.9±0.5% (N=3 assay plates).

The total oxidant status (TOS) was measured by a colorimetric method and reflects the overall content of circulating oxidizing agents in plasma (Erel, 2005). The oxidants in the plasma oxidize the ferrous ion-o-dianisidine complex to the ferric ion, which reacts with Xylenol Orange to give a coloured (blue) complex. The intensity of colour can be measured by a spectrophotometer at λ=535 nm and is proportional to the total amount of oxidants in the plasma. The assay was calibrated by drawing a standard curve with serial dilution of hydrogen peroxide ($H_2O_2$) and the results were expressed as nmol $H_2O_2$ equivalent ml$^{-1}$. The mean TOS intra-assay CV was 2.4±1.2% (N=3 replicates) and the inter-assay CV was 4.1±2.2% (N=3 assay plates). The oxidative status index (OSI) was calculated as the (TOS×10)/TAC ratio for each individual, whereby high values of the ratio reflect high oxidative stress.

#### Determination of circulating GSH
GSH, the most abundant and probably most important intracellular non-enzymatic antioxidant, directly neutralizes some free radicals (e.g. superoxide radical and hydroxyl radical) as well as peroxynitrite and is the electron donor for the reduction of hydroperoxides (including lipid hydroperoxides) in the reaction catalysed by GSH peroxidase (Wu et al., 2004; Dalle-Donne et al., 2008). In such reactions, GSH is oxidized to form glutathione disulphide (GSSG), which is then reduced to GSH by the NADPH-dependent GSSG reductase (GR) (Hayes and McLellan, 1999; Wu et al., 2004). Lipid hydroperoxides are major oxidants generated during aerobic metabolism and are regarded as intermediate derivatives of oxidative damage (Costantini, 2014).

We measured both total GSH (tGSH, i.e. the sum of GSH and GSSG concentration) and GSSG concentrations in whole blood as follows. Starting from ca. 80 μl whole blood, we took 20, 10 and 40 μl aliquots for the determination of tGSH, blood haemoglobin (Hb) and GSSG concentrations, respectively. tGSH and GSSG were

determined spectrophotometrically according to Giustarini et al. (2013). For tGSH determination, each whole-blood aliquot was precipitated with an equal volume of 15% trichloroacetic acid (TCA), shaken vigorously and centrifuged at room temperature (14,000 $g$ for 2 min). The supernatant was first diluted 1:100 with water. We then added to a cuvette 945 μl of 200 mmol l$^{-1}$ PBS, pH 7.4, 5 μl of 20 mmol l$^{-1}$ 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB; in 200 mmol l$^{-1}$ PBS, pH 7.4), 10 μl of sample and 20 μl of 4.8 mmol l$^{-1}$ NADPH (in 0.5% NaHCO$_3$). The contents of the cuvette were mixed and 20 μl of a 20 IU ml$^{-1}$ GR solution (in 50 mmol l$^{-1}$ phosphate buffer, pH 7.4) was added. After rapid mixing, we immediately recorded absorbance at 412 nm for 1 min. We then built a calibration curve, substituting the unknown sample with 10, 25, 50, 75 and 100 μmol l$^{-1}$ GSH standard solutions and used it to determine the tGSH concentration in our samples. Samples were measured in duplicate (CV=11.01±0.97%, mean± s.e.m.) and the mean value was used in the analyses.

For GSSG determination, 40 μl of whole blood was mixed as soon as possible after collection with 4 μl of 310 mmol l$^{-1}$ N-ethylmaleimide (NEM) by slow tilting for 1 min. NEM-treated aliquots were then precipitated with an equal volume of 15% TCA, shaken vigorously and centrifuged (14,000 $g$ for 2 min) at room temperature. The supernatant was collected and excess NEM was removed by extraction with three volumes of dichloromethane; the mixture was moderately vortex mixed for 5 min and then centrifuged at 14,000 $g$ for 30 s. We then proceeded by adding to a cuvette 925 μl of 200 mmol l$^{-1}$ PBS, pH 7.4, 5 μl of 20 mmol l$^{-1}$ DTNB (in 200 mmol l$^{-1}$ PBS, pH 7.4), 20 μl of NEM-free supernatant and 20 μl of 4.8 mmol l$^{-1}$ NADPH (in 0.5% NaHCO$_3$). The contents were mixed, 20 μl GR was added, mixed again and absorbance was recorded at 412 nm for 1 min. Afterwards, we added 10 μl of 10 μmol l$^{-1}$ GSSG, mixed it with the stirrer rod and recorded absorbance again at 412 nm for 1 min. To determine blood GSSG concentration, we first calculated $S$ (slope sample−slope blank) and $S_t$ [(slope sample+GSSG)−slope sample]. Blood GSSG was expressed as $S×[GSSG_c]/S_t×49.5×2$, where [GSSG$_c$] is the final concentration of the GSSG standard in the cuvette (0.1 μmol l$^{-1}$), 49.5 is the sample dilution factor in the cuvette and 2 is the dilution due to acidification. Samples were measured in duplicate (CV=9.99±0.68%, mean±s.e.m.) and the mean value was used in the analyses.

tGSH and GSSG concentrations were normalized to Hb concentration, determined by Drabkin's cyanmethaemoglobin method according to the manufacturer's instructions for Drabkin's Reagent (D5941 Sigma-Aldrich). Hb concentration was determined from 3 μl whole blood. A calibration curve ranging from 0 to 3 mg ml$^{-1}$ Hb was prepared. After 15 min of incubation at room temperature, samples were centrifuged (10,000 $g$, 1 min) and the supernatant read in the spectrophotometer at 540 nm. In the analyses, we included normalized tGSH (μmol l$^{-1}$ g$^{-1}$ Hb) and the GSH:GSSG ratio (higher values reflect low oxidative stress conditions) (e.g. Raja-aho et al., 2012).

#### Analysis of plasma protein oxidative damage
As proxies of oxidative damage, we measured plasma protein carbonyl content (PCO) and concentration of protein thiol groups (PSH) (Rubolini et al., 2012). Protein carbonylation constitutes a mostly irreversible type of severe oxidative damage that often results in the loss of protein structure and/or functional efficiency (Dalle-Donne et al., 2003; Barreiro and Hussain, 2010; Barreiro, 2014). Hence, high PCO levels reveal high protein oxidative damage (Dalle-Donne et al., 2003; Cabiscol et al., 2014).

**Table 1. Effects of dietary flavonoids on redox status, condition indices and moult**

| Traits | Control diet | Flavonoid diet | Estimate | F/z | d.f. | P |
|---|---|---|---|---|---|---|
| Redox status | | | | | | |
| TAC (µmol HClO neutralized ml$^{-1}$) | 208.08±10.29 | 205.24±12.67 | 2.24±17.44 | 0.02 | 1, 66 | 0.90 |
| TOS (nmol H$_2$O$_2$ equivalents ml$^{-1}$)[a] | 11.88±0.59 | 11.69±0.56 | 0.014±0.022 | 0.45 | 1, 65 | 0.51 |
| OSI[a,b] | 0.67±0.08 | 0.65±0.05 | −0.002±0.049 | 0.01 | 1, 66 | 0.97 |
| tGSH (µmol l$^{-1}$ g$^{-1}$ Hb) | 10.10±0.34 | 10.27±0.28 | −0.13±0.44 | 0.08 | 1, 66 | 0.77 |
| GSH:GSSG ratio | 97.52±14.57 | 75.88±12.40 | −11.52±20.81 | 0.31 | 1, 66 | 0.58 |
| PCO (a.u.) | 0.027±0.002 | 0.031±0.002 | 0.004±0.003 | 1.58 | 1, 65 | 0.21 |
| PSH (a.u.) | 0.43±0.03 | 0.39±0.04 | 0.037±0.054 | 0.45 | 1, 63 | 0.50 |
| Body condition indices | | | | | | |
| Body mass (40 days) (g)[c] | 72.88±0.97 | 74.31±1.44 | 0.27±1.66 | 0.03 | 1, 63 | 0.87 |
| Body mass (55 days) (g)[c] | 73.70±1.19 | 77.08±1.70 | 1.92±2.05 | 0.87 | 1, 44 | 0.36 |
| Fat score (40 days) | 1.69±0.13 | 1.63±0.14 | −0.24±0.21 | 1.27 | 1, 66 | 0.26 |
| Fat score (55 days) | 1.78±0.12 | 1.69±0.14 | −028±0.20 | 2.02 | 1, 48 | 0.16 |
| Moult | | | | | | |
| Onset (40 days) | 9/36±0.25[d] | 21/35±0.60[d] | 2.24±0.70 | 3.21 | | **0.002** |
| Onset (55 days) | 23/36±0.64[d] | 31/35±0.89[d] | 1.97±0.74 | 2.65 | | **0.010** |
| Advancement score (40 days) | 0.36±0.12 | 1.00±0.17 | 0.63±0.19 | 11.39 | 1, 65 | **0.001** |
| Advancement score (55 days) | 0.89±0.15 | 1.63±0.18 | 0.77±0.18 | 18.90 | 1, 65 | **<0.001** |

TAC, total non-enzymatic antioxidant capacity; TOS, total oxidant status; OSI, oxidative status index; tGSH, total glutathione; GSH, glutathione; GSSG, glutathione disulphide; PCO, protein carbonyl content; PSH, protein thiol groups.
[a]Variable was log$_{10}$-transformed before inclusion in the model. [b]Expressed as (TOS×10)/TAC. [c]Model also included the effect of tarsus length to account for skeletal size effect on body mass. [d]Moulting birds/total birds per group (proportion of moulting birds).
Mean±s.e.m. values for both treatments are reported together with estimated effects (±s.e.m.) from mixed models (see Materials and methods) including age class and sex as fixed effects. In models of redox and condition indices, moult onset was included as a further fixed effect. Two-way interactions between treatment and sex (all models) and between treatment and moult (models of body mass, fat score and physiological variables) were never statistically significant and were removed from all models (details not shown for brevity). Sample size is 36 individuals for the control diet and 35 for the flavonoid-supplemented diet. For binomial mixed models of moult onset, z-scores are reported as test statistics. For linear mixed models, d.f. were estimated according to the Kenward–Roger method. Statistically significant P-values are highlighted in bold.

Conversely, protein thiols are highly susceptible to oxidation and a decrease of plasma PSH concentration may indicate oxidative stress (Rossi et al., 2009; Zinellu et al., 2016).

To determine plasma PCO, carbonylated proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH) (Colombo et al., 2016). Briefly, 200 µg of plasma protein was mixed with 40 µl of 10 mmol l$^{-1}$ DNPH in 2 mol l$^{-1}$ HCl and incubated for 60 min in the dark. Protein samples were then mixed with an equal volume of 20% TCA and incubated for 10 min in ice. After centrifugation at 20,000 $g$ for 15 min at 4°C, protein pellets were washed three times with 1:1 ethanol:ethylacetate to remove free DNPH. Air-dry protein pellets were resuspended in 2×reducing Laemmli sample buffer. Derivatized protein samples (40 µg) were blotted onto a PVDF membrane under vacuum using a slot-blot apparatus. After blocking, the membrane was incubated with anti-DNP antibody (anti-dinitrophenyl–KLH antibody, rabbit IgG fraction; A6430, Molecular Probes, Eugene, OR, USA) and then with horseradish

peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG; G21234, Molecular Probes) (for further methodological details, see Dalle-Donne et al., 2007; Colombo et al., 2016). Protein carbonylation signal was visualized by enhanced chemiluminescence (ECL) (Dalle-Donne et al., 2007; Colombo et al., 2016) and normalized to Amido Black staining of the slot blot for total transferred proteins. A single determination was made for each sample. Plasma PCO content was expressed in arbitrary units (a.u.).

To determine PSH concentration, plasma proteins (100 µg) were diluted to 1 µg µl$^{-1}$ in PBS and incubated for 1 h with 15 µmol l$^{-1}$ biotin-maleimide. The reaction was stopped with 2×reducing Laemmli sample buffer. Derivatized protein samples (40 µg) were slot-blotted onto a PVDF membrane as described above, probed with streptavidin–HRP (Amersham), at 1:5000 dilution, for 2 h and visualized by ECL. A single determination was made for each sample. Plasma PSH content was expressed in a.u. All

**Table 2. Effects of redox status and body condition on moult onset and intensity after 40 days of dietary treatment**

| | Moult onset | | | Moult intensity | | | |
|---|---|---|---|---|---|---|---|
| | Estimate | z | P | Estimate | F | d.f. | P |
| TAC | −0.004±0.005 | 0.80 | 0.43 | −0.001±0.001 | 0.11 | 1, 65 | 0.74 |
| TOS | −11.6±6.01 | 1.93 | 0.06 | −2.54±1.10 | 5.34 | 1, 65 | **0.024** |
| OSI | 0.13±1.70 | 0.08 | 0.94 | −0.35±0.54 | 0.42 | 1, 65 | 0.52 |
| tGSH | 0.45±0.22 | 2.02 | **0.048** | 0.14±0.05 | 6.16 | 1, 65 | **0.016** |
| GSH:GSSG ratio | −0.006±0.004 | 1.29 | 0.20 | 0.001±0.001 | 0.01 | 1, 65 | 0.99 |
| PCO | 13.8±29.6 | 0.46 | 0.64 | 2.39±8.89 | 0.07 | 1, 65 | 0.79 |
| PSH | −6.02±2.00 | 3.00 | **0.004** | −1.09±0.47 | 5.30 | 1, 62 | **0.025** |
| Body mass | 0.06±0.05 | 1.20 | 0.24 | 0.003±0.014 | 0.05 | 1, 65 | 0.82 |
| Fat score | 0.74±0.45 | 1.63 | 0.11 | 0.07±0.12 | 0.34 | 1, 65 | 0.56 |

Parameter estimates (±s.e.m.) were obtained from mixed models (moult onset: binomial mixed model; moult intensity: linear mixed model) where we also included the effects of dietary flavonoid treatment, sex and age class. For binomial mixed models of moult onset, z-scores are reported as test statistics. For linear mixed models, d.f. were estimated according to the Kenward–Roger method. Statistically significant P-values are highlighted in bold.
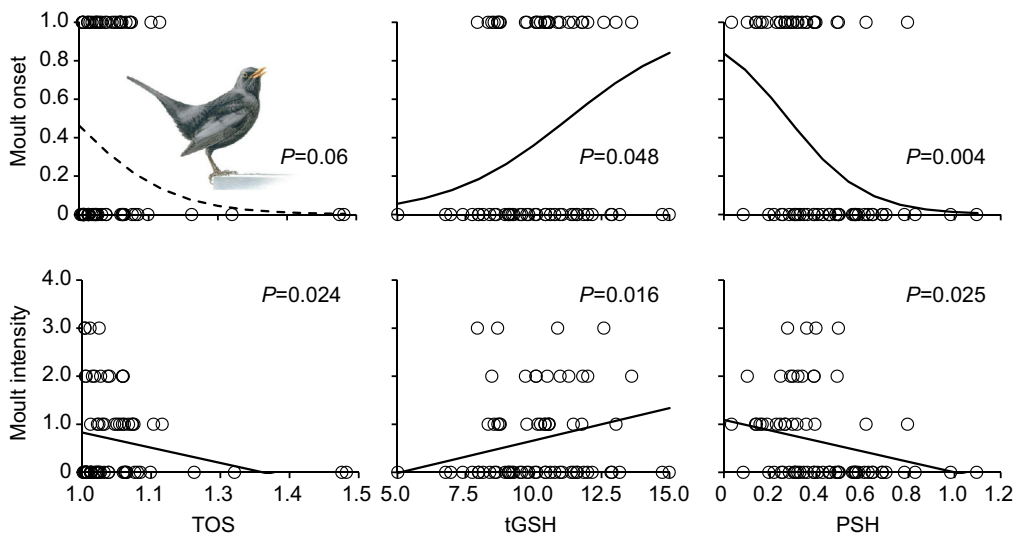
**Fig. 1. Effects of total oxidant status (TOS), total glutathione (tGSH) and concentration of protein thiol groups (PSH) on moult onset and intensity.** *P*-values and lines representing model-predicted values (while holding other model covariates at their mean value) for the variable of interest were derived by the mixed models reported in Table 2. Dashed lines denote non-significant effects.

densitometric analyses were performed using ImageJ 1.40d software (National Institutes of Health, Bethesda, MD, USA).

### Statistical analyses

The effect of dietary flavonoid supplementation on redox endpoints, condition variables and moult was tested by mixed models where we included dietary treatment (0=control diet, 1=flavonoid-supplemented diet), sex (0=female, 1=male) and age class (0=not fully grown rectrices, 1=fully grown rectrices when recruited for the experiment) as fixed effects. Batch and rehabilitation centre of provenance were included as random intercept effects. The treatment×sex interaction was included in all models, and removed if non-significant. We assumed a binomial error distribution for moult onset and a Gaussian error distribution for all other traits. For linear mixed models we checked that model residuals were normally distributed, and this was the case for all traits (details not shown for brevity). To control for the possible effects of moult on physiology, in linear mixed models of redox variables and condition we included moult onset as a further fixed effect, together with the treatment×moult interaction. TOS and OSI were $log_{10}$-transformed before the analyses to reduce the effect of a few high values on the outcome of the analyses.

The effect of each redox and condition variable on moult onset and advancement was first investigated by binomial and linear mixed models, respectively, where treatment, sex and age class were included as fixed effects, while batch and rehabilitation centre of provenance were included as random intercept effects. To assess the independent effects of condition and redox variables on moult onset

and intensity, we then included in mixed models all those variables (besides treatment, sex and age class) that had a *P*-value <0.15 in previous models.

Mixed models were fitted using SAS 9.3 PROG GLIMMIX and PROC MIXED (SAS Institute Inc., Cary, NC, USA). Means and parameter estimates are reported with their associated s.e.m. unless otherwise stated.

### RESULTS

#### Effects of dietary flavonoids on redox status, condition and moult

Redox endpoints, body mass and fat score were not significantly affected by dietary flavonoids (Table 1). In contrast, dietary flavonoids significantly affected both onset and advancement of moult: a larger proportion of flavonoid-supplemented birds had started to moult and these birds had a higher moult advancement score compared with controls (Table 1). After 40 days of dietary treatment, 60% of flavonoid-supplemented birds versus 25% of control birds had started feather replacement. The pattern persisted after 55 days (89% versus 64%; Table 1). Two-way interactions were never statistically significant and were removed from all models reported in Table 1 (details not shown for brevity).

In the mixed models reported in Table 1, no significant sex difference in any variable was detected, with the exception of tGSH, which was significantly lower in males than in females (estimate: tGSH, $-0.92\pm0.43$, $F_{1,66}=4.70$, $P=0.033$). In addition, compared with those that had not yet started moult, moulting birds had a significantly higher fat score ($0.50\pm0.21$, $F_{1,48}=5.49$, $P=0.023$) and

**Table 3. Mixed models of moult onset and intensity after 40 days of dietary treatment**

| | Moult onset | | | Moult intensity | | | |
|---|---|---|---|---|---|---|---|
| | Estimate | *z* | *P* | Estimate | *F* | d.f. | *P* |
| Dietary treatment | 4.76±1.67 | 2.84 | **0.006** | 0.57±0.17 | 10.97 | 1, 62 | **0.002** |
| Sex | 1.66±1.61 | 1.43 | 0.16 | 0.17±0.18 | 0.87 | 1, 62 | 0.35 |
| Age class | 1.92±1.34 | 1.34 | 0.16 | 0.54±0.25 | 4.76 | 1, 56 | **0.033** |
| TOS | −16.81±7.09 | 2.37 | **0.021** | −2.26±1.04 | 4.70 | 1, 63 | **0.034** |
| tGSH | 3.23±1.56 | 2.07 | **0.043** | 0.12±0.05 | 5.72 | 1, 63 | **0.019** |
| PSH | −6.94±2.91 | 2.38 | **0.020** | −0.82±0.44 | 3.53 | 1, 63 | 0.065 |
| Fat score | 0.55±0.65 | 0.85 | 0.40 | | | | |

All variables with *P*<0.15 listed in Table 2 were included in the models in addition to the effects of dietary flavonoid treatment, sex and age class. Parameter estimates are given ±s.e.m. For binomial mixed models of moult onset, *z*-scores are reported as test statistics. For linear mixed models, d.f. were estimated according to the Kenward–Roger method.

tGSH (1.15±0.53, $F_{1,63}$=4.78, $P$=0.033) but showed a decreased concentration of PSH (−0.21±0.07, $F_{1,22}$=8.45, $P$=0.008). Age class significantly affected moult advancement after 55 days and tGSH levels: older birds had higher moult advancement scores (0.72±0.21, $F_{1,67}$=11.5, $P$=0.001), but lower tGSH (−1.32±0.50, $F_{1,61}$=6.83, $P$=0.011) than younger ones.

### Redox and condition variables as predictors of moult status

In binomial mixed models where each redox and condition variable was entered separately, moult onset after 40 days of dietary treatment was significantly predicted by higher tGSH and lower PSH (Table 2, Fig. 1). Moult intensity after 40 days significantly increased with higher tGSH, but decreased with increasing PSH and TOS (Table 2, Fig. 1). Mixed models of both moult onset and intensity where we had included all variables with $P$<0.15 in previous analyses showed that both moult variables were significantly positively affected by tGSH and negatively affected by TOS and PSH, though the latter was marginally non-significant in the model of moult intensity (Table 3).

## DISCUSSION

Flavonoids are widespread and abundant in fruits and berries and are largely recognized as potent antioxidants *in vitro* (Vinson et al., 1995). Hence, flavonoids acquired through the diet have the potential to affect an individual's health state via their effect on redox balance. Previous evidence suggests that non-exclusively frugivorous birds may gain fitness advantages by feeding on flavonoid-rich berries, in terms of, for example, improved plumage quality and immune function (Catoni et al., 2008, 2009). To our knowledge, however, the direct effect of dietary flavonoids on redox status has never been assessed in any bird species. This is surprising as previously documented effects of flavonoids on fitness traits of birds may have been mediated by beneficial effects of flavonoids on redox status. Contrary to expectations, we did not observe any statistically significant difference in the redox status of juvenile blackbirds that were maintained on a flavonoid-enriched diet for 40 days post-fledging compared with those maintained on a control diet. However, dietary flavonoid supplementation affected the timing and advancement of the transition moult from juvenile to adult plumage (i.e. post-fledging moult): flavonoid-supplemented birds markedly advanced the onset of moult and had larger moult scores after both 40 and 55 days of dietary treatment compared with controls. In addition, we found that moulting birds suffered from larger protein oxidative damage (reduced PSH), and that higher circulating tGSH and lower TOS predicted onset and advancement of moult. Finally, males had lower tGSH levels than females at the end of the experiment, irrespective of dietary treatment and moult status.

### Effects of dietary flavonoids on redox status and moult

The lack of effect of dietary flavonoids on blood non-enzymatic antioxidants (both TAC and GSH) may not come as a surprise considering the ongoing debate over the direct antioxidant effects of flavonoids *in vivo* (Halliwell, 2008). In contrast to *in vitro* studies, it is indeed possible that flavonoids do not directly modulate antioxidant activity *in vivo*, as they appear to be extensively metabolized and their metabolites may not be very effective as free radical scavengers (Chen et al., 2014; Rodriguez-Mateos et al., 2014). However, flavonoids and their metabolites may promote up-regulation of antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase, whose biological activity may efficiently contribute to prevent oxidative insults (Halliwell and Gutterridge, 2003).

Additionally, we did not observe any statistically significant difference in protein oxidative damage between flavonoid-supplemented and control blackbirds. Such a lack of effect, coupled with the observations of both a higher level of oxidative damage (decreased PSH) in moulting versus non-moulting birds and a greater proportion of moulting birds among flavonoid-supplemented individuals, may suggest that dietary flavonoids could be helpful in buffering the redox imbalance induced by post-juvenile moult, either directly or via their metabolites.

In spite of the lack of effect of dietary flavonoids on overall redox status, the proportion of flavonoid-supplemented birds starting the post-juvenile moult was almost twice that observed among controls after both 40 and 55 days of dietary treatment. Moreover, we found that flavonoid-supplemented blackbirds showed a greater proportion of plumage with moulted feathers compared with controls: hence, not only the onset of moult but also its advancement was enhanced by dietary flavonoids. The mechanistic links between dietary flavonoids and moult onset may be manifold. As recalled in the Introduction, flavonoids may interact with the biosynthesis of eumelanin, the most abundant pigment in blackbird feathers (McGraw, 2006). A key role in the link between flavonoids and moult may be played by a flavonoid-mediated increase of calcium availability (Kanter et al., 2007; Yamaguchi et al., 2007). Melanin biosynthesis by melanocytes critically depends on limiting mineral elements, including calcium. For instance, calcium may upregulate the activity of tyrosinase (Buffey et al., 1993), a fundamental enzyme in the melanin biosynthesis pathway (McGraw, 2007), thus promoting melanogenesis and possibly triggering the onset of moult. A further hypothesized mechanism linking development of melanized feathers to flavonoids, namely the buffering of the low GSH levels required for melanogenesis by the antioxidant properties of flavonoids, seems less likely to apply because of the absence of detectable effects of flavonoids on antioxidant defences.

### Moult, oxidative status and sex differences

Feather replacement is a crucial process in the annual cycle of birds, as feathers are subjected to wear and tear due to mechanical abrasion and ectoparasite- and UV-mediated damage, and the maintenance of an efficient plumage is of paramount importance for fitness. However, moult is a highly energy-demanding activity (Lindström et al., 1993). This is highlighted by several studies where feather moult has been shown to be traded against competing energy-demanding processes, such as reproduction, migration and pre-migratory fuel accumulation (e.g. Rubolini et al., 2002; Echeverry-Galvis and Hau, 2013; Saino et al., 2014). Energy is required for feather keratin and pigment synthesis, for buffering the temporary loss of insulation during body feather replacement, and (in the case of flight feather moult) to compensate for the reduced foraging efficiency due to impaired mobility (Murphy, 1996). Moreover, incorporation of melanin pigments into feathers requires lowered circulating GSH levels (Galván and Alonso-Alvarez, 2008). Hence, moult may result in redox imbalance, but this topic has not been thoroughly addressed. A previous study showed that barn swallows (*Hirundo rustica*) at initial stages of flight feather moult had higher glutathione *S*-transferase activity and lipid hydroperoxides than birds that had nearly terminated moult, suggesting that moult, either directly or indirectly via increased energy expenditure due to handicapped flight, may cause oxidative stress (Raja-aho et al., 2012). Our findings strongly suggest that moult resulted in higher protein oxidative damage (reduced PSH). In addition, higher tGSH levels and reduced TOS positively

predicted moult onset and advancement. However, the direction of the causal flow of moult–redox status relationships is unclear. While the interpretation of higher PSH of moulting versus non-moulting birds is rather straightforward, the interpretation of the association between GSH and TOS is more complex. For instance, TOS did not significantly differ between moulting and non-moulting birds ($P$=0.07) while it significantly predicted moult onset and advancement. Hence, lower TOS may be a cause rather than a consequence of moult: birds with lower levels of circulating oxidizing molecules in plasma are probably in better condition and this could promote feather replacement. The same may apply to tGSH, which was significantly higher in moulting birds versus those that had not yet initiated moult. Alternatively, as low levels of GSH promote melanin biosynthesis, it may be speculated that birds that are about to start moult require lower tGSH levels to activate melanogenesis in melanocytes, while the same may not be the case once the moulting process has been initiated. Analyses of the time course of tGSH levels during moult and/or after induced feather replacement may be helpful in clarifying the link between this endogenous antioxidant and moult.

No sex differences emerged in the timing of post-juvenile moult or redox status, with the single exception of GSH, which was at a significantly lower level in males. Blackbirds show conspicuous sexual differences in melanin-based plumage coloration (Cramp, 1998). As low GSH levels can enhance melanin synthesis via several biochemical pathways (see Galván and Alonso-Alvarez, 2008), lower tGSH levels among males may be related to the higher degree of eumelanization of their body feathers compared with females. Interestingly, decreased tGSH levels among males did not translate into any sex difference in oxidative damage, suggesting that males were able to compensate for their lower degree of GSH-mediated cellular antioxidant activity, perhaps by exploiting the antioxidant properties of melanins (McGraw, 2006).

## Concluding remarks and ecological implications

Our study suggests that flavonoid consumption does not directly modulate organismal redox status in juvenile European blackbirds. However, it enhanced the process of feather renewal, probably by affecting melanin biosynthesis via several possible biochemical pathways. An effect of dietary flavonoids on eumelanin-based coloration has previously been observed in blackcaps, where the visibility against the background of the black head feather coloration of males after moult was enhanced in flavonoid-supplemented individuals versus controls. From a functional, ultimate perspective, our study suggests that natural selection should favour the consumption of flavonoid-rich food during the critical period of feather replacement. Observational evidence suggests that this may be the case, as most non-exclusively frugivorous birds of temperate areas consume large amounts of ripe flavonoid-rich berries, such as *Sambucus*, *Rubus* and *Prunus* spp., during summer–early autumn (e.g. Wheelwright, 1988; Boddy, 1991), in a period when alternative animal (invertebrate) food is abundant and when all species undergo feather replacement. Moreover, Berthold (1976) suggested that two *Sylvia* warbler species showed a preference for fruits during moult. Most previous research has concentrated on the role of berry consumption during migration or pre-migratory periods and on its effect in promoting fat accumulation and improving oxidative status during or after endurance flight, whereas the role of flavonoid-rich fruit consumption during moult has largely been overlooked. We suggest that the topic deserves more attention, as it hints at a possible contribution of fruit flavonoids in the evolution of plant fruiting time according to seasonal variation

in dietary preferences of birds. Interestingly, birds appear to discriminate among berries based on their flavonoid content, preferring those providing the highest flavonoid reward (Schaefer et al., 2008). Food choice experiments investigating preferences for flavonoid-rich food during the moulting and non-moulting periods of the life cycle, together with analyses of the plumage quality of birds fed flavonoid-enriched food compared those fed a flavonoid-poor diet, may shed further light on the possible role of flavonoids in affecting moult and plumage quality.

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