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Plumage ornaments and reproductive investment in relation to oxidative status in the Iberian Pied Flycatcher (*Ficedula hypoleuca iberiae*)

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Abstract: A key aspect in the study of plumage traits with a potential role in communication is the cost associated with trait production and maintenance, expressed in terms of oxidative stress. In the Iberian Pied Flycatcher (*Ficedula hypoleuca iberiae* (Witherby, 1928)), males and some females exhibit a white forehead patch and both sexes present conspicuous white patches on the wings. We examined associations between these plumage ornaments and their ability to cope with oxidative stress. Furthermore, we explored oxidative costs of reproductive investment. Total antioxidant status (TAS) in plasma and glutathione (tGSH) levels in red blood cells, as well as a marker of oxidative damage in plasma lipids (malondialdehyde (MDA)), were assessed simultaneously for the first time in studies of avian reproduction. We found negative associations between antioxidants and ornaments in incubating females, although this relationship was positive while feeding nestlings. For males, MDA levels were negatively associated with ornaments, while TAS showed a positive relation. Female MDA showed a positive correlation with intensity of incubation attendance, while this relation was negative for tGSH levels. These results indicate that multiple achromatic plumage ornaments signal the individual capacity to cope with costs related to oxidative stress. Moreover, this study highlights the critical role of incubation for avian life histories.

Key words: egg attendance, *Ficedula hypoleuca iberiae*, glutathione, malondialdehyde (MDA), Iberian Pied Flycatcher, provisioning rates, total antioxidant status (TAS), achromatic plumage patches.

Résumé : Un aspect clé de l'étude des caractères du plumage pouvant jouer un rôle dans la communication est le coût associé à la production et au maintien des caractères, exprimé en termes de stress oxydatif. Les gobemouches noirs ibériques (*Ficedula hypoleuca iberiae* (Witherby, 1928)) mâles et certaines femelles présentent une tache frontale blanche et les deux sexes présentent des taches blanches bien en évidence sur les ailes. Nous avons examiné les associations entre ces ornements du plumage et la capacité des oiseaux à faire face au stress oxydatif. Nous avons en outre examiné les coûts oxydatifs de l'investissement reproducteur. La capacité antioxydante totale (TAS) dans le plasma et les concentrations de glutathion (tGSH) dans les globules rouges, ainsi qu'un marqueur des dommages oxydatifs dans les lipides plasmatiques (le malondialdéhyde (MDA)) ont été évalués simultanément pour la première fois dans une étude de la reproduction des oiseaux. Nous avons constaté des associations négatives entre les antioxydants et l'ornementation des ailes chez les femelles couveuses, cette relation étant toutefois positive quand les femelles nourrissaient des oisillons. En ce qui concerne les mâles, les concentrations de MDA étaient négativement associées aux ornements, alors que la TAS présentait une relation positive. Le MDA des femelles présentait une corrélation positive avec l'intensité de l'assiduité à couver, alors que cette relation était négative pour les concentrations de tGSH. Ces résultats indiquent que des ornements achromatiques multiples du plumage signalent la capacité des individus de faire face aux coûts associés au stress oxydatif. De plus, l'étude met en relief le rôle clé de la couvaison dans les cycles biologiques des oiseaux. [Traduit par la Rédaction]

Mots-clés : assiduité à couver, *Ficedula hypoleuca iberiae*, glutathion, malondialdéhyde (MDA), gobemouche noir ibérique, taux d'approvisionnement, capacité antioxydante totale (TAS), taches achromatiques du plumage.

Introduction

Ornamental plumage in birds is one of the most studied types of sexually selected traits (Hill and McGraw 2006) and often shows condition-dependent variation (Cotton et al. 2004 and references therein). Plumage colour may be due to pigmentation, feather structure, or a combination of both (McGraw et al. 2002). Although feather colouration can be produced by various pigments, melanins are the most prevalent and can also be deposited in all avian integuments (Prum and Williamson 2002; Leskinen et al. 2012). However, apart from these colouration traits, some plum-age ornaments consist of patches of white plumage that produce a scattering of light in all directions by unmelanized feather keratin (Prum et al. 1999). Perhaps because the production of achromatic plumage requires neither pigments nor a precisely ordered feather nanostructure, producing a small amount of unmelanized feathers has traditionally been considered to be noncostly in terms of resource allocation. However, proposed mechanisms for maintaining the honesty of unpigmented signals have usually focused not on production costs but on various costs of maintaining the trait (McGlothlin et al. 2007), such as a higher risk of

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physical abrasion (e.g., Barrowclough and Sibley 1980) or biotic degradation by feather-degrading bacteria or ectoparasites (Kose et al. 1999; Goldstein et al. 2004; Gunderson et al. 2008; Burtt et al. 2011; Ruiz-de-Castañeda et al. 2012).

A key aspect in the study of the function and evolution of plumage traits with a potential role in communication is the cost associated with trait production and maintenance. Handicap Theory predicts that the honesty of sexual ornaments reflects individual capacity to withstand signalling costs (Zahavi 1977; Andersson and Iwasa 1996). These costs have been traditionally associated with resource limitation (Siefferman and Hill 2007), impaired immunocompetence (Folstad and Karter 1992), or energetic constraints (Hill 2000). However, more recently, some of these costs have been considered in terms of oxidative stress (von Schantz et al. 1999; Alonso-Alvarez et al. 2007). Oxidative stress is usually defined as the imbalance between levels of reactive oxygen and nitrogen species and the state of the antioxidant machinery in the organism (Halliwell and Gutteridge 2007; Metcalfe and Alonso-Alvarez 2010). Oxidative stress 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). However, research on oxidative costs of sexual signalling has been foremost focused on carotenoid-based ornaments (Pérez-Rodríguez 2009; Simons et al. 2012) and melanin-based ornaments (e.g., Galván and Alonso-Alvarez 2008; Roulin et al. 2011), but little is known about achromatic ornaments. Apart from the particular case of signal expression, oxidative stress has important health-related implications (Costantini et al. 2006) and is considered to be a mediator of life-history trade-offs between growth, reproduction, and self-maintenance (Dowling and Simmons 2009; Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010). For instance, there is evidence that nestling provisioning rates, the most frequently used measure of parental effort (Moreno et al. 1999), are related to high metabolic rates (Moreno et al. 2001; Nilsson 2002) and these high levels of metabolism may cause oxidative stress by the increased production of pro-oxidant metabolites and free radicals (von Schantz et al. 1999).

In the Iberian Pied Flycatcher (*Ficedula hypoleuca iberiae* (Witherby, 1928)), a small passerine bird, males and females differ in the expression of melanin-based dorsal plumage; males are conspicuously black, while females are brown (Lundberg and Alatalo 1992). In Iberian populations, males and some females exhibit a distinctive white forehead patch (Potti 1993; Morales et al. 2007) and both sexes have conspicuous white patches on their wings (Lundberg and Alatalo 1992). A large number of studies have shown that the presence and extent of these white-plumage patches in both sexes of Pied Flycatchers are associated with reproductive success (Morales et al. 2007), resistance to infections (Potti and Merino 1996), physiological stress (Lobato et al. 2010), hormonal levels (Moreno et al. 2014), or genetic quality (related Collared Flycatchers (*Ficedula albicollis* (Temminck, 1815)); Török et al. 2003). Likewise, blackness of males has shown associations with timing of breeding and reproductive success (Galván and Moreno 2009; Sirkiä et al. 2010), predation risk (Slagsvold et al. 1995), condition (Slagsvold and Lifjeld 1992), and immune response (Kerimov et al. 2013). White-plumage patches (but not plumage blackness) of flycatchers have been also related to individual and mate oxidative stress in the genus *Ficedula* Brisson, 1760 (Markó et al. 2011; Moreno et al. 2011, 2013a, 2013b).

Our main goal was to examine possible associations between plumage sexual ornaments of Iberian Pied Flycatchers and their oxidative status during the reproductive period. Previous studies have shown that the sign of relationships between reproduction and oxidative status can change depending on reproductive phase because oxidative stress can reflect both costs of and constraints on reproduction (Stier et al. 2012). For these reasons, we aim to study the covariation between oxidative traits and before and after hatching reproductive investment in this species,

something which has not been previously done. 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 have used plasma malondialdehyde (MDA) levels, a by-product of peroxidative decomposition of unsaturated lipids (Halliwell and Gutteridge 2007), as a measure of oxidative damage and a presumptive marker of oxidative stress (Mateos et al. 2005; Halliwell and Gutteridge 2007; Sepp et al. 2012). To monitor antioxidant defences, we have measured simultaneously the total antioxidant status (TAS) of plasma and total glutathione (tGSH) levels in red blood cells. TAS measures the pooled effect of all extracellular antioxidant compounds of the blood (Miller et al. 1993; Cohen et al. 2007; Costantini 2011). Glutathione is a tripeptide thiol found in animal cells functioning in the protection of cells against free radicals, being often considered to be the most important intracellular antioxidant (Meister 1991; Wu et al. 2004). No previous study has, to our knowledge, included these three variables simultaneously in analyses of avian ornamentation and reproductive investment.

Given the potential costs of investment in ornamentation and reproduction in terms of oxidative stress, we predicted that (*i*) higher levels of signalling (i.e., larger achromatic patches in both sexes or more melanised dorsal plumage in males) would be related to a higher capacity to cope with oxidative stress (more antioxidant defences and (or) lower levels of oxidative damage), and (*ii*) there would be a relationship between reproductive investment (incubation attendance, provisioning rates) and oxidative status of breeding individuals. However, in a correlative study such as this, causes and effects are difficult to separate, preventing us from making unique predictions. Thus, if higher oxidative stress limits investment in reproduction, then we would predict negative relationships between reproductive investment and oxidative stress, whereas the opposite is expected if higher oxidative levels result from increased reproductive investment. Moreover, the sign and intensity of these associations could change between the incubation and the nestling phases as mentioned above.

Materials and methods

General field methods

The study was conducted during the springs of 2012 and 2013 in a montane forest of Pyrenean oak (*Quercus pyrenaica* Willd.) at 1200 m above sea level in Valsaín, central Spain (40°54′N, 04°01′W), where long-term studies on cavity-nesting birds have been ongoing since 1991. In the study area, there are 570 nest boxes (for dimensions, structure, and placement of nest boxes see appendix in Lambrechts et al. 2010) occupied by Iberian Pied Flycatchers, Great Tits (*Parus major* L., 1758), Eurasian Nuthatches (*Sitta europaea* L., 1758), and Blue Tits (*Cyanistes caeruleus* (L., 1758)).

We followed breeding activities from the early stages of nest construction to fledging in nest boxes occupied by Iberian Pied Flycatchers. Egg laying in the Iberian Pied Flycatcher population under study typically begins in late May and modal clutch size is six and chicks usually fledge at the age of 17 days. The female incubates and broods alone and receives part of her food from her mate (Moreno et al. 2011). Breeding activities were followed routinely and laying and hatching dates and brood sizes at fledging were determined.

In 2012 and 2013, males and females were captured in their nest boxes with traps while provisioning nestlings of 7–8 days, ringed if necessary, or identified and weighed to the nearest 0.01 g with a digital balance. As experiments were carried out in 2012 and 2013, we have only included unmanipulated control birds in this study. We took a blood sample of about 120 μL from the brachial vein that was collected in heparinized microcapillaries. Females were blood-sampled in 2012 (*n* = 37) and 2013 (*n* = 33), whereas males were only blood-sampled in 2013 (*n* = 32). Blood samples were

stored in Eppendorf tubes in an icebox until returning to the laboratory in the same day. Plasma was separated from blood by centrifugation (10 min at 12 000 rev·min⁻¹) and then both fractions were stored at −80 °C until analysed for assaying MDA, TAS, and uric acid from plasma, and tGSH in red blood cells (see below). If haemolysis occurs during sampling, then a possible efflux of intracellular pro-oxidants and antioxidant molecules into plasma could alter levels of oxidative markers measured in blood samples, thereby confounding interpretation of results. Thus, haemolysis levels in plasma samples were noted by a visual detection of red colour of plasma, as a consequence of release of haemoglobin from red blood cells, in a continuous gradient from 0 (no haemolysis) to 2 (high degree of haemolysis). Only one person noted haemolysis degree to minimize interobserver variability.

Additionally, in 2013 some females belonging to this sample ($n = 30$) were also captured during incubation (7 or 8 days after clutch completion) in the nest box during daytime without traps, as they are not easily frightened away from the nest when incubating. They were ringed if necessary, identified, weighed, and blood-sampled in the same way as above. The whole procedure took less than 5 min and no female deserted after manipulation.

All captured individuals were photographed at the nestling stage to analyze their ornamentation: head from above with a ruler below the chin for reference and folded wing from above with ruler beside for reference (Moreno et al. 2011). Then, we determined areas of forehead and wing white patches analyzing photographs with Photoshop CS4 (version 11.0; Adobe Inc., San Jose, California, USA) according to Moreno et al. (2014). In the case of males, we also determined the blackness of dorsal plumage as the percentage of black feathers on the back of the head and mantle (Galván and Moreno 2009).

Lipid peroxidation assays

Plasma concentrations of MDA were calculated following Agarwal and Chase (2002) with some modifications made by Mougeot et al. (2009). Assays were carried out in 2 mL capacity 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 stock solution (10 µmol·L⁻¹ in 40% ethanol) serially diluted using 40% ethanol. Twenty-five microlitres butylated hydroxytoluene solution (0.05% m/v in 95% ethanol), 200 µL phosphoric acid solution (0.44 mol·L⁻¹), and 50 µL thiobarbituric acid solution (42 mmol·L⁻¹) were added to 25 µL of plasma samples (1:2.5 dilution in water) or standards. Samples were mixed using a vortex for 5 s and then heated at 100 °C for 1 h on a dry bath incubator to allow 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 mixed using a vortex for 1 min. Tubes were then centrifuged at 14 000 rev·min⁻¹ 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 Technologies, Santa Clara, California, USA) fitted with a 5 µm ACE guard column and 5 µm ODS 100 mm × 4.6 mm column (Advanced Chromatography Technologies Ltd., Aberdeen, Scotland) maintained at 37 °C. The mobile phase was methanol-buffered (40:60 v/v), the buffer being a 50 mmol·L⁻¹ anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5 mol·L⁻¹ 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 (reference No. G1321A; Agilent Technologies) set at 515 nm (excitation) and 553 nm (emission). Repeatability showed by a set of samples assayed in duplicate and TEP standards was high ($R = 0.722$, $N = 66$, $p < 0.001$ and $R = 0.962$, $N = 6$, $p < 0.001$, respectively).

Total antioxidant status (TAS)

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 µmol·L⁻¹ myoglobin (reference No. M0630-250MG; Sigma-Aldrich, St. Louis, Missouri, USA) and 740 µmol·L⁻¹ potassium ferricyanate, then passing the mixture through a column of Sephadex (reference No. G15-120; Sigma-Aldrich, St. Louis, Missouri, USA). The chromogen, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), was mixed in phosphate-buffered saline (PBS) to 153 µmol·L⁻¹. The standard was made by dissolving a water-soluble α-tocopherol derivative, Trolox, in PBS to 1.7 mmol·L⁻¹. The assay was run in 96-well flat-bottomed clear microplates on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, Vermont, USA). 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 were put separately into the wells. Next, 15 µL metmyoglobin and 250 µL ABTS were sequentially added to each well. A multichannel pipette was used to simultaneously add 50 µL of 300 µmol·L⁻¹ H_2O_2 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 H_2O_2) manually using a timer. The reaction runs for around 10 min. Most of samples were assayed in duplicate and showed high repeatability ($R = 0.981$, $N = 60$, $p < 0.001$).

Intracellular total glutathione (tGSH) level

tGSH levels in red blood cells were determined as described in López-Arrabé et al. (2014). Briefly, samples of red blood cells were homogenized in a stock buffer and mixed with 10% trichloroacetic acid. The mixture was mixed using a vortex, centrifuged, and the supernatant was separated. Three working solutions were made up in a reaction buffer as follows: (1) 0.3 mmol·L⁻¹ NADPH, (2) 6 mmol·L⁻¹ DTNB, and (3) 50 U GSH reductase·mL⁻¹. The next steps were performed on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc.). To 75 µL of sample (supernatant), we added 240 µL of the mixture of solutions 1 and 2. Afterward, 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. A set of samples assayed in duplicate showed high repeatability ($R = 0.983$, $N = 106$, $p < 0.001$).

Measurement of levels of uric acid

Uric acid is the main form of nitrogen excretion in birds and an indicator of amino acid catabolism. But, in addition, 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 TAS values corrected for uric acid are recommended over raw TAS levels (Cohen et al. 2007). The uricase/peroxidase method was used for measuring levels (kit reference No. 11522; Biosystems, Barcelona, Spain) and using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc.). Reagent volumes and further assay details were implemented according to manufacturer instructions. A set of samples assayed in duplicate showed high repeatability ($R = 0.99$, $N = 45$, $p < 0.001$).

Behavioural data

In 2012 and 2013, 5 days after clutch completion (day 6 of incubation) and 2 and 8 days after hatching date, we recorded nest activity inside nest boxes for about 95 min (97.28 ± 1.83 min (mean ± SE), $N = 110$) with a cold white light (LED; 5 mm) powered by a 3 V battery and a camera (GoPro HD Hero1) mounted on the roof inside the nest box (Cantarero et al. 2013). From records taken

during incubation, we estimated the proportion of time spent by the female inside the nest box or "egg attendance", which includes the time allocated to incubating and turning the eggs (Cantarero et al. 2013). In addition, we estimated hourly provisioning rates by males to incubating females. From films taken during the early and late nestling periods, we obtained hourly provisioning rates by males and females to nestlings. At the early nestling phase, we also estimated "brooding attendance", which is the proportion of time spent by the female inside the nest box.

Statistical analyses

Physiological variables were normally distributed and were therefore analyzed with general linear models (GLM) using the STATISTICA version 8.0 package (StatSoft, Inc., Tulsa, Oklahoma, USA) and final models were selected by backward elimination of nonsignificant terms to improve the models.

To establish differences in oxidative parameters, we used Student's t test analyses between males and females and between reproductive phases. Moreover, we explored correlation matrices for possible associations between these variables.

In both males and females, the association between wing and forehead patches was weak (males: $r^2 = 0.03$; females: $r^2 = 0.13$). To determine if incubating female oxidative parameters were related to their own ornaments, we applied GLM models with MDA, TAS, or tGSH levels measured in incubation as dependent variables and laying date, clutch size, incubating female body mass, female forehead and wing white patches as covariates. For female physiological parameters measured during the nestling phase, we applied similar models but substituted laying date, clutch size, and incubating female body mass with hatching date, brood size at the age of 9 days, and female body mass during the nestling phase, respectively, and included year as fixed factor. Since Moreno et al. (2013a) found a strong correlation between oxidative damage levels of females and blackness of their mates, we included this male trait in female MDA models. In the case of males, models included physiological variables as dependents variables, while hatching date, brood size, male body mass, blackness of dorsal plumage, and male forehead and wing white patches were introduced as covariates.

We analyzed if female oxidative parameters during incubation were related to incubating behaviour, including laying date, clutch size, egg attendance, and male provisioning rate during incubation as covariates. During the nestling phase, we analyzed the associations between parental care and physiological condition for both females and males. For females, we included oxidative variables as dependent variables, year as fixed factor, and brooding attendance and provisioning rates by females to nestlings as covariates. In the case of males, models included oxidative parameters as dependents and male provisioning rates to nestlings as covariates. In both cases, we controlled by hatching date and brood size.

In all cases, we controlled MDA, TAS, and tGSH models by including haemolysis degree and TAS analyses by levels of uric acid (see above) as covariates.

Results

There were no differences between male and female oxidative levels (Student's t test: all $p > 0.05$; Table 1) and between reproductive phases for female variables (Student's t test: all $p > 0.05$; Table 1). No correlations were found between both female and male MDA, TAS, and tGSH levels (all $p > 0.05$). In all cases, haemolysis degree was positively correlated with MDA levels (Tables 2, 3, 4) and only for females during the nestling stage was haemolysis negatively correlated with tGSH levels (Tables 2, 3, 4). In all cases, levels of uric acid were positively correlated with TAS values (Tables 2, 3, 4).

Oxidative status and plumage ornamentation

At incubation female TAS and tGSH levels were negatively related to folded wing-patch area and forehead white patch, respectively (Table 2). tGSH was higher in heavier females (Table 2).

Table 1. Levels (mean ± SE) of malondialdehyde (MDA), total antioxidant status (TAS) of plasma, and total glutathione (tGSH) in male and female Iberian Pied Flycatchers (*Ficedula hypoleuca iberiae*) during the incubation and nestling phases.

Sex	Variable	Incubation phase	Nestling phase
Female	MDA (μmol·mL^{-1})	10.00±0.77	11.21±0.64
	TAS (mmol·L^{-1})	2.60±0.16	1.62±0.08
	tGSH (μmol·g^{-1})	3.36±0.16	3.27±0.09
Male	MDA (μmol·mL^{-1})	—	14.24±1.15
	TAS (mmol·L^{-1})	—	1.83±0.08
	tGSH (μmol·g^{-1})	—	3.55±0.12

Table 2. Results of general linear models (GLM) for female physiological variables (levels of malondialdehyde (MDA), total antioxidant status (TAS) of plasma, and total glutathione (tGSH)) measured during the incubation phase of the Iberian Pied Flycatcher (*Ficedula hypoleuca iberiae*).

	df	β	F	p	Partial η^2
MDA (μmol·mL^{-1})					
Full model					
Laying date	1, 13	−0.093	0.194	0.667	0.015
Clutch size	1, 13	0.297	1.762	0.207	0.119
Body mass (g)	1, 13	0.317	2.806	0.118	0.177
Forehead-patch area (cm²)	1, 13	0.057	0.072	0.792	0.005
Wing-patch area (cm²)	1, 13	0.062	0.081	0.780	0.006
Male blackness (%)	1, 13	−0.070	0.098	0.759	0.007
Haemolysis	1, 13	0.484	5.126	0.041*	0.283
Final model					
Haemolysis	1, 25	0.609	14.761	0.001*	0.371
TAS (mmol·L^{-1})					
Full model					
Laying date	1, 10	0.088	0.220	0.649	0.022
Clutch size	1, 10	−0.004	<0.001	0.984	<0.001
Body mass (g)	1, 10	0.153	0.737	0.411	0.069
Forehead-patch area (cm²)	1, 10	0.133	0.485	0.502	0.046
Wing-patch area (cm²)	1, 10	−0.495	5.534	0.040*	0.356
Uric acid (mg·dL^{-1})	1, 10	0.910	22.748	<0.001*	0.695
Haemolysis	1, 10	−0.088	0.234	0.639	0.023
Final model					
Wing-patch area (cm²)	1, 17	−0.397	8.517	0.010*	0.334
Uric acid (mg·dL^{-1})	1, 17	0.915	45.375	<0.001*	0.727
tGSH (μmol·g^{-1})					
Full model					
Laying date	1, 18	0.302	2.692	0.118	0.130
Clutch size	1, 18	0.169	0.745	0.399	0.039
Body mass (g)	1, 18	0.411	5.495	0.031*	0.234
Forehead-patch area (cm²)	1, 18	−0.403	4.513	0.048*	0.200
Wing-patch area (cm²)	1, 18	−0.116	0.370	0.550	0.020
Haemolysis	1, 18	−0.362	3.199	0.090	0.151
Final model					
Body mass (g)	1, 24	0.339	4.333	0.048*	0.153
Forehead-patch area (cm²)	1, 24	−0.489	9.014	0.006*	0.273

*Significant difference ($\alpha = 0.05$).

While feeding nestlings, females showed a positive association between TAS and presence of a forehead patch (Table 3). Females that bred earlier showed higher levels of TAS (Table 3). While tGSH level was once again positively related to female body mass, it showed a negative association with the number of nestlings (Table 3). Female MDA levels were not related to ornamental traits or condition at any stage (Tables 2, 3).

In the case of males, wing-patch area was negatively and positively associated with MDA and tGSH levels, respectively (Table 4), while TAS showed no associations with ornaments (Table 4). MDA levels also showed a positive correlation with brood size (Table 4).

Table 3. Results of general linear models (GLM) for female physiological variables (levels of malondialdehyde (MDA), total antioxidant status (TAS) of plasma, and total glutathione (tGSH)) measured during the nestling phase of the Iberian Pied Flycatcher (*Ficedula hypoleuca iberiae*).

	df	β	F	p	Partial η^2
MDA (μmol·mL^{-1})					
Full model					
Hatching date	1, 37	−0.155	0.388	0.537	0.010
Brood size	1, 37	0.004	<0.001	0.977	<0.001
Body mass (g)	1, 37	−0.010	0.004	0.949	<0.001
Forehead-patch area (cm²)	1, 37	−0.006	0.001	0.970	<0.001
Wing-patch area (cm²)	1, 37	−0.216	1.681	0.203	0.043
Male blackness (%)	1, 37	0.028	0.031	0.860	<0.001
Haemolysis	1, 37	0.440	6.687	0.014*	0.153
Year	1, 37	−0.005	<0.001	0.984	<0.001
Final model					
Haemolysis	1, 65	0.491	20.595	<0.001*	0.241
TAS (mmol·L^{-1})					
Full model					
Hatching date	1, 25	−0.499	4.865	0.037*	0.162
Brood size	1, 25	−0.176	1.975	0.172	0.073
Body mass (g)	1, 25	0.185	1.630	0.213	0.061
Forehead-patch area (cm²)	1, 25	0.312	6.484	0.017*	0.206
Wing-patch area (cm²)	1, 25	0.087	0.392	0.537	0.015
Uric acid (mg·dL^{-1})	1, 25	0.756	35.067	<0.001*	0.584
Haemolysis	1, 25	0.034	0.079	0.780	0.003
Year	1, 25	−0.356	2.128	0.157	0.078
Final model					
Hatching date	1, 34	−0.213	4.569	0.039*	0.119
Forehead-patch area (cm²)	1, 34	0.211	4.143	0.049*	0.109
Uric acid (mg·dL^{-1})	1, 34	0.824	63.494	<0.001*	0.651
tGSH (μmol·g^{-1})					
Full model					
Hatching date	1, 51	0.004	<0.001	0.984	<0.001
Brood size	1, 51	−0.289	5.340	0.024*	0.095
Body mass (g)	1, 51	0.426	9.713	0.003*	0.160
Forehead-patch area (cm²)	1, 51	0.013	0.011	0.917	<0.001
Wing-patch area (cm²)	1, 51	0.154	1.224	0.274	0.023
Haemolysis	1, 51	−0.354	7.162	0.010*	0.123
Year	1, 51	−0.165	0.684	0.412	0.013
Final model					
Brood size	1, 61	−0.309	7.228	0.009*	0.106
Body mass (g)	1, 61	0.308	6.901	0.011*	0.102
Haemolysis	1, 61	−0.272	5.516	0.022*	0.083

*Significant difference (α = 0.05).

Table 4. Results of general linear models (GLM) for male physiological variables (levels of malondialdehyde (MDA), total antioxidant status (TAS) of plasma, and total glutathione (tGSH)) measured during the nestling phase of the Iberian Pied Flycatcher (*Ficedula hypoleuca iberiae*).

	df	β	F	p	Partial η^2
MDA (μmol·mL^{-1})					
Full model					
Hatching date	1, 21	0.010	0.008	0.930	<0.001
Brood size	1, 21	0.291	5.212	0.033*	0.199
Body mass (g)	1, 21	0.086	0.495	0.489	0.023
Blackness (%)	1, 21	−0.079	0.379	0.545	0.018
Forehead-patch area (cm²)	1, 21	0.024	0.036	0.852	0.002
Wing-patch area (cm²)	1, 21	−0.245	4.065	0.057	0.162
Haemolysis	1, 21	0.626	19.119	<0.001	0.476
Final model					
Brood size	1, 25	0.296	7.614	0.011*	0.233
Wing-patch area (cm²)	1, 25	−0.261	5.533	0.027*	0.181
Haemolysis	1, 25	0.627	30.163	<0.001*	0.547
TAS (mmol·L^{-1})					
Full model					
Hatching date	1, 16	−0.172	1.788	0.200	0.100
Brood size	1, 16	0.089	0.387	0.542	0.024
Body mass (g)	1, 16	0.073	0.327	0.575	0.020
Blackness (%)	1, 16	−0.176	1.680	0.213	0.095
Forehead-patch area (cm²)	1, 16	0.233	3.207	0.092	0.167
Wing-patch area (cm²)	1, 16	0.194	2.535	0.131	0.137
Uric acid (mg·dL^{-1})	1, 16	0.606	14.520	0.001*	0.476
Haemolysis	1, 16	0.202	1.718	0.208	0.097
Final model					
Uric acid (mg·dL^{-1})	1, 23	0.833	52.357	<0.001*	0.695
tGSH (μmol·g^{-1})					
Full model					
Hatching date	1, 23	−0.032	0.037	0.849	0.001
Brood size	1, 23	0.237	1.656	0.211	0.067
Body mass (g)	1, 23	−0.171	0.929	0.345	0.039
Blackness (%)	1, 23	−0.240	1.688	0.207	0.068
Forehead-patch area (cm²)	1, 23	0.242	1.706	0.204	0.069
Wing-patch area (cm²)	1, 23	0.478	7.382	0.012*	0.243
Haemolysis	1, 23	−0.130	0.364	0.552	0.015
Final model					
Wing-patch area (cm²)	1, 29	0.554	12.863	0.001*	0.307

*Significant difference (α = 0.05).

Oxidative status and reproductive investment

During incubation, female MDA showed a positive correlation with intensity of egg attendance ($F_{[1,21]}$ = 6.088, p = 0.022, partial η^2 = 0.225; Fig. 1), although it was not related to male provisioning rate to females ($F_{[1,17]}$ = 1.358, p = 0.260, partial η^2 = 0.074). While tGSH levels negatively covaried with egg attendance ($F_{[1,24]}$ = 7.445, p = 0.012, partial η^2 = 0.237; Fig. 2), this was not related to male provisioning rate to females ($F_{[1,18]}$ = 0.659, p = 0.427, partial η^2 = 0.035). TAS levels of incubating females were not related to attendance variables (all p > 0.05).

Neither female nor male oxidative parameters were related to parental-care investment during the nestling phase (all p > 0.05).

Discussion

We have conducted an observational study to establish possible associations between oxidative measures, plumage ornamentation, and parental-care behaviours in breeding individuals of an Iberian Pied Flycatcher population. Our results show that female antioxidant defences were associated with the expression of multiple achromatic plumage ornaments. Both measured ornaments, forehead and wing white patches, were correlated with tGSH and TAS. However, the sign of the association between ornamentation and tGSH in females differed between reproductive stages, being negative during the incubation phase and positive at the nestling stage. Males with larger wing patches also showed lower levels of oxidative stress. On the other hand, oxidative status of incubating female was related to egg-attendance behaviour, although there was a lack of association between oxidative stress parameters of both males and females and their provisioning rates at any stage of nestling care.

Evidence for an adaptive function of sexual signalling in females has been based on positive relationships between ornament expression and traits indicative of quality (Kraaijeveld et al. 2007; Morales et al. 2008). Patches of white plumage in females could express a metabolically and oxidatively costly female strategy to obtain resources necessary for breeding (Rosvall 2011; Moreno et al. 2013b), which may be beneficial in highly competitive circumstances (Midamegbe et al. 2011). Thus, less ornamented females could suffer less physiological costs while breeding, but could also suffer competitive exclusion from breeding resources in some circumstances. Recently, it has been shown in Iberian Pied Flycatchers that female–female competition is influenced by forehead-patch expression (Morales et al. 2014) and that the area of the wing white patch in females is associated with higher circulating testosterone levels during the incubation phase (Moreno et al. 2014) that can lead to weaker resistance to oxidative damage (Alonso-Alvarez et al. 2007; Mougeot et al. 2009). Moreover, Moreno et al. (2013b)

Fig. 1. Association between residuals of female Iberian Pied Flycatcher (*Ficedula hypoleuca iberiae*) malondialdehyde (MDA) levels measured during incubation corrected by haemolysis degree and egg attendance.
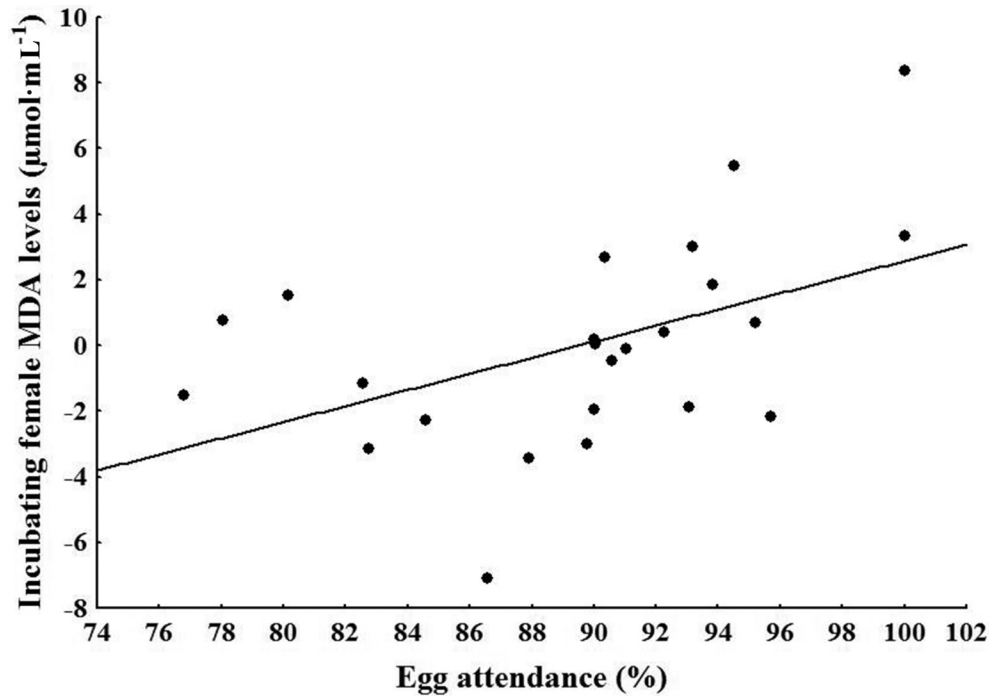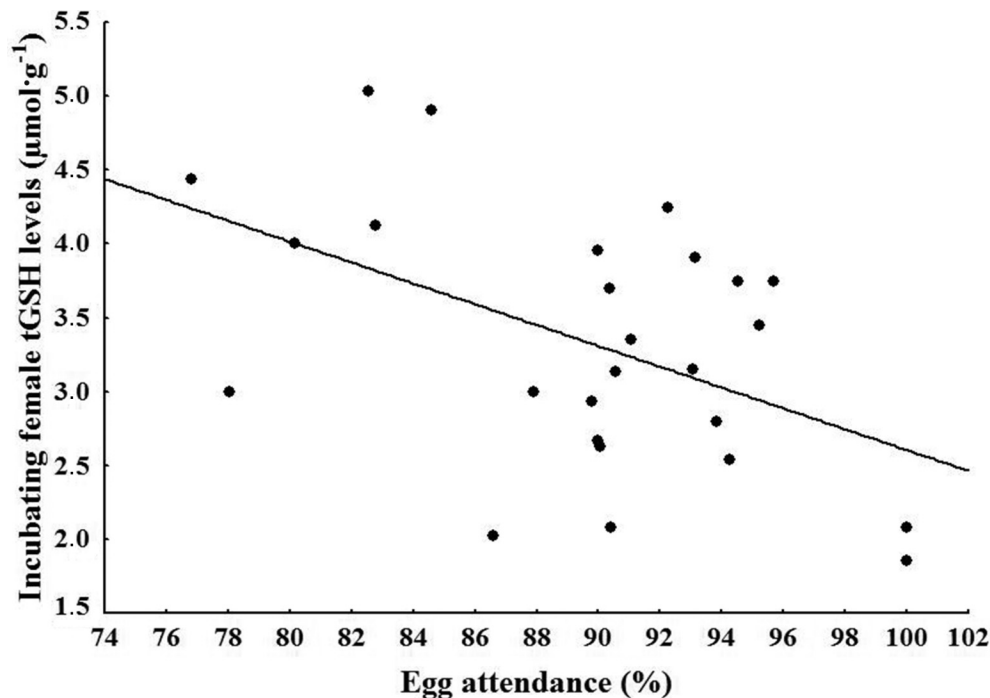

Fig. 2. Association between female Iberian Pied Flycatcher (*Ficedula hypoleuca iberiae*) total glutathione (tGSH) levels measured during incubation and egg attendance.

showed that the addition of a forehead white patch to Iberian Pied Flycatcher females without one leads to increased oxidative damage. Although we did not find associations between female ornaments and oxidative damage during incubation, our results are in accordance with these assumptions by indicating that females with larger ornaments experience a depletion in antioxidant defences at the beginning of the reproductive period possibly due to female–female competitive interactions. This depletion of anti-

oxidants could result in higher levels of oxidative stress. During the nestling phase, competitive interactions between females may be rare and ornamented females would express their capacity to cope with increased levels of oxidative stress. Alternatively, the contrasting patterns of association between female achromatic ornaments and antioxidant capacity at both phases of the reproductive period could be mediated by the capacity of the female to allocate resources to egg production. Thus, highly orna-

mented females would be able to invest relatively higher amounts of antioxidants into eggs, thus down-regulating their own circulating defences, but quickly recover afterwards, during the nestling period. Consistent with this possibility, Potti et al. (2013) demonstrated that forehead patches of female Iberian Pied Flycatchers are related to some fitness advantages, as increased fecundity, thereby compensating the costs of ornament expression, supporting the possibility that more ornamented females are indeed of higher quality than those with smaller ornaments. Finally, in contrast to Moreno et al. (2013a), we have detected no relationship between oxidative damage of females and plumage darkness of males, possibly due to a lack of males with percentages of black plumage below 50% in the study years compared with the year of the former study.

The size of the white forehead patch of males, but not wing-patch size, has been shown to be positively associated with plasma antioxidant capacity, although not with oxidative damage during the nestling phase, in the closely related Collared Flycatcher (Markó et al. 2011). Moreover, Moreno et al. (2011) showed a similar relationship between forehead-patch size and antioxidants, as well as a negative covariation between levels of MDA and this ornament in males of Iberian Pied Flycatchers. In our study, we found such associations between oxidative damage, glutathione levels, and wing ornamentation, suggesting that multiple plumage ornaments act as a signalling complex that provide information about individual oxidative status, decreasing the likelihood that individual quality will be improperly assessed (Guindre-Parker et al. 2013). Which plumage trait (forehead or wing patch) turns up significant may depend on the amount of variation in the different traits present in the sample studied. Male achromatic signals may function both as mating signals (Hegyi et al. 2010) and as social signals in territorial contexts (Järvistö et al. 2013). Our study confirms previous results, with multiple measures of oxidative status, that achromatic signals may reveal the capacity to cope with oxidative stress in these birds.

Females that bred earlier showed higher levels of antioxidants. Although early breeding is usually associated with individual quality (Verhulst and Nilsson 2008 and references therein), producing eggs early in the season could also result in an increase in physiological stress (Lobato et al. 2010) and in energetic costs due to cold weather conditions under which foraging efficiency is low (Stevenson and Bryant 2000), and this might have negative effects on current and future reproductive success (te Marvelde et al. 2012). This factor combined with costs derived from signalling involves stressful conditions for females that might thereby compromise their self-maintenance. Thus, our results are consistent with the idea that only those females that are better able to cope with oxidative stress may be able to sustain these costs. On the other hand, brood size was negatively associated with tGSH levels in females and positively with oxidative damage in males. Moreover, female body mass also predicted the antioxidant status expressed as tGSH in both the incubation and the nestling stages. These findings indicate that oxidative status is related to resource allocation (Wiersma et al. 2004) and to conditions dependent on reproductive effort, which in turn is affected by parental metabolic exertion.

We found a correlation between oxidative traits of laying females and their investment in egg attendance. Those females that spent a higher proportion of time incubating suffered a higher oxidative stress, expressed as higher levels of MDA and less intracellular tGSH. Females with higher oxidative damage may decrease the time spent outside the nest box to reduce energetic demands derived from flight, because it has been shown in birds that flight effort may increase oxidative damage and deplete antioxidant status (Costantini et al. 2008, 2012). However, incubation has been shown in this species to be energetically costly (Moreno and Sanz 1994), so higher nest attendance could also contribute to oxidative stress through metabolic costs of incubation. Neverthe-

less, Moreno et al. (2013b) showed that female oxidative damage measured when feeding nestlings was not correlated with incubation attendance in unmanipulated females. This suggests that the effects of incubation attendance on oxidative status may vanish some time after hatching of the young.

Unexpectedly, we found no relationship between provisioning rates and oxidative stress of parents despite the association between provisioning rates and metabolic exertion found in this population (Moreno et al. 2001). This may be due to the fact that under natural conditions (i.e., when breeding effort is not experimentally enhanced), adults adjust their parental investment to maintain redox balance without compromising their welfare (Metcalfe and Monaghan 2013).

To conclude, the evidence presented here, although observational, strengthens the idea that multiple signalling ornaments in the form of achromatic plumage patches involve costs related to oxidative stress (Morales et al. 2008; Moreno et al. 2011, 2013a, 2013b) for both male and female Iberian Pied Flycatchers. Moreover, although the importance of reproductive costs in terms of oxidative stress remains controversial (Selman et al. 2012; Metcalfe and Monaghan 2013; Speakman and Garratt 2014), our study highlights the key role of the specific phase of the breeding period in these links, showing the association between reproductive effort during incubation and female oxidative status under natural conditions. This supports the critical role of incubation in avian life-history evolution (Reid et al. 2002).

Acknowledgements

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