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Nest-dwelling ectoparasites reduce antioxidant defences in females and nestlings of a passerine: a field experiment

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Abstract Ectoparasites may imply a cost in terms of oxidative stress provoked by inflammatory responses in hosts. Ectoparasites may also result in costs for nestlings and brooding females because of the direct loss of nutrients and reduced metabolic capacity resulting from parasite feeding activities. These responses may involve the production of reactive oxygen and nitrogen species that may induce oxidative damage in host tissues. Our goal was to examine the effect of ectoparasites in terms of oxidative stress for nestlings and adult females in a population of pied flycatchers *Ficedula hypoleuca*. We manipulated the entire nest ectoparasite community by reducing ectoparasite loads in some nests through a heating treatment and compared them with a control group of nests with natural loads. A marker of total antioxidant capacity (TAS) in plasma and total levels of glutathione (tGSH) in red blood cells as well as a marker of oxidative damage in plasma lipids (malondialdehyde; MDA) were assessed simultaneously. Levels of tGSH were higher in heat-treated nests than in controls for both females and nestlings. Higher TAS values were observed in females from heat-treated nests. In nestlings there was a negative correlation between TAS and MDA. Our study supports the hypothesis that ectoparasites expose cavity-nesting birds to an oxidative challenge. This could be paid for in the long term, ultimately compromising individual fitness.

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Introduction

Parasites and pathogens are important evolutionary forces (Atkinson and van Riper 1991; Møller 1997) contributing to the emergence of different protection mechanisms such as behavioural defences, physical barriers (e.g. skin, scales, etc.) and the immune system (Hõrak et al. 2006; Sepp et al. 2012). The activation of some of these defences is energetically costly and this can play an important role in physiological and life history strategies of the host. In contrast to endoparasites that live in intimate contact with their host, ectoparasites are characterized by free-living stages whose key habitat is the surface of the host or their immediate environment. It has previously been shown that environmental stressors, such as parasite exposure and infection (Sorci and Faivre 2009; Costantini 2008; Saino et al. 2002) can result in oxidative stress on hosts because of the resulting upregulation of the immune system, which is the main physiological defence mechanism against parasites (Zuk and Stoehr 2002). In this context, we should consider that ectoparasites could be costly, at least in part, because they induce oxidative stress in their hosts, which is associated with long-term effects, including accelerated aging (e.g. Golden et al. 2002; Metcalfe and Alonso-Alvarez 2010). Oxidative stress is usually defined as the imbalance between the rate of production of reactive oxygen and nitrogen species (RONS) by the organism and state (levels, integrity or activity) of the antioxidant machinery

(Halliwell and Gutteridge 2007; Metcalfe and Alonso-Alvarez 2010). Endogenous antioxidants, like uric acid or glutathione, are synthesized by the organism. Exogenous antioxidants, like vitamins A, C and E, and carotenoids, must be obtained from food (Halliwell and Gutteridge 2007). When an imbalance in antioxidants occurs, it may lead to oxidative damage in important biomolecules (lipids, proteins and DNA), which could impair their functionality (Finkel and Holbrook 2000; Halliwell and Gutteridge 2007).

The effect of any type of parasite on oxidative stress is mediated by the immune response of the host. In the case of ectoparasites, small wounds are produced when they bite the host, through which an oral secretion is introduced into the skin tissues. These secretions have antigenic properties that induce an inflammatory response (Baron and Weintraub 1987; Owen et al. 2010). Different immune cells generate RONS in order to destroy pathogens (Halliwell and Gutteridge 2007; Sorci and Faivre 2009; Costantini and Møller 2009). However, when uncontrolled, these RONS may also affect the host tissues, leading to oxidative stress (Sorci and Faivre 2009). Furthermore, the induction of an immune response due to ectoparasite activity may also increase metabolic activity (Møller et al. 1994; Demas et al. 1997), thereby raising RONS levels, and hence, favour an imbalance in antioxidant status (Finkel and Holbrook 2000). This, combined with an energetically costly adjustment of other physiological and behavioural traits (Richner et al. 1993; Christe et al. 1996; Cantarero et al. 2013a), may also result in higher levels of tissue oxidative damage under stressful conditions (von Schantz et al. 1999; van de Crommenacker et al. 2012). In summary, oxidative stress can be considered as a proximate mechanism involved in the cost of parasitism, and is key to understanding life history trade-offs between growth, reproduction and self-maintenance (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010; Metcalfe and Monaghan 2013).

Our goal is to examine the effect of ectoparasites in terms of oxidative stress in a population of wild birds. Birds are suitable for the study of oxidative stress as they are generally long lived, in comparison to mammals of similar size, and they apparently have strategies to cope with a much higher metabolic rate and energy expenditure than mammals (Costantini 2008). Bird-ectoparasite associations have also provided many influential examples of parasite-mediated evolution and ecology (Proctor and Owens 2000). Many ectoparasite species live and reproduce within the nest material, feeding intermittently or continuously on hosts. Therefore, nest properties can influence host and parasite reproductive success and the outcome of host-parasite interactions (Heeb et al. 2000). In cavity-nestling birds in particular, the microclimatically stable environment of nests and the presence of an abundant food supply offer excellent breeding conditions for ectoparasites (Collias and Collias 1984).

Ectoparasites may result in costs for nestlings in terms of compromised growth and condition (Richner et al. 1993; Heeb et al. 1998; Heeb et al. 2000; Tomás et al. 2008; Brommer et al. 2011; Cantarero et al. 2013a) because of the direct loss of nutrients and reduced metabolic capacity resulting from parasite feeding activities (Simon et al. 2004). Moreover, in iteroparous species, the effect of parasites on offspring development may, in turn, affect reproductive decisions of parents when they face the classic trade-off in the investment of limiting resources between current and future reproductive events in order to maximise their own individual fitness (reviewed in Edward and Chapman 2011 and Alonso-Alvarez and Velando 2012). In this context, increased reproductive effort to save parasitized offspring might impair parental future reproductive success or survival (e.g. Richner and Tripet 1999). On the other hand, adults might also suffer direct effects of ectoparasite blood sucking, as in the case of incubating and brooding females, which lead to energetic constraints and loss of condition (Oppliger et al. 1994). Ectoparasites may additionally induce costly immune, inflammatory responses (Møller et al. 2005; Owen et al. 2009) and physiological stress (Martínez-de la Puente et al. 2011).

Only two studies have tested the effect of nest-dwelling ectoparasites on nestling oxidative stress levels (de Coster et al. 2012; Wegmann et al. 2015), manipulating the abundance of a single ectoparasite, the hen flea *Ceratophyllus gallinae*, in great tit *Parus major* nests. Here we study the effect of nest-dwelling ectoparasite loads on the oxidative balance of breeding adults and nestlings in another well-known cavity-nesting passerine species, the pied flycatcher *Ficedula hypoleuca*. The most common ectoparasites in nests of Iberian pied flycatchers are mites *Dermanyssus gallinoides* (more than 60 % prevalence), blowfly larvae *Protocalliphora azurea* and fleas (both between 40 % and 50 % prevalence). Strong effects on nestling growth and mortality in our and other populations has been reported for three types of ectoparasites (Merino and Potti 1995, 1996, 1998; Potti et al. 1999, 2002; Potti 2007; Lobato et al. 2005, 2008; Moreno et al. 1999, 2008, 2009; Tomás et al. 2007, 2012; Martínez-de la Puente et al. 2009, 2010; Cantarero et al. 2013b). Moreover, we must take into account that nest ectoparasites are a community of species and the decline of one species may allow the proliferation of others (Heeb et al. 2000). This, together with potential multiplicative combined effects of ectoparasites, as in the case of the pied flycatcher, makes it biologically meaningful to study the effects of the entire community of ectoparasites on host physiological parameters related to oxidative stress. Therefore, here we have reduced the abundance of

all ectoparasites by a heat treatment of nest boxes. We have previously shown that this procedure significantly reduces ectoparasite abundance and exerts a positive effect on nestling wing and tarsus length (Cantarero et al. 2013a).

In order to properly evaluate the redox balance of individuals, measures of antioxidant capacity and oxidative damage must be obtained simultaneously (Costantini and Verhulst 2009; Monaghan et al. 2009; Pérez-Rodríguez 2009). Here we use plasma malondialdehyde (MDA) levels for the assessment of potential oxidative damage accompanying ectoparasite loads. MDA is a by-product of peroxidative decomposition of unsaturated lipids (Halliwell and Gutteridge 2007). MDA is often considered a presumptive marker of oxidative stress (Mateos et al. 2005; Halliwell and Gutteridge 2007; Sepp et al. 2012). To monitor antioxidant defences of pied flycatchers, we used two independent markers: total antioxidant status of plasma (TAS) and total glutathione (tGSH) levels in red blood cells. TAS measures the capacity of plasma samples to inhibit a redox reaction induced by free radicals (Miller et al. 1993; Cohen et al. 2007) and is primarily the result of the pooled effect of all extracellular antioxidant compounds of the blood (Costantini 2011). Glutathione is the predominant low-molecular-weight tripeptide thiol found in animal cells, functioning in the reduction of the disulphide linkages of proteins and in the protection of cells against free radicals, and is often considered as the most important intracellular antioxidant (Meister 1991; Wu et al. 2004). In addition, to test whether ectoparasites impaired the nutritional condition of adult females and nestlings, we measured levels of triglycerides (marker of lipid amounts) and uric acid [a marker of protein breakdown (e.g. Alonso-Alvarez and Ferrer 2001; Hõrak et al. 2006; Costantini et al. 2008)]. Uric acid and triglycerides in plasma are correlated with TAS and MDA values, so we additionally used these biochemical parameters as covariates for oxidative stress markers (Cohen et al. 2007; Romero-Hinstaro and Alonso-Alvarez 2014).

Here we examine the effects of a complete natural ectoparasite fauna on oxidative stress of both nestlings and breeding females in cavity-nesting birds. We predicted that:

1. Individuals in control nests would show higher levels of MDA (oxidative damage) than in experimental nests.
2. Ectoparasites would have an effect on antioxidant markers (TAS and tGSH). However, it is difficult to predict the sign of this latter effect, as antioxidants may be exhausted due to their involvement in tissue protection or, rather, through being mobilized to protect against free radicals (Calabrese 2007; Costantini and Verhulst 2009; Costantini et al. 2010). Therefore, we also explored the relationship between oxidative damage and antioxidant markers.
3. Individuals in control nests would show an impaired nutritional state due to the direct effects of ectoparasitism.

Materials and methods

General field methods

The study was conducted during the spring 2012 in a montane forest of Pyrenean oak *Quercus pyrenaica*, at 1200 m a.s.l. in Valsaín, central Spain (40°54′N, 4°01′W) where long-term studies on cavity-nesting birds have been ongoing since 1991 (see Cantarero et al. 2013a for general description). In the study area there are 552 nest boxes [see Lambrechts et al. (2010) for dimensions, structure and placement of nest boxes] occupied by pied flycatchers, great tits, nuthatches *Sitta europaea* and blue tits *Cyanistes caeruleus*.

We followed breeding activities from the early stages of nest construction to fledging in nest boxes occupied by pied flycatchers. Egg laying in the pied flycatcher population typically begins in late May and the modal clutch size is six. No brooding is observed after nestlings attain 7 days of age (Sanz and Moreno 1995), and chicks usually fledge at the age of 17 days. Breeding activities are followed routinely every year and laying and hatching dates and brood sizes at hatching and fledging are determined.

Shortly before fledging, at day 13 (hatching date = day 1), nestlings were ringed, weighed and measured. Body mass was obtained with a Pesola spring balance to the nearest 0.25 g and tarsal length was measured with digital callipers (precision 0.01 mm). We took a blood sample of about 120 µl from the brachial vein that was collected in heparinised microcapillaries. Blood samples ($n = 268$) were stored in Eppendorf tubes in an ice-box until returning to the lab in the same day. Plasma was separated from blood by centrifugation (10 min at 12,000 rpm) and then both fractions were stored at −80 °C until analysed for assaying TAS, MDA, tGSH, uric acid and triglycerides (see below).

Parental individuals were captured in their nest boxes with traps while feeding nestlings of 7–8 days, ringed if necessary or identified by their ring and measured. Due to their higher exposition to ectoparasite infestations, only females ($n = 52$) were blood-sampled in the same way as nestlings to test the effect of treatment on their physiological parameters.

Haemolysis levels in plasma samples were noted by a visual detection of the red colour of plasma, as a consequence of the release of haemoglobin from red blood cells, in a gradient from 0 (no haemolysis) to 2 (high degree of haemolysis). Only one person noted haemolysis degree in order to minimize inter-observer variability. Of the 320

blood samples collected, 71 from nestlings and 18 from females were moderately to highly haemolysed.

Because the total volume of some plasma samples was not sufficient to carry out all physiological or biochemical analyses, we established a priority order of assays as follows: MDA, triglycerides, TAS and uric acid levels. This explains why sample sizes for different measures differ.

Experimental reduction of ectoparasites protocol

Of the 91 nest boxes occupied by pied flycatchers we selected 56 whose laying date was between dates 45 and 51 (1 April = day 1) and assigned nests randomly to two groups. Nests in the control group ($n = 37$) maintained their natural ectoparasite loads, while in the experimental group ($n = 19$ nests), we reduced the number of ectoparasites by a heat treatment for 30 s at 750 W using a microwave oven. This treatment ensured that experimental nests did not contain live arthropods when placed in the nest box (Rendell and Verbeek 1996). To avoid the loss of water during the heat treatment, the nests were placed into a hermetic plastic container. Furthermore, before returning the original nest, the flame from a butane jet torch lighter (Microtorch GT-3000) was passed across the walls of the nest box to kill ectoparasites that might remain there. Only for the time that the original nests were treated (around 30 min) was a fresh substitute nest introduced into the nest box (these nests had been collected in previous seasons after being abandoned prior to laying and kept frozen at −20 °C until use). To prevent ectoparasite recolonisation of experimental nests a total of three heat treatments were made in the experimental group: 7 days after clutch completion, when nestlings were 2 days old, and when nestlings were 8 days old. Nests in the control group were visited on the same days and handled in a similar way to experimental ones but without applying any treatment. The efficiency of our treatment to eliminate or significantly reduce ectoparasites in heat-treated nests and abundances of ectoparasite species in control nests has been previously reported in Cantarero et al. (2013a) for the same set of nests.

Lipid peroxidation assays

Plasma concentrations of total MDA were calculated following Agarwal and Chase (2002) with some modifications made by Mougeot et al. (2009). Assays were carried out in 2-mL screw-top microcentrifuge tubes and all chemical solutions were prepared using ultra pure water (Milli-Q Synthesis; Millipore, Watford, UK). For calibration, a standard curve was prepared using a 1,1,3,3-tetraethoxypropane (TEP) stock solution (10 μM in 40 % ethanol) serially diluted using 40 % ethanol. Butylated hydroxytoluene solution (25 μl; 0.05 % w/v in 95 % ethanol), 200 μl phosphoric acid solution (0.44 M) and 50 μl thiobarbituric acid (TEP) solution (42 mM) were added to 25 μl of plasma samples (1:2.5 dilution in water) or standards. Samples were vortex mixed for 5 s and then heated at 100 °C for 1 h in a dry bath incubator to allow the formation of MDA–TBA adducts. The reaction was then stopped by placing samples on ice for 5 min before 125 μl n-butanol was added and tubes were vortex mixed for 1 min. Tubes were then centrifuged at 14,000 r.p.m. and 4 °C for 3 min, before the upper (n-butanol) phase was collected and transferred into a high performance liquid chromatography (HPLC) vial for analysis. Samples (10 μl) were injected into an Agilent 1200 HPLC system (Agilent, Santa Clara, CA) fitted with a 5-μm ACE guard column and 5-μm octadecylsilane 100 × 4.6-mm column (Advanced Chromatography Technologies, Aberdeen, Scotland) maintained at 37 °C. The mobile phase was methanol buffer (40:60 v/v), the buffer being 50 mM anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5 M potassium hydroxide solution), running isocratically over 3.5 min at a flow rate of 1 mL min^{-1}. Data were collected using a fluorescence detector (G1321A; Agilent) set at 515 nm (excitation) and 553 nm (emission). High repeatability (Lessells and Boag 1987, here and hereafter) was shown by both a subset of samples assayed in duplicate and TEP standards ($R = 0.706$, $n = 22$, $p < 0.001$ and $R = 0.968$, $n = 6$, $p < 0.001$, respectively).

Total antioxidant status

TAS was assayed following Miller et al. (1993) with some modifications made by Cohen et al. (2007). Metmyoglobin was generated by mixing equal volumes of 400 µM myoglobin (M0630-250MG; Sigma-Aldrich, St Louis, MO) and 740 µM potassium ferrocyanide, then passing the mixture through a column of Sephadex (G15-120; Sigma-Aldrich). The chromogen, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was mixed in PBS to 153 µM. The standard was made by dissolving a water-soluble α-tocopherol derivative, Trolox, in PBS to 1.7 mM. The assay was run in 96-well flat-bottomed clear microplates on a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT). Temperature was maintained at 37°C, and readings were taken at 660 nm. Only one 12-well row was used from the plate at a time. Five microlitres of standard (Trolox) or samples was put separately into the wells. Next, 15 µL metmyoglobin and 250 µL ABTS were sequentially added to each well. A multi-channel pipette was used to simultaneously add 50 µL of 300 µM hydrogen peroxide to all the wells, starting the reaction. Kinetic measurements using the spectrophotometer were taken at 10 s intervals; readings were synchronized to the start of the reaction (i.e., injection of hydrogen peroxide) manually using a timer.

The reaction runs for around 10 min. Most of samples were assayed in duplicate and showed high repeatability ($R = 0.912$, $n = 209$, $p < 0.001$).

Intracellular tGSH level

tGSH levels in red blood cells were determined according to Galván and Alonso-Alvarez (2008) with some particular modifications. Red blood cell samples were diluted (1:20 w/v) and homogenized in a stock buffer [0.01 M phosphate-buffered saline (PBS) and 0.02 M ethylenediaminetetraacetic acid (EDTA)] using a Mini-BeadBeater (BioSpec; Bartlesville, OK) and mixed with an equal volume of 10 % trichloroacetic acid. The mixture was vortexed three times during 5 s each bout within a 10-min period. Afterwards, the mixture was centrifuged (3000 *g*, 15 min, 6°C), and the supernatant separated. Three working solutions were made up in a reaction buffer (125 mM sodium phosphate and 6.3 mM EDTA) as follows: (1) 0.3 mM dihydronicotinamide-adenine dinucleotide phosphate, (2) 6 mM 5,5′-dithio-bis(2-nitrobenzoic acid), and (3) 50 U GSH reductase mL^{-1}. Solutions 1 and 2 were mixed at 7:1 volume. The next steps were performed on a Synergy HT Multi-Mode Microplate Reader (BioTek). To 75 µl of sample (supernatant) we added 240 µl of the mixture of solutions 1 and 2. Then, 20 mL of solution 3 was added after 15 s and the absorbance at 405 nm was monitored after 15 and 45 s. The change in absorbance was used to determine the intracellular tGSH concentration by comparing the output with the results from a standard curve generated by serial dilution of GSH from 0.5 to 0.031 mM. Only one 12-well row was used from the plate at a time. A subset of samples assayed in duplicate showed a high repeatability ($R = 0.929$, $n = 44$, $p < 0.001$).

Measurement of uric acid and triglyceride levels

Uric acid is the main form of nitrogen excretion in birds and an indicator of amino acid catabolism. Uric acid is also a powerful antioxidant whose concentration is frequently positively related to TAS values (Cohen et al. 2007; Hõrak et al. 2007; Pérez-Rodríguez et al. 2008), potentially confounding the interpretation of this marker (Cohen et al. 2007; Costantini 2011). For this reason uric acid-corrected TAS values are recommended over raw TAS levels (Cohen et al. 2007). Triglyceride concentrations reflect the individual´s state of fattening by indicating the amount of lipids absorbed during the few hours before blood sampling (Jenni-Eiermann and Jenni 1998). However, triglyceride levels can be related to MDA levels, either because of an effect of diet (MDA is also present in food) or because MDA may also be influenced by the amount of circulating lipids susceptible to oxidation (Pérez-Rodríguez et al. 2015). For this reason, analyses of MDA levels would benefit from entering triglyceride levels as a covariate (Romero-Haro and Alonso-Alvarez 2014). Thus, in this study we analysed the effect of our treatment both on raw and on triglycerides-corrected MDA (Pérez-Rodríguez et al. 2015).

The uric acid oxidase/peroxidase method and the glycerol phosphate oxidase/peroxidase method were used for measuring uric acid and triglyceride levels, respectively (kits 11522 and 11529; Biosystems, Barcelona) and using a Synergy HT Multi-Mode Microplate Reader (BioTek). Reagent volumes and further assay details were implemented according to manufacturer instructions. A subset of samples assayed in duplicate showed a high repeatability for both variables (uric acid, $R = 0.99$, $p < 0.001$; triglycerides, $R = 0.94$, $p < 0.001$; $n = 45$ in both cases).

Statistical analyses

Statistical analyses were conducted using STATISTICA (version 7.0; StatSoft). MDA and triglyceride levels of nestlings were normalized through logarithmic transformation. The remaining physiological variables were normally distributed.

Although degree of haemolysis in plasma of nestlings and females was not affected by treatment (Mann–Whitney *U*-test—females, $U = 276.500$, $n = 52$, $p = 0.485$; nestlings, $U = 8142.000$, $n = 266$, $p = 0.837$), haemolysis may affect physiological variables due to a possible efflux of intracellular pro-oxidants and antioxidant molecules in plasma that could alter levels of oxidative markers measured in serum, thereby confounding interpretation of results. Thus, we controlled for this factor in all physiological analyses.

To control for variations in oxidative levels within the control group due to differences in specific ectoparasite abundances, we explored ectoparasite prevalences and possible associations between abundances, as well as correlations between physiological parameters of hosts and ectoparasite abundances within the control group.

Then, we tested the effect of ectoparasite removal on MDA concentration, TAS and tGSH and for triglycerides, uric acid levels and body condition. For female analyses we used treatment as an explanatory factor. In the case of nestling analyses, we included nest identity nested within treatment as a random factor and treatment as a fixed factor. In both cases we controlled for uric acid in TAS analyses. Effects of treatment on MDA levels were also controlled and non-controlled for variability in triglyceride levels (see above). The models testing the effect of the treatment on body condition included body mass as a dependent variable and tarsus length as a covariate (García-Berthou 2001).

Cantarero et al. (2013a) found negative effects of ectoparasites on tarsus and wing lengths of nestlings, but no correlation between brood size and treatment. However, brood sizes might introduce possible confounding effects on physiological levels. Thus, we performed preliminary tests to control for possible effects of brood size and body condition (tarsus length and body mass) on females and nestling data. Only in cases in which some of these had an effect in physiological variables, were they finally introduced as covariates in initial models (only nestling body mass for triglyceride models and brood size and body mass for female tGSH models).

Finally, we tested covariation between antioxidants and MDA levels by generalised linear model (GLM) analyses, controlling by haemolysis degree and treatment in all cases and uric acid for TAS analyses. Moreover, we used Student's *t*-tests to analyse differences between physiological female levels and intra-brood means of nestling levels. Effect sizes were calculated as partial η^2, i.e. the ratio of variance accounted for by an effect plus its associated error variance within the model.

G*Power (version 3.1.9.2; University of Kiel, Germany) was used to estimate the power to detect large effects ($f = 0.40$) with our samples sizes. Moreover, we calculated observed power and effect sizes for the effect of treatment.

Results

Within the control group, 26 nests (70 %) were parasitized by more than one ectoparasite species at time. Mites had the highest prevalence (36 control nests, 97 %), blowflies were present in 23 of these control nests (62 %) and fleas were found in nine nests (24 %). There were no correlations between abundances of three ectoparasite species (all $p > 0.05$) in control nests. There were no correlations between mean levels of oxidative parameters and three type of ectoparasite abundances within the control group for both females and nestlings (all $p > 0.05$).

Although female oxidative damage, as measured by MDA levels, was similar for both groups (Fig. 1a; Table 1), females in experimental nests showed significantly higher levels of extracellular antioxidants measured by TAS (Fig. 1b), and higher concentrations of the endogenous antioxidant tGSH in red blood cells (Fig. 1c) than those in control nests. For nestlings, significant differences between treatments were only found for tGSH levels (Table 2), these being higher in treated nests (Fig. 1). Results for effects of treatment on MDA levels (Tables 1, 2) did not change when plasma triglyceride levels were entered as a covariate.

For females, there were no correlations between physiological parameters analysed (GLM analyses, all $p > 0.30$).

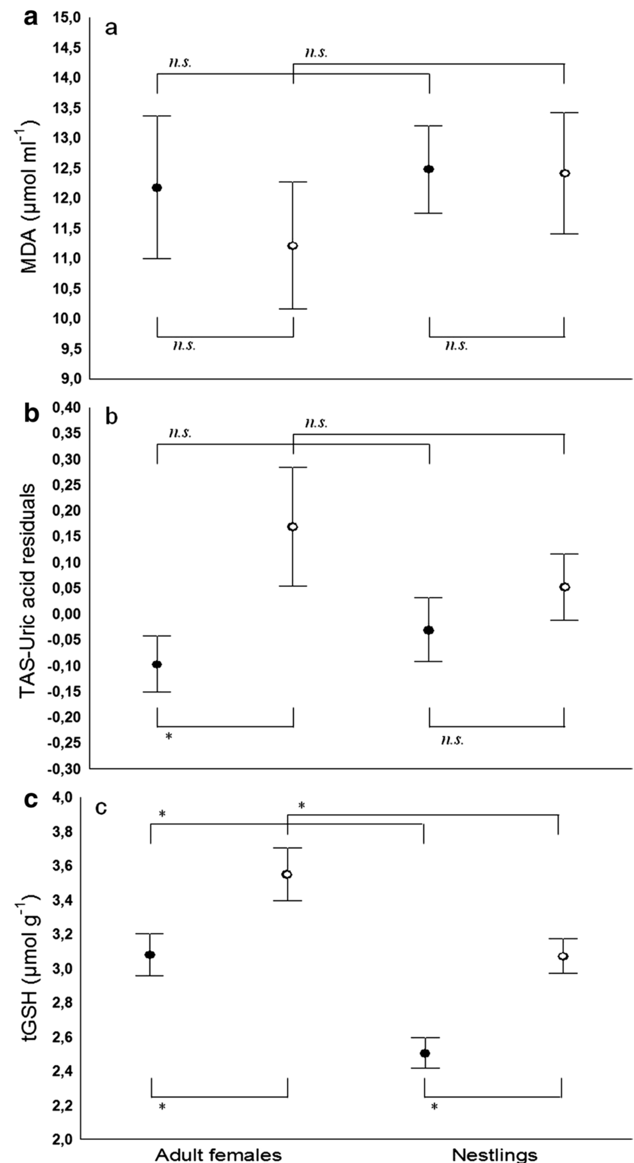

Fig. 1 Effect of experimental removal of nest-dwelling ectoparasites on **a** plasma malondialdehyde (*MDA*), **b** plasma total antioxidant capacity (*TAS*; corrected for uric acid levels), and **c** total glutathione (*tGSH*) in erythrocytes. *Filled circles* Control nests, *open circles* heat-treated nests. Values are mean ± SE levels. *Asterisk* indicates significant differences, *n.s.* non-significant differences

Although MDA and TAS levels of nestlings were not affected by treatment, MDA negatively covaried with TAS as dependent variable (GLM analyses, $F_{1,134} = 5.47$, $p = 0.021$; $\beta = -0.014$) after controlling by uric acid levels, degree of haemolysis, treatment and nest identity. The rest of the covariations between physiological parameters of nestlings were not significant (GLM analyses, all $p > 0.10$).

Triglycerides and uric acid levels of females were not affected by treatment (Table 3). The same was found for

Table 1 Results of generalised linear model (GLM) analyses of adult female malondialdehyde (*MDA*), total antioxidant capacity (*TAS*) and total glutathione (*tGSH*) levels (minimal models selected by backward elimination of non-significant terms to improve models)

	Num. *df*	*F*	*p*	Power ($f = 0.40$)	Observed effect size	Observed power
MDA (μmol mL^{-1})						
Full model						
Treatment	1	0.008	0.927	0.669	0.013	0.051
Haemolysis	2	8.122	<0.001*			
Error	48					
Minimal model						
Haemolysis	2	8.484	<0.001*			
Error	49					
TAS (mmol l^{-1})						
Full model						
Treatment	1	3.746	0.064	0.427	0.386	0.403
Uric acid (mg dl^{-1})	1	39.213	<0.001*			
Haemolysis	2	1.643	0.214			
Error	25					
Minimal model						
Treatment	1	5.543	0.026*	0.429	0.453	0.523
Uric acid (mg dl^{-1})	1	37.596	<0.001*			
Error	27					
tGSH (μmol g^{-1})						
Full model						
Treatment	1	10.299	0.003*	0.616	0.495	0.797
Haemolysis	2	3.015	0.059			
Brood size	1	5.012	0.030*			
Body mass (g)	1	3.369	0.073			
Error	42					
Minimal model						
Treatment	1	6.584	0.014*	0.618	0.386	0.587
Haemolysis	2	3.952	0.026*			
Error	44					

Statistical power calculated a priori to detect large effects ($f = 0.40$), observed effect sizes (based on partial η^2) and observed power are included for the effect of the treatment

*Significant difference ($\alpha = 0.05$)

nestling uric acid levels (Table 3). Triglycerides of nestlings were not affected by treatment (Table 3), although they showed a positive association with body mass (GLM analysis, $F_{1,239} = 6.49$, $p = 0.011$; $\beta = 0.250$). Body condition, measured as tarsus length-corrected body mass, of females and nestlings was not affected by treatment (Table 3).

Independently of treatment, levels of triglycerides and uric acid were lower in females than in nestlings (Table 3; *t*-test—triglycerides, $t_{92} = -7.111$, $p < 0.001$; uric acid, $t_{78} = -2.997$, $p = 0.004$). By contrast, tGSH levels (Fig. 1c) were higher in females than in nestlings (*t*-test, tGSH, $t_{101} = 4.213$, $p < 0.001$), while there were no differences for TAS (Fig. 1b) and MDA (Fig. 1a) (*t*-test, all $p > 0.500$).

Discussion

Our results support the hypothesis that ectoparasites impose a physiological cost for cavity-nesting birds. Although there was a lack of significant differences in MDA levels between the two treatments (prediction 1), our prediction about the negative effects of ectoparasites on host antioxidants (2) was supported by the lower tGSH levels in both females and nestlings exposed to ectoparasites and by the lower TAS values of females in control nests. These results indicate that ectoparasites exposed cavity-nesting birds to an oxidative challenge that depleted antioxidant defences. Moreover, exploring associations between oxidative damage and antioxidants, we found a negative relation between antioxidant status and MDA levels in nestlings, but not in

Table 2 Results of GLM analyses of nestling MDA, TAS and tGSH levels (minimal models are selected by backward elimination of non-significant terms to improve models)

	Effect	*df*	*F*	*p*	Power ($f = 0.40$)	Observed effect size	Observed power
MDA (μmol mL^{-1})							
Full model							
Treatment	Fixed	1, 52.4	0.091	0.764	0.668	0.041	0.057
Haemolysis	Fixed	2, 193	39.709	<0.001*			
Nest (Treatment)	Random	51, 193	3.298	<0.001*			
Error		193					
Minimal model							
Haemolysis	Fixed	2, 193	39.709	<0.001*			
Nest (Treatment)	Random	52, 193	3.235	<0.001*			
Error		193					
TAS (mmol l^{-1})							
Full model							
Treatment	Fixed	1, 51.2	0.085	0.772	0.642	0.095	0.085
Uric acid (mg dl^{-1})	Fixed	1, 117	210.546	<0.001*			
Haemolysis	Fixed	2, 117	1.686	0.189			
Nest (treatment)	Random	48, 117	6.359	<0.001*			
Error		117					
Minimal model							
Uric acid (mg dl^{-1})	Fixed	1, 119	205.219	<0.001*			
Nest (Treatment)	Random	49, 119	6.139	<0.001*			
Error		119					
tGSH (μmol g^{-1})							
Full model							
Treatment	Fixed	1, 50.9	13.854	<0.001*	0.668	0.522	0.878
Haemolysis	Fixed	2, 211	8.503	<0.001*			
Nest (Treatment)	Random	51, 211	3.809	<0.001*			
Error		211					

Statistical power calculated a priori to detect large effects ($f = 0.40$), observed effect sizes (based on partial η^2) and observed power are included for the effect of the treatment. For abbreviations, see Table 1

*Significant difference ($\alpha = 0.05$)

Table 3 Mean ± SE (sample size *in parentheses*) of female and nestling indicators of nutritional condition in relation to treatment

	Control	Heat-treated	*F*	*p*	Power ($f = 0.40$)	Observed effect size	Observed power
Adult females							
Triglycerides (mg dl^{-1})	138.03 ± 4.69 (25)	128.14 ± 5.86 (16)	1.74	0.195	0.591	0.211	0.211
Uric acid (mg dl^{-1})	10.42 ± 0.35 (19)	10.17 ± 0.46 (11)	0.59	0.447	0.431	0.146	0.100
Body mass[a] (g)	12.71 ± 0.214 (35)	12.45 ± 0.20 (19)	1.20	0.279	0.669	0.153	0.151
Nestlings							
Triglycerides (mg dl^{-1})	227.32 ± 3.94 (153)	197.63 ± 4.96 (91)	2.46	0.125	0.669	0.217	0.256
Uric acid (mg dl^{-1})	12.18 ± 0.26 (96)	12.59 ± 0.29 (69)	1.89	0.176	0.645	0.204	0.222
Body mass[a] (g)	14.05 ± 0.05 (170)	14.05 ± 0.07 (100)	0.00	0.982	0.669	0.022	0.052

Statistical power calculated a priori to detect large effects ($f = 0.40$), observed effect sizes (based on partial η^2) and observed power are included for the effect of the treatment

[a] Tarsus length corrected; least squares means

females. Finally, and contrary to our prediction (3), those biochemical parameters related to nutritional state (triglycerides and uric acid levels) were not affected by treatment.

Cellular GSH levels reflect a steady-state balance between synthesis and loss. At normal levels of oxidative stress there is essentially no net loss of GSH through oxidation (Griffith 1999). However, if pro-oxidant levels increase sufficiently, GSH protects cells, reacting rapidly with peroxides and producing [glutathione disulphide (GSSG), an oxidized form of GSH]. Although the enzyme GSH reductase reduces GSSG back to GSH, GSSG is rather degraded extracellularly and GSH is not taken up intact by cells (Griffith 1999; Wu et al. 2004). The depletion of cellular tGSH levels in parasitized individuals may indicate an increase in RONS produced during inflammatory responses to ectoparasite bites (Baron and Weintraub 1987; Owen et al. 2010) or as a consequence of an increase in metabolic activity (Finkel and Holbrook 2000). Accordingly, Cantarero et al. (2013a) found that ectoparasites increase begging intensity in nestlings, and they detected a positive correlation between begging intensity and provisioning rates of adults. This presumably raised energetic costs and metabolic activity in both cases, potentially leading to an oxidative challenge (Moreno-Rueda et al. 2012).

Another piece of evidence about the possible costs of ectoparasitism on hosts is a decrease in plasma antioxidant status, a measure that assesses the levels of non-enzymatic antioxidants, such as vitamins A, C and E, and carotenoids. All of these are progressively used up to protect the cell membrane and prevent lysis (Lesgards et al. 2002; Bertrand et al. 2006). Low molecular weight and lipid-soluble antioxidants like tocopherol (vitamin E), have been linked mainly to lipid peroxidation, while the water-soluble ascorbic acid (vitamin C) has been suggested to be part of a first line of defence (Dotan et al. 2004) and can also protect membranes against peroxidation by enhancing the activity of tocopherol (Sies and Stahl 1995). In this line of reasoning, the negative correlation between MDA and TAS in nestling values supports the role of TAS in protection against lipid peroxidation. Our results showed that adult females, but not nestlings, had less antioxidant capacity in parasitized than heat-treated nests. The fact that only females showed this effect would suggest a stronger challenge imposed by ectoparasites for the parents compared to nestlings. This may be a consequence of the intense flight activity during nestling feeding. In fact, it has been recently shown in birds that flight effort may contribute to the depletion of the antioxidant defences in order to maintain redox homeostasis (Costantini et al. 2008). In contrast, nestlings experience very low activity levels in nests and when measured have been shown to have passed the peak of tissue growth and cell proliferation (Lundberg and Alatalo 1992), which may also be sources of oxidative stress (Metcalfe and Alonso-Alvarez 2010 and references therein). These results are in accordance with studies in which parasite-mediated depletion of antioxidant defences have been reported (Hõrak et al. 2004a; Mougeot et al. 2009; van de Crommenacker et al. 2012). However, there are some studies that report opposite results or a lack of effects (Hõrak et al. 2004b; Pap et al. 2011; de Coster et al. 2012; Wegmann et al. 2015), which can be explained by hormetic compensation of the antioxidant machinery (Hõrak et al. 2007), or due to differential adjustments in maternal investment that can affect self-maintenance and offspring physiological status (de Coster et al. 2012; Wegmann et al. 2015).

In birds a decline in erythrocyte tGSH levels was also detected under exposure to experimentally high levels of RONS, though no effect in TAS values was found (Galván and Alonso-Alvarez 2009; Alonso-Alvarez and Galván 2011). Although we have not found effects of ectoparasites on TAS in nestlings, Cantarero et al. (2013a) showed that the same nestlings exposed to ectoparasites reduced their growth compared with nestlings from the treated group, which suggests that the resources invested by cells in maintaining oxidative stress under the control may have affected cell proliferation and thereby tissue growth. Furthermore, the decrease in nestling tGSH in control nests is consistent with an incomplete compensation of antioxidant defences. However, we cannot discount that our manipulation could have had some effect on nestling morphological traits used as signals in parent-offspring communication, such as the carotenoid-based colour of the nestling mouth (e.g. de Ayala et al. 2007; Dugas 2012). These carotenoid-based traits can be particularly susceptible to alterations in the oxidative status of the individual (von Schantz et al. 1999; Pérez-Rodríguez 2009), which could have influenced sibling and parent behaviour during nestling and post-fledging periods. Unfortunately, we have not assessed these ornamental traits in our birds. However, we have previously shown a lack of effect of the ectoparasite abundance treatment on provisioning rates to nestlings during development (Cantarero et al. 2013a).

Although we have not found effects of ectoparasites on MDA, this may be due to adjustments in the antioxidant system (tGSH and TAS) induced by ectoparasites. Similar patterns have been observed in coccidian infection in mammals (Rakhshandehroo et al. 2013), studies of which report a depletion in antioxidant defences to maintain a safe level of oxidative damage after a peak of infection. Our results may therefore suggest that individuals have the ability to cope, at least to some degree, with any increase in oxidative stress due to parasitism. However, the lack of effects of the treatment on some biomarkers may also be due to our limited sample size. A priori G*Power tests were performed to estimate the minimum sample size required to detect effects of our treatment on physiological variables. Minimum

sample sizes required to detect (α = 0.05; 1-β = 0.80) large (f = 0.40), medium (f = 0.25) and small (f = 0.10) effects of our treatment on physiological variables were 52, 128 and 787 nests, respectively. Accordingly, Cohen's conventions (1992) proposes at least 26 cases per group to test differences between two treatments in order to detect, at least, large size effects at $p < 0.05$. Here, however, we had a maximum of 19 nests in the manipulated group, which a priori implies intermediate power levels to detect large effects (range, 0.43–0.67; Tables 1, 2 and 3). We also estimated a low observed power for both adult female and nestling analyses, only reaching the 0.80 threshold in the case of tGSH (Table 2). The variable sensitivity to the effect of treatment may be due to the large differences of variance among response variables. Therefore, an interpretation of these null results might be highly speculative.

Triglyceride concentrations reflect the individual's state of fattening by indicating the amount of lipids absorbed during the few hours before blood sampling (Jenni-Eiermann and Jenni 1998) and are positively related to body mass, as reported in the Results. Uric acid levels are also good indicators of nutritional state due to active protein catabolism during the last phase of fasting (Alonso-Alvarez and Ferrer 2001; McCue 2010). Thus, these blood metabolites may reflect variation in the basic nutritional state (Hõrak et al. 2006), and the lack of effects of our experiment on these may be related to the lack of differences in body condition as shown in the Results. This could be explained by conditions during the year of study (2012), which were especially favourable, as nestlings attained their largest masses since the inception of the long-term study (Cantarero et al. 2013a).

Interestingly, lower levels of triglycerides of adult females when compared with nestlings may reflect costs of flight, causing tissues to increased hydrolysis of triglycerides from adipose free fatty acids and glycerol and oxidation of free fatty acids by muscle activity (Schwilch et al. 1996). Additionally, high uric acid levels reported for nestlings, when compared with females, may be a consequence of nestling diet due to plasma uric acid levels in birds increasing a few hours after feeding (Romero-Haro and Alonso-Alvarez 2014 and references therein). On the other hand, similar to our findings, Isaksson et al. (2005) reported higher levels of tGSH in adult females than in great tit nestlings. They explained this as an adaptive response [i.e. hormesis (Calabrese 2007; Costantini et al. 2010)] to the physical strain and other stressors experienced by breeding adults, as compared to nestlings sitting mostly immobile, warm, and safe in the nest box along with a not fully developed defence system (Isaksson et al. 2005). In females, tGSH levels are negatively associated with dependent brood size and positively correlated with their body mass. This might indicate that tGSH in females is related to effort-dependent condition, which in turn is affected by parental metabolic exertion.

To summarize, we have reported here negative effects of nest ectoparasites on nestling physiological traits related to oxidative stress. These may contribute to explain the detrimental effects on nestling development and fledging success demonstrated for the same set of nests (Cantarero et al. 2013a). We also show that breeding females are also affected physiologically by nest-dwelling ectoparasites, as shown in other studies with respect to other health parameters (e.g. Tomás et al. 2007). Here we demonstrated that ectoparasites may impose an oxidative challenge to cavity-nesting birds: breeding females and nestlings from parasitized nests managed to maintain oxidative damage levels, but at the cost of an impaired antioxidant system. Though the potential long-term effects of our findings are still being studied, these parasite-induced oxidative challenges can lead to reduced survival or resource allocation to future reproduction (Richner and Tripet 1999; Fitze et al. 2004a, 2004b). A large number of studies have shown the links between oxidative stress and antioxidant defences with fitness traits including fecundity, egg-laying capacity and short- and long-term viability (Blount et al. 2004; reviews in Monaghan et al. 2009; reviews in Dowling and Simmons 2009; Helfenstein et al. 2010; Metcalfe and Alonso-Alvarez 2010; Saino et al. 2011). Moreover, associations between recruitment probability and pre-fledging oxidative damage (Noguera et al. 2011) and between antioxidant defences and winter survival (Norte et al. 2008) have been reported in wild birds. Here, we acknowledge that the use of microwaves to reduce ectoparasite loads may kill microorganisms present in nests, both beneficial and pathogenic (Goodenough and Stallwood 2010; González-Braojos et al. 2012), and that we therefore cannot exclude possible interactions of their presence/absence on our results. However, contrary to the idea that nest-dwelling ectoparasites are of minor importance for breeding success and survival (e.g. Eeva et al. 1994; Bauchau 1997), we here report detectable costs of ectoparasitism not only on behavioural and developmental traits (Cantarero et al. 2013a), but also on key physiological parameters, which may compromise host fitness.

Author contribution statement J. L.-A., A. C., L. P.-R. and J. M. conceived and designed the experiments. J. L.-A., A. C., S. G.-B. and J. M. conducted fieldwork. J. L.-A., L. P.-R., A. P. and C. A.-A., performed the laboratory analyses. J. L.-A., L. P.-R., C. A.-A. and J. M. analysed the data and wrote the manuscript.

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References

Agarwal R, Chase SD (2002) Rapid, fluorimetric-liquid chromatographic determination of malondialdehyde in biological samples. J Chromatogr B 775:121–126

Alonso-Alvarez C, Ferrer M (2001) A biochemical study of fasting, subfeeding, and recovery processes in yellow-legged gulls. Physiol Biochem Zool 74:703–713

Alonso-Alvarez C, Galván I (2011) Free radical exposure creates paler carotenoid-based ornaments: a possible interaction in the expression of black and red traits. PloS One 6:e19403

Alonso-Alvarez C, Velando A (2012) Benefits and costs of parental care. In: Royle NJ, Smiseth PT, Kolliker M (eds) The evolution of parental care. Oxford University Press, Oxford, pp 40–61

Atkinson CT, van Riper C (1991) Pathogenicity and epizootiology of avian haematozoa: *Plasmodium*, *Leucocytozoon*, and *Haemoproteus*. In: Loye JE, Zuk M (eds) Bird-parasite interactions: ecology, evolution and behaviour. Oxford University Press, New York, pp 19–48

Baron RW, Weintraub J (1987) Immunological responses to parasitic arthropods. Parasitol Today 3:77–82

Bauchau V (1997) Do parasitic mites decrease growth of nestling pied flycatchers *Ficedula hypoleuca*? Ardea 85:243–247

Bertrand S, Criscuolo F, Faivre B, Sorci G (2006) Immune activation increases susceptibility to oxidative tissue damage in zebra finches. Funct Ecol 20:1022–1027

Blount JD, Houston DC, Surai PF, Møller AP (2004) Egg–laying capacity is limited by carotenoid pigment availability in wild gulls *Larus fuscus*. Proc R Soc Lond B Biol Sci 271:S79–S81

Brommer JE, Pitala N, Siitari H, Kluen E, Gustafsson L (2011) Body size and immune defense of nestling blue tits (*Cyanistes caeruleus*) in response to manipulation of ectoparasites and food supply. Auk 128:556–563

Calabrese EJ (2007) Threshold dose-response model-RIP: 1911 to 2006. BioEssays 29:686–688

Cantarero A, López-Arrabé J, Redondo AJ, Moreno J (2013a) Behavioural responses to ectoparasites in pied flycatchers *Ficedula hypoleuca*: an experimental study. J Avian Biol 44:591–599

Cantarero A, López-Arrabé J, Rodríguez-García V, González-Braojos S, Ruiz-de-Castañeda R, Redondo AJ, Moreno J (2013b) Factors affecting the presence and abundance of generalist ectoparasites in nests of three sympatric hole-nesting bird species. Acta Ornithol 48:39–54

Christe P, Richner H, Oppliger A (1996) Of great tits and fleas: sleep baby sleep. Anim Behav 52:1087–1092

Cohen J (1992) A power primer. Psychol Bull 112:155–159

Cohen A, Klasing K, Ricklefs R (2007) Measuring circulating antioxidants in wild birds. Comp Biochem Physiol B 147:110–121

Collias NE, Collias EC (1984) Nest building and bird behavior. Princeton University Press, Princeton

Costantini D (2008) Oxidative stress in ecology and evolution: lessons from avian studies. Ecol Lett 11:1238–1251

Costantini D (2011) On the measurement of circulating antioxidant capacity and the nightmare of uric acid levels. Method Ecol Evol 2:321–325

Costantini D, Møller AP (2009) Does immune response cause oxidative stress in birds? A meta-analysis. Comp Biochem Physiol A 153:339–344

Costantini D, dell'Ariccia G, Lipp HP (2008) Long flights and age affect oxidative status of homing pigeons (*Columba livia*). J Exp Biol 211:377–381

Costantini D, Monaghan P, Metcalfe NB (2010) Ecological processes in a hormetic framework. Ecol Lett 13:1435–1447

de Ayala RM, Saino N, Møller AP, Anselmi C (2007) Mouth coloration of nestlings covaries with offspring quality and influences parental feeding behavior. Behav Ecol 18:526–534

de Coster G, de Neve L, Verhulst S, Lens L (2012) Maternal effects reduce oxidative stress in female nestlings under high parasite load. J Avian Biol 43:177–185

Demas GE, Chefer V, Talan MI, Nelson RJ (1997) Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6 J mice. Am J Physiol 273:R1631–R1637

Dotan Y, Lichtenberg D, Pinchuk I (2004) Lipid peroxidation cannot be used as a universal criterion of oxidative stress. Prog Lipid Res 43:200–227

Dowling DK, Simmons LW (2009) Reactive oxygen species as universal constraints in life-history evolution. Proc R Soc Lond B Biol Sci 276:1737–1745

Dugas MB (2012) Cross-fostering reveals that among-brood differences in ornamental mouth coloration mostly reflect rearing conditions in nestling house sparrows. Biol J Linn Soc 106:169–179

Edward DA, Chapman T (2011) Mechanisms underlying reproductive trade-offs: costs of reproduction. In: Flatt T, Heyland A (eds) Mechanisms of life history evolution. Oxford University Press, Oxford, pp 137–152

Eeva T, Lehikoinen E, Nurmi J (1994) Effects of ectoparasites on breeding success of great tits (*Parus major*) and pied flycatchers (*Ficedula hypoleuca*) in an air-pollution gradient. Can J Zool 72:624–635

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239–247

Fitze PS, Clobert J, Richner H (2004a) Long-term life-history consequences of ectoparasite-modulated growth and development. Ecology 85:2018–2026

Fitze PS, Tschirren B, Richner H (2004b) Life history and fitness consequences of ectoparasites. J Anim Ecol 73:216–226

Galván I, Alonso-Alvarez C (2008) An intracellular antioxidant determines the expression of a melanin-based signal in a bird. PLoS One 3:e3335

Galván I, Alonso-Alvarez C (2009) The expression of melanin-based plumage is separately modulated by exogenous oxidative stress and a melanocortin. Proc R Soc Lond B Biol Sci 276:3089–3097

García-Berthou E (2001) On the misuse of residuals in ecology: testing regression residuals vs. the analysis of covariance. J Anim Ecol 70:708–711

Golden TR, Hinerfeld DA, Melov S (2002) Oxidative stress and aging: Beyond correlation. Aging Cell 1:117–123

González-Braojos S, Vela AI, Ruiz-de-Castañeda R, Briones V, Cantarero A, Moreno J (2012) Is nestling growth affected by nest

reuse and skin bacteria in pied flycatchers *Ficedula hypoleuca*? Acta Ornithol 47:119–127

Goodenough AE, Stallwood B (2010) Intraspecific variation and interspecific differences in the bacterial and fungal assemblages of blue tit (*Cyanistes caeruleus*) and great tit (*Parus major*) nests. Microb Ecol 59:221–232

Griffith OW (1999) Biologic and pharmacologic regulation of mammalian glutathione synthesis. Free Radical Bio Med 27:922–935

Halliwell B, Gutteridge J (2007) Free radicals in biology and medicine. Oxford University Press, Oxford

Heeb P, Werner I, Kölliker M, Richner H (1998) Benefits of induced host responses against an ectoparasite. Proc R Soc Lond B Biol Sci 265:51–56

Heeb P, Kölliker M, Richner H (2000) Bird-ectoparasite interactions, nest humidity, and ectoparasite community structure. Ecology 81:958–968

Helfenstein F, Losdat S, Møller AP, Blount JD, Richner H (2010) Sperm of colourful males are better protected against oxidative stress. Ecol Lett 13:213–222

Hõrak P, Saks L, Karu U, Ots I, Surai PF, McGraw KJ (2004a) How coccidian parasites affect health and appearance of greenfinches. J Anim Ecol 73:935–947

Hõrak P, Surai PF, Ots I, Møller AP (2004b) Fat soluble antioxidants in brood-rearing great tits *Parus major*: relations to health and appearance. J Avian Biol 35:63–70

Hõrak P, Zilmer M, Saks L, Ots I, Karu U, Zilmer K (2006) Antioxidant protection, carotenoids and the costs of immune challenge in greenfinches. J Exp Biol 209:4329–4338

Hõrak P, Saks L, Zilmer M, Karu U, Zilmer K (2007) Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. Am Nat 170:625–635

Isaksson C, Örnborg J, Stephensen E, Andersson S (2005) Plasma glutathione and carotenoid coloration as potential biomarkers of environmental stress in great tits. EcoHealth 2:138–146

Jenni-Eiermann S, Jenni L (1998) What can plasma metabolites tell us about the metabolism, physiological state and condition of individual birds? An overview. Biol Conserv Fauna 102:312–319

Lambrechts MM, Adriaensen F, Ardia DR, Artemyev AV, Atiénzar F, Bánbura J, Barba E, Bouvier J-C, Camprodon J, Cooper CB, Dawson RD, Eens M, Eeva T, Faivre B, Garamszegi LZ, Goodenough AE, Gosler AG, Grégoire A, Griffith SC, Gustafsson L, Scott-Johnson L, Kania W, Keišs O, Llambias PE, Mainwaring MC, Mänd R, Massa B, Mazgajski TD, Møller AP, Moreno J, Naef-Daenzer B, Nilsson J-A, Norte AC, Orell M, Otter KA, Park CR, Perrins CM, Pinowski J, Porkert J, Potti J, Remeš V, Richner H, Rytkönen S, Shiao M-T, Silverin B, Slagsvold T, Smith HG, Sorace A, Stenning MJ, Stewart I, Thompson CF, Török J, Tryjanowski P, van Noordwijk AJ, Winkler DW, Ziane N (2010) The design of artificial nestboxes for the study of secondary hole-nesting birds: a review of methodological inconsistencies and potential biases. Acta Ornithol 45:1–26

Lesgards JF, Durand P, Lassarre M, Stocker P, Lesgards G, Lanteaume A, Prost M, Lehucher-Michel MP (2002) Assessment of lifestyle effects on the overall antioxidant capacity of healthy subjects. Environ Health Persp 110:479

Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: a common mistake. Auk 104:116–121

Lobato E, Moreno J, Merino S, Sanz JJ, Arriero E (2005) Haematological variables are good predictors of recruitment in nestling pied flycatchers (*Ficedula hypoleuca*). Écoscience 12:27–34

Lobato E, Merino S, Moreno J, Morales J, Tomás G, Martínez-de la Puente J, Osorno JL, Kuchar A, Möstl E (2008) Corticosterone metabolites in blue tit and pied flycatcher droppings: effects of brood size, ectoparasites and temperature. Horm Behav 53:295–305

Lundberg A, Alatalo RV (1992) The pied flycatcher. Poyser, London

Martínez-de la Puente J, Merino S, Lobato E, Rivero-de Aguilar J, Del Cerro S, Ruiz-de-Castañeda R, Moreno J (2009) Does weather affect biting fly abundance in avian nests? J Avian Biol 40:653–657

Martínez-de la Puente J, Merino S, Lobato E, Rivero-de Aguilar J, del Cerro S, Ruiz-de-Castañeda R, Moreno J (2010) Nest-climatic factors affect the abundance of biting flies and their effects on nestling condition. Acta Oecol 36:543–547

Martínez-de la Puente J, Merino S, Tomás G, Moreno J, Morales J, Lobato E, Martínez J (2011) Nest ectoparasites increase physiological stress in breeding birds: an experiment. Naturwissenschaften 98:99–106

Mateos R, Lecumberri E, Ramos S, Goya L, Bravo L (2005) Determination of malondialdehyde (MDA) by high-performance liquid chromatography in serum and liver as a biomarker for oxidative stress: Application to a rat model for hypercholesterolemia and evaluation of the effect of diets rich in phenolic antioxidants from fruits. J Chromatogr B 827:76–82

McCue MD (2010) Starvation physiology: reviewing the different strategies animals use to survive a common challenge. Comp Biochem Physiol A 156:1–18

Meister A (1991) Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy. Pharmacol Ther 51:155–194

Merino S, Potti J (1995) Mites and blowflies decrease growth and survival in nestling pied flycatchers. Oikos 73:95–103

Merino S, Potti J (1996) Weather dependent effects of nest ectoparasites on their bird hosts. Ecography 19:107–113

Merino S, Potti J (1998) Growth, nutrition and blow fly parasitism in nestling pied flycatchers. Can J Zool 76:936–941

Metcalfe NB, Alonso-Alvarez C (2010) Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Funct Ecol 24:984–996

Metcalfe NB, Monaghan P (2013) Does reproduction cause oxidative stress? An open question. Trends Ecol Evol 28:347–350

Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A (1993) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci 84:407

Møller AP (1997) Parasitism and the evolution of host life history. In: Clayton DH, Moore J (eds) Host-parasite evolution: general principles and avian models. Oxford University Press, Oxford, pp 105–127

Møller AP, de Lope F, Moreno J, González G, Pérez JJ (1994) Ectoparasites and host energetics: house martin bugs and house martin nestlings. Oecologia 98:263–268

Møller AP, Christe P, Garamszegi LZ (2005) Coevolutionary arms races: increased host immune defense promotes specialization by avian fleas. J Evol Biol 18:46–59

Monaghan P, Metcalfe NB, Torres R (2009) Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecol Lett 12:75–92

Moreno J, Merino S, Potti J, de León A, Rodríguez R (1999) Maternal energy expenditure does not change with flight costs or food availability in the pied flycatcher (*Ficedula hypoleuca*): costs and benefits for nestlings. Behav Ecol Sociobiol 46:244–251

Moreno J, Lobato E, Morales J, Merino S, Martínez-de La Puente J, Tomás G (2008) Pre-laying nutrition mediates maternal effects on offspring immune capacity and growth in the pied flycatcher. Oecologia 156:727–735

Moreno J, Merino S, Lobato E, Ruiz-de-Castañeda R, Martínez-de La Puente J, del Cerro S, Rivero-de Aguilar J (2009) Nest-dwelling ectoparasites of two sympatric hole-nesting passerines in relation to nest composition: An experimental study. Écoscience 16:418–427

Moreno-Rueda G, Redondo T, Trenzado CE, Sanz A, Zúñiga JM (2012) Oxidative stress mediates physiological costs of begging in magpie (*Pica pica*) nestlings. PLoS One 7:e40367

Mougeot F, Martínez-Padilla J, Webster LM, Blount JD, Pérez-Rodríguez L, Piertney SB (2009) Honest sexual signalling mediated by parasite and testosterone effects on oxidative balance. Proc R Soc Lond B Biol Sci 276:1093–1100

Noguera JC, Kim SY, Velando A (2011) Pre-fledgling oxidative damage predicts recruitment in a long-lived bird. Biol Lett 8:61–63

Norte AC, Ramos JA, Araujo PM, Sousa JP, Sheldon BC (2008) Health-state variables and enzymatic biomarkers as survival predictors in nestling great tits (*Parus major*): effects of environmental conditions. Auk 125:943–952

Oppliger A, Richner H, Christe P (1994) Effect of an ectoparasite on lay date, nest-site choice, desertion, and hatching success in the great tit (*Parus major*). Behav Ecol 5:130–134

Owen JP, Delany ME, Cardona CJ, Bickford AA, Mullens BA (2009) Host inflammatory response governs fitness in an avian ectoparasite, the northern fowl mite (*Ornithonyssus sylviarum*). Int J Parasitol 39:789–799

Owen JP, Nelson AC, Clayton DH (2010) Ecological immunology of bird-ectoparasite systems. Trends Parasitol 26:530–539

Pap PL, Vágási CI, Czirják GÁ, Titilincu A, Pintea A, Osváth G, Fülöp A, Barta Z (2011) The effect of coccidians on the condition and immune profile of molting house sparrows (*Passer domesticus*). Auk 128:330–339

Pérez-Rodríguez L, Romero-Haro AA, Sternalski A, Muriel J, Mougeot F, Gil D, Alonso-Alvarez C (2015) Measuring oxidative stress: the confounding effect of lipid concentration in measures of lipid peroxidation. Physiol Biochem Zool. doi:10.1086/680688

Pérez-Rodríguez L (2009) Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. BioEssays 31:1116–1126

Pérez-Rodríguez L, Mougeot F, Alonso-Alvarez C, Blas J, Viñuela J, Bortolotti GR (2008) Cell-mediated immune activation rapidly decreases plasma carotenoids but does not affect oxidative stress in red-legged partridges (*Alectoris rufa*). J Exp Biol 211:2155–2161

Potti J (2007) Variation in the hematocrit of a passerine bird across life stages is mainly of environmental origin. J Avian Biol 38:726–730

Potti J, Moreno J, Merino S, Frías O, Rodríguez R (1999) Environmental and genetic variation in the haematocrit of fledgling pied flycatchers *Ficedula hypoleuca*. Oecologia 120:1–8

Potti J, Dávila JA, Tella JL, Frias O, Villar S (2002) Gender and viability selection on morphology in fledgling pied flycatchers. Mol Ecol 11:1317–1326

Proctor H, Owens I (2000) Mites and birds: diversity, parasitism and coevolution. Trends Ecol Evol 15:358–364

Rakhshandehroo E, Razavi SM, Nazifi S, Farzaneh M, Mobarraei N (2013) Dynamics of the enzymatic antioxidants during experimental caprine coccidiosis. Parasitol Res 112:1437–1441

Rendell WB, Verbeek NAM (1996) Are avian ectoparasites more numerous in nest boxes with old nest material? Can J Zool 74:1819–1825

Richner H, Tripet F (1999) Ectoparasitism and the trade-off between current and future reproduction. Oikos 86:535–538

Richner H, Oppliger A, Christe P (1993) Effect of an ectoparasite on reproduction in great tits. J Anim Ecol 62:703–710

Romero-Haro AA, Alonso-Alvarez C (2014) Covariation in oxidative stress markers in the blood of nestling and adult birds. Physiol Biochem Zool 87:353–362

Saino N, Bertacche V, Ferrari RP, Martinelli R, Møller AP, Stradi R (2002) Carotenoid concentration in barn swallow eggs is influenced by laying order, maternal infection and paternal ornamentation. Proc R Soc Lond B Biol Sci 269:1729–1733

Saino N, Caprioli M, Romano M, Boncoraglio G, Rubolini D, Ambrosini R, Bonisoli-Alquati A, Romano A (2011) Antioxidant defenses predict long-term survival in a passerine bird. PLoS One 6:e19593

Sanz JJ, Moreno J (1995) Mass loss in brooding female pied flycatchers *Ficedula hypoleuca*: no evidence for reproductive stress. J Avian Biol 26:313–320

Schwilch R, Jenni L, Jenni-Eiermann S (1996) Metabolic responses of homing pigeons to flight and subsequent recovery. J Comp Physiol B 166:77–87

Sepp T, Karu U, Blount JD, Sild E, Männiste M, Hõrak P (2012) Coccidian infection causes oxidative damage in greenfinches. PLoS One 7:e36495

Sies H, Stahl W (1995) Vitamins E and C, beta-carotene, and other carotenoids as antioxidants. Am J Clin Nutr 62:1315S–1321S

Simon A, Thomas D, Blondel J, Perret P, Lambrechts MM (2004) Physiological ecology of Mediterranean blue tits (*Parus caeruleus* L.): effects of ectoparasites (*Protocalliphora* spp.) and food abundance on metabolic capacity of nestlings. Physiol Biochem Zool 77:492–501

Sorci G, Faivre B (2009) Inflammation and oxidative stress in vertebrate host-parasite systems. Philos Trans R Soc Lond B Biol Sci 364:71–83

Tomás G, Merino S, Moreno J, Morales J (2007) Consequences of nest reuse for parasite burden and female health and condition in blue tits, *Cyanistes caeruleus*. Anim Behav 73:805–814

Tomás G, Merino S, Martínez-de la Puente J, Moreno J, Morales J, Lobato E (2008) Determinants of abundance and effects of blood-sucking flying insects in the nest of a hole-nesting bird. Oecologia 156:305–312

Tomás G, Merino S, Martínez-de la Puente J, Moreno J, Morales J, Lobato E, Rivero-de Aguilar J, del Cerro S (2012) Interacting effects of aromatic plants and female age on nest-dwelling ectoparasites and blood-sucking flies in avian nests. Behav Process 90:246–253

van de Crommenacker J, Richardson DS, Koltz AM, Hutchings K, Komdeur J (2012) Parasitic infection and oxidative status are associated and vary with breeding activity in the Seychelles warbler. Proc R Soc Lond B Biol Sci 279:1466–1476

von Schantz T, Bensch S, Grahn M, Hasselquist D, Wittzell H (1999) Good genes, oxidative stress and condition-dependent sexual signals. Proc R Soc Lond B Biol Sci 266:1–12

Wegmann M, Boegeli B, Richner H (2015) Physiological responses to increased brood size and ectoparasite infestation: Adult great tits favour self-maintenance. Physiol Behav 141:127–134

Wu G, Fang YZ, Yang S, Lupton JR, Turner ND (2004) Glutathione metabolism and its implications for health. J Nutr 134:489–492

Zuk M, Stoehr AM (2002) Immune defense and host life history. Am Nat 160:S9–S22