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Oxidative status of blue tit nestlings varies with habitat and nestling size

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ABSTRACT

Oxidative status has been proposed as an important ecological and evolutionary force given that pro-oxidant metabolites damage molecules, cells and tissues, with fitness consequences for organisms. Consequently, organisms usually face a trade-off between regulating their oxidative status and other physiological traits. However, environmental stressors and the availability of dietary-derived antioxidants vary according to local conditions and, thus, organisms inhabiting different habitats face different oxidative pressures. Still, there is little information on how different environmental conditions influence the oxidative status of animals inhabiting terrestrial environments. In this work, we examined the variation in oxidative status in the blue tit (*Cyanistes caeruleus*), a bird species with hatching asynchrony. Specifically, we examined the oxidative status of the largest and the smallest nestlings in the brood, inhabiting four forests differing in food availability and ectoparasite prevalence. We measured lipid peroxidation (malondialdehyde; MDA) as a marker of oxidative damage, total antioxidant capacity (Trolox-equivalent antioxidant capacity; TEAC) and antioxidant enzymatic activity (catalase, glutathione S-transferase, glutathione peroxidase) in blood samples. The glutathione peroxidase (GPX) activity differed among the forests, being the highest in the pine forest and the lowest in a mixed oak (*Quercus*) forest in the most humid area. Lipid peroxidation was higher in larger nestlings, suggesting higher oxidative damage with an increasing growth rate. Neither brood size, laying date, nor ectoparasites were related to the oxidative status of nestlings. These results suggest that nest rearing conditions might shape the oxidative status of birds, having consequences for habitat-dependent variation in regulation of oxidative status.

1. Introduction

Oxidative stress is defined as the imbalance between the production of pro-oxidant substances and antioxidant defences in favour of the former (Costantini, 2014). Pro-oxidant molecules comprise reactive oxygen species (ROS), including both radical and non-radical species (Jones, 2008), which react with different biomolecules in the cell, such as proteins, lipids, and nucleic acids, typically provoking cellular damage (Halliwell, 2007). To counteract the oxidative damage produced by ROS, organisms possess several antioxidant defences that can be produced endogenously (e.g. glutathione or antioxidant enzymes) or can be dietary-derived (e.g. polyphenols, carotenoids, or vitamins C and E) (Costantini et al., 2010). These antioxidant defences balance the ROS concentration maintaining the oxidative status of the organism in equilibrium. However, when the oxidative status is altered and the antioxidant system is unable to effectively counteract the oxidative damage, the organism experiences oxidative stress (Jones, 2008). To regulate the oxidative status is important for fitness (Costantini, 2019),

Abbreviations: ROS, Reactive Oxygen Species; MDA, Malondialdehyde; TEAC, Trolox-equivalent antioxidant capacity; CAT, Catalase; GST, Glutathione S-transferase; GPX, Glutathione peroxidase; GSH, Reduced glutathione; SOD, Superoxide dismutase.; Hb, Haemoglobin; TBARS, Thiobarbituric acid-reacting substances; ABTS, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid; CDNB, 1-chloro-2,4-dinitrobenzene; TMB, 3,3′,5,5′-tetramethylbenzidine hydrochloride.

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but, typically, organisms face a trade-off between combating oxidative stress and investing in other physiological traits (Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010).

ROS are mainly produced by aerobic metabolism and the immune system, so ecological situations in which organisms should raise their metabolism or mounting an immune response typically conduce to an increased ROS production. Hence, ROS production is expected to augment when organisms deploy aggressive behaviours (Mentesana and Adreani, 2021) or an immune response (Costantini and Møller, 2009). The capacity of organisms to counteract ROS production depends on the endogenous defences, such as antioxidant enzymes, and the acquisition of dietary-derived antioxidants. In this way, organisms suffering a shortage of exogenous antioxidants might show a perturbed oxidative status, in the form of increased oxidative damage or of up-regulated endogenous antioxidant defences.

In nestling birds, the oxidative status may be modulated by several environmental factors. Nest-dwelling ectoparasites result in costs for nestlings in terms of diminished growth, condition, or haematocrit (e.g. Hurtrez-Boussès et al., 1997; Pitala et al., 2009; Brommer et al., 2011), but ectoparasites also may expose chicks to an oxidative challenge (Hanssen et al., 2013; López-Arrabé et al., 2015). Ectoparasites typically provoke an inflammatory immune response, with a concomitant rising in ROS production (Sorci and Faivre, 2009). Moreover, parasitism stimulates the hypothalamic–pituitary–adrenal axis, resulting in an elevated metabolism and so an increase of pro-oxidants (Beaulieu and Costantini, 2014).

Brood size and nestling rank position within the nest also alter the oxidative status of chicks. In some bird species, the larger the brood size, the higher the oxidative stress suffered by nestlings, probably because of competition for food resources (Costantini et al., 2006; Bourgeon et al., 2011). In asynchronous hatched birds, size hierarchies among nestlings are commonly established, implying the existence of marginal (smaller) and core (larger) nestlings within broods (Forbes et al., 1997). In these broods, parents typically feed more frequently core nestlings than marginal ones (Moreno-Rueda et al., 2007); consequently, core nestlings grow faster. Given that accelerated growth in nestlings weakens the antioxidant capacity (Alonso-Alvarez et al., 2007) and increases the oxidative damage (Hall et al., 2010; Moreno-Rueda et al., 2012; Stier et al., 2014), core nestlings, or those nestlings growing larger body sizes, might suffer an unbalanced oxidative status.

Laying date also may affect nestling oxidative status. Environmental conditions tend to be increasingly severe as the breeding season progresses (Verhulst and Nilsson, 2008). Consequently, one may expect increased oxidative stress with the advance of the laying date, mainly due to diminished food availability and so in the intake of dietary-derived antioxidants. The effect of laying date on nestling oxidative status seems complex; some studies report changes in antioxidants with laying date (Norte et al., 2009a; López-Arrabé et al., 2016), but these changes may be year-dependent (Losdat et al., 2010, 2011) or even habitat-dependent (Salmón et al., 2018).

Moreover, inter-habitat variation in oxidative status is expected, especially if habitats differ in food availability, particularly in dietary antioxidants. For example, great tit (*Parus major*) nestlings present higher endogenous antioxidants in habitats with lower availability of antioxidants (Salmón et al., 2018). Similar findings have been reported in adult great tits, which show higher plasmatic concentrations of glutathione (an endogenous antioxidant) when inhabiting forests poor in dietary antioxidants (Isaksson, 2013). In adult Seychelles warblers (*Acrocephalus sechellensis*), birds harbour more pro-oxidant metabolites when inhabiting territories with low food availability, presumably as a consequence of increased physical effort deploy for foraging (van de Crommenacker et al., 2011).

Hence, the local environment and nest rearing conditions alter the oxidative status of nestling birds in different ways. Indeed, cross-fostering experiments in wild birds have found that both genetics and the environment can explain the variation in ROS and antioxidant capacity in nestling birds (Costantini and Dell'Omo, 2006; Norte et al., 2009b; Kim et al., 2010; Losdat et al., 2014).

In this work, we examine how oxidative damage and antioxidant enzymatic and non-enzymatic capacity vary in blue tit (*Cyanistes caeruleus*) nestlings inhabiting four different forests in southern Spain and, therefore, facing distinct environmental conditions. The four forests are grouped into two main areas consisting of: (1) a dry area where two forests are situated, one comprising holm oaks (*Quercus ilex*) and another of Pyrenean oaks (*Quercus pyrenaica*); and (2) a humid area containing the remaining two forests, one of Scots pine (*Pinus sylvestris*) and another mixed woodland with holm oaks and Pyrenean oaks. In the humid area, there is a river (Río Chico) crossing the Scots pine forest and a stream (Acequia Almiar) that cross both the Scots pine and mixed forests (Fig. 1). Compared to the dry area, the humid area has a lower mean temperature, higher humidity, lower irradiation and insolation time, more canopy cover, and a higher prevalence of certain ectoparasites (Supplementary Material S1). Moreover, some forests also differ in food availability, in terms of caterpillar abundance (Supplementary Material S1).

We predicted that: (1) If ectoparasites alter the oxidative status of nestlings (Hanssen et al., 2013; López-Arrabé et al., 2015), blue tit nestlings from nests with a higher prevalence of ectoparasites will have higher oxidative damage and/or a reduction in enzymatic and non-enzymatic antioxidant levels. (2) If sibling competition for food resources and large broods increase pro-oxidants production (Costantini et al., 2006; Bourgeon et al., 2011), blue tit nestlings from larger broods will suffer more oxidative damage or lower antioxidant defence than those from smaller broods. (3) Given that core nestlings receive more food than their marginal siblings and invest the extra food in growth (Hall et al., 2010), larger blue tit nestlings are expected to suffer more from oxidative damage, or to show reduced antioxidant defences, than smaller nestlings. (4) If environmental conditions tend to be more severe as the season progresses, a negative correlation between laying date and antioxidants in blue tit nestlings is expected (Norte et al., 2009b). (5) If the various forests involve different selective and pro-oxidant pressures for the blue tit (e.g. food availability, ectoparasites, etc.), the oxidative status of nestlings will differ according to forest type. Nestlings inhabiting poor-quality habitats are expected to upregulate some antioxidant enzymes as dietary-derived antioxidants are scarce (Monaghan et al., 2009).

Because oxidative stress increases unevenly according to the characteristics of a particular ROS (Halliwell and Gutteridge, 1995), and there is no single biomarker for oxidative stress (Monaghan et al., 2009;

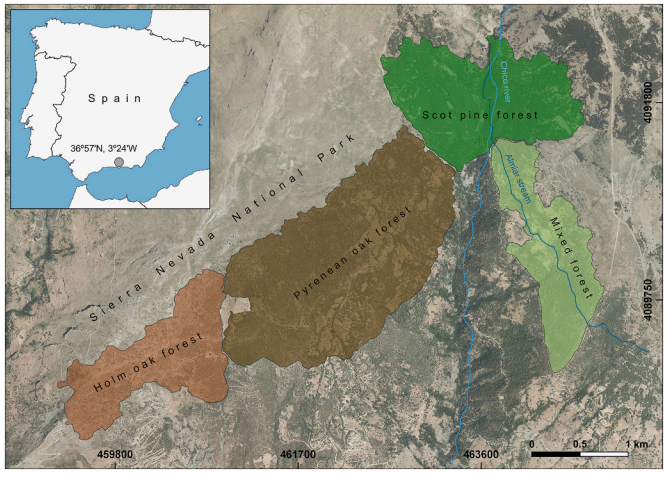

Fig. 1. Location of the four forests (holm oak, Pyrenean oak, Scots pine, mixed) where sampling was performed (Sierra Nevada National Park, SE Spain). Notice the presence of a river and a stream crossing the humid area, comprising the Scots pine and mixed forests.

Hõrak and Cohen, 2010), we measured oxidative damage, total antioxidant capacity, and the activity of various antioxidant enzymes. To do this, we quantified the oxidative damage in blood, based on the levels of malondialdehyde (MDA), the main product of lipid peroxidation (Hõrak and Cohen, 2010), and we examined the Trolox-equivalent antioxidant capacity (TEAC), a parameter that assesses the cumulative action of all the antioxidants present in plasma (Somogyi et al., 2007). Also, we examined the activity of several antioxidant enzymes: catalase (CAT), glutathione peroxidase (GPX) and glutathione S-transferase (GST). The activity of these antioxidant enzymes in the blood, and also MDA levels, reflects their level in other tissues, hence they can be considered good indicators of the oxidative status of an individual (Margaritelis et al., 2015).

2. Material and methods

2.1. Study area and sampling

The blue tit is an insectivorous forest passerine inhabiting the western Palearctic, widely distributed across the Iberian Peninsula (Salvador, 2016). Clutch size is, on average, 6–9 in this geographic area (Salvador, 2016). The eggs typically hatch asynchronously, leading to a size hierarchy within the brood and resulting in a brood comprising core and marginal nestlings (Stenning, 2018). Sampling was performed in 2017 in four different forests located between 1700 and 1800 m asl (meters above sea level) in the Sierra Nevada National Park (SE Spain, 36°57′N; 3°24′W) (see Introduction). In our study area, the blue tit breeding season begins in April, when birds start to build their nests, and ends in July, when the last nestling leave the nest. We used nest boxes (ICONA C model) with the following characteristics: basal area, 196 cm^2; height, 20 cm; hole diameter, 3 cm; material, wood with outer plastic paint layer (more details in Moreno-Rueda, 2003). The nest boxes were hung from a tree branch attached to a metal hook and placed at 100 m intervals in each forest. We determined hatching day and brood size by inspecting the nest boxes.

In total, we sampled 35 nest-boxes: 7 from the holm oak forest, 15 from the Pyrenean oak forest, 8 from the Scots pine forest, and 5 from the mixed forest. For each brood, we weighed and measured the nestlings when they were 13 days old (day 0 = hatching day), the age when the body mass of blue tits reaches asymptotic growth (Björklund, 1996). We used a digital portable scale (accurate to 0.1 g) and identified the largest and the smallest nestling within the brood. We also measured the tarsus length of nestlings using a digital calliper (accurate to 0.01 mm). Also when 13 days old, we took a 100 μL sample of blood (approximately 1% of nestling body mass) from the jugular vein of the nestlings within each nest, using disinfected and heparinised insulin syringes (following Owen, 2011). This quantity of blood has been shown to have a negligible effect on tit nestling survival (review in Sheldon et al., 2008). The handling time was also minimised, as far as possible (always less than 1 min), to reduce nestling stress and avoid possible artefacts in subsequent biochemical analyses (de Jong, 2019). We took the blood samples in the field, preserved the samples in a portable fridge, and then transported them to the laboratory (the maximum time delay was 2 h from the time of blood collection).

Once the fledglings had left their nests, we carefully revised the nest material searching for nest-dwelling ectoparasites. We recorded the presence or absence of puparia and larvae of the blowfly *Protocalliphora azurea* in each nest, as well the presence or absence of hen flea (*Ceratophyllus gallinae*) adults and larvae. *P. azurea* larvae parasitise nestlings while in their nests (Bennett and Whitworth, 1991), and their blood-sucking feeding negatively affects the growth rate and body condition of blue tit nestlings (Hurtrez-Boussès et al., 1997). The adults of *C. gallinae* take blood from both adult birds and nestlings, and this haematophagous activity reduces the haematocrit, feather growth, body condition and immune response of blue tit nestlings (Tripet and Richner, 1997; Pitala et al., 2009; Brommer et al., 2011).

2.2. Oxidative stress analyses

Blood samples were mixed with a cold buffered solution (20 mM Tris-HCl, 10% glycerol and 0.1% Triton X-100 (*v*/v), pH 8.0) in a ratio of 1:3 (v/v), frozen for 48 h at −80 °C (to break down cell membranes), and then centrifuged at 5000 *g* for 10 min at 4 °C in a Sigma 3 K30 centrifuge. The supernatant was distributed into aliquots of 100 μL and frozen at −80 °C until analysis. All the enzymatic assays were conducted at 25 ± 0.5 °C using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA) in duplicate in 96-well microplates (UVStar®, Greiner Bio-One, Germany). All the enzymatic reactions were started by adding the supernatant, and all biochemical assays had their controls, which consisted of all components except the blood sample. The specific assay conditions were as described below.

Lipid peroxidation levels were determined following the thiobarbituric acid assay of Buege and Aust (1978), based on the MDA levels generated as the main product of lipid peroxidation. In the presence of thiobarbituric acid, MDA reacts to produce red thiobarbituric acid reactive substances (TBARS) that can be measured spectrophotochemically at 535 nm. The net absorbance (sample reaction – sample blank) was converted to a MDA concentration from the corresponding standard curve (0–35 μM MDA). The measures were expressed as μM MDA. Although TBARS can react with other aldehydes, most of the chromogen formed is ascribed to the complex MDA-TBARS even when the MDA concentration in the sample is low (Gutteridge and Quinlan, 1983). We added 0.01% *w*/*v* butylated hydroxytoluene to the reaction mixture to neutralize the possible aldehydes that can be generated during the heating procedure.

The total antioxidant capacity, assayed as TEAC, was measured according to Erel (2004). This assay consists of oxidising ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) with hydrogen peroxide (H_2O_2) in an acidic medium, where the change to an emerald-green colour is determined through spectrophotometry at 595 nm. For this purpose, the extract was added to a reaction mixture containing 0.35 M acetate buffer (pH 5.8), 1.3 mM ABTS, and 0.25 mM H_2O_2 in 4 mM acetate buffer (pH 3.6). To eliminate the interference of the colour of haemoglobin, for each sample we used a control consisting of the blood with the buffer. Antioxidant activity refers to the equivalent of a water-soluble analogue of vitamin E (Trolox, Sigma 23001-3) dissolved in phosphate buffer, 0.1 M, pH 7.4, and which was used as a standard. The results were expressed in terms of Trolox equivalent antioxidant capacity (μM).

Catalase (CAT; EC 1.11.1.6) activity was determined spectrophotochemically by measuring the decrease in the H_2O_2 concentration over a 3 min period at 240 nm, according to Aebi (1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and freshly prepared 10.6 mM H_2O_2.

Glutathione peroxidase (GPX; EC 1.11.1.9) activity was measured according to Flohé and Günzler (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.1), 1 mM EDTA, 3.9 mM GSH, 3.9 mM sodium azide, 1 IU/mL glutathione reductase, 0.2 mM NADPH, and 0.05 mM cumene hydroperoxide. After the addition of cumene hydroperoxide, the NADPH consumption rate was determined spectrophotochemically at 340 nm.

Glutathione S-transferase (GST; EC 2.5.1.1.8) activity was determined following the method of Habig et al. (1974), but adapted to a microplate. A reaction mixture consisting of 0.1 M phosphate buffer (pH 6.5), 1.2 mM GSH, and 1.23 mM solution of 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol, was prepared just before the assay. GST activity was then measured spectrophotochemically at 340 nm by the formation of glutathione-CDNB-conjugate.

For all the enzymatic activities, one unit of activity is defined as the amount of enzyme required to transform 1 μmol of substrate/min under the described assay conditions. To estimate the specific enzyme activity, the haemoglobin levels in the extracts were determined using the Drabkin colorimetric method (Spinreact, Spain), with animal-origin

haemoglobin (15 g/dL; Spinreact, Spain) as the standard. For all the biochemical variables, two measurements were taken from each aliquot, and the average of these was used in the subsequent statistical analyses. All the biochemical reagents, including substrates, coenzymes, and enzymes, were obtained from Roche (Mannheim, Germany), Sigma Aldrich Chemical Co. (USA), or Merck (Darmstadt, Germany).

2.3. Statistical analyses

We used Cleveland plots to check whether the oxidative status biomarkers (MDA, TEAC, CAT, GST and GPX) and haemoglobin concentrations had outliers, and we graphically inspected these for normality (following Zuur et al., 2010). Because none of the biomarkers followed a normal distribution, all of the variables were log-transformed. An outlier was detected for the GST activity of a larger nestling from the holm oak forest. The outlier was probably an artefact as it far exceeded the confidence interval at 95% for GST activity (CI-95% = 14.31–16.80 mU/mg Hb, $n = 68$; outlier = 74.10 mU/mg Hb). For this reason, we performed the analyses using both the original full dataset and the dataset without this outlier. Herein we report the results without the outlier, in which we have greater confidence. The complete dataset is available in Supplementary Material S2.

To determine differences in laying date and brood size between the forests, we used a one way ANOVA test. To check for variability in the prevalence of blowflies and fleas with forest type, given that these variables are frequencies, we used the chi-squared test. We also used a paired Student's *t*-test to check for differences in laying date and brood size in nests that were infested and non-infested with blowflies and fleas. The correlation between the brood size and laying date was established using the Pearson product-moment correlation. We used Linear Models (LM) to check whether haemoglobin concentrations varied with forest type, nestling rank, laying date, brood size and the prevalence of blowflies and fleas. Because the haemoglobin concentration did not differ between any of these variables (forest: $F_{3,65} = 1.69$, $p = 0.177$; nestling rank: $F_{1,67} = 0.21$, $p = 0.644$; laying date: $F_{1,67} < 0.01$, $p = 0.994$; brood size: $F_{1,67} = 1.03$, $p = 0.314$; blowflies: $F_{1,67} = 0.40$, $p = 0.529$; fleas: $F_{1,67} = 1.57$, $p = 0.215$), we used the specific enzymatic activity standardised to the total haemoglobin concentration for the subsequent statistical analyses.

To examine whether body mass and tarsus length of the larger and the smaller nestlings varied with forest type, we used Linear Mixed Effects Models of Restricted Maximum Likelihood (REML-LMM) (Zuur et al., 2009) with nest identity as a random factor, and nestling rank (larger versus smaller), forest type (holm oak, Pyrenean oak, Scots pine, and mixed), and the interaction forest*nestling as predictors. We also used REML-LMMs to test for variation in all oxidative status biomarkers; for this, we first ran full models with the following structure: each biomarker was a dependent variable, the nest identity as a random factor, and forest, nestling rank, interaction forest*nestling, laying date, brood size, the prevalence of fleas, the prevalence of blowflies, tarsus length (log-transformed), and weight (log-transformed) as predictors. Results of full models are given in Supplementary Material S3. Given that tarsus length and body mass were correlated ($r = 0.68$, $p < 0.001$), the simultaneous inclusion of the two variables in the same models could produce collinearity. For this reason, we calculated the Variance Inflation Factor (VIF) for each full model. VIF values were below 10, except for the interaction forest*nestling in a single full model.

To be sure that the results from full models remain robust and were not affected by problems of collinearity or overfitting, we also applied a model selection approach. To select the best models among all possible models, we used the Akaike information criterion (AIC), and we chose those models with a ΔAIC value under 2 (Quinn and Keough, 2002). The normality and homoscedasticity of the model residuals were checked following Zuur et al. (2010). The basic statistics are given as mean ± SE (standard error). All the analyses were performed using the packages "nlme" (Pinheiro et al., 2019) and "MuMIn" (Bartoń, 2020) in the R software environment, version 4.0.0. (R Development Core Team, 2020).

3. Results

3.1. Descriptive statistics of the study system

Laying occurred between 6 and 24 May (average: 11 May ±0.58 days). Brood size ranged from 2 to 8 nestlings (mean: 5.49 ± 0.19). Laying date and brood size did not significantly differ between forests (respectively, $F_{3,31} = 2.59$, $p = 0.071$; $F_{3,31} = 2.50$, $p = 0.078$). Brood size tended, although not significantly, to decrease with laying date ($r = -0.33$, $p = 0.056$). Eight out of 35 nests were infested with fleas, but this frequency did not differ with forest type ($\chi^2_3 = 1.22$, $p = 0.749$). Flea infestation did not differ with laying date or brood size (in the two cases, $|t_{33}| < 0.10$, $p > 0.90$). However, blowfly nest infestation differed significantly between forests ($\chi^2_3 = 12.89$, $p = 0.005$). All the nests sampled in the Scots pine ($n = 8$) and mixed ($n = 5$) forests were infested with blowflies, while 7 out of 15 nests in the Pyrenean oak forest and only 2 out of 7 nests in the holm oak forests were infested. Overall, 22 out of 35 nests were infested with blowflies. Blowfly infestation did not vary with laying date or brood size (in the two cases, $|t_{33}| \leq 1.20$, $p > 0.20$). The tarsus length of blue tit nestlings did not differ between the forests (REML-LMM; forest: $\chi^2_3 = 1.81$, $p = 0.61$; nestling rank (larger versus smaller): $\chi^2 = 7.79$, $p = 0.005$; interaction forest*nestling: $\chi^2_3 = 1.10$, $p = 0.78$). The body mass of blue tit nestlings neither differed among the forests (forest: $\chi^2_3 = 6.34$, $p = 0.09$; nestling rank: $\chi^2 = 22.01$, $p < 0.0001$; interaction forest*nestling: $\chi^2_3 = 3.74$, $p = 0.29$).

3.2. Biomarkers of oxidative stress

Table 1 summarises the MDA level, TEAC, the activity of the antioxidant enzymes and the haemoglobin concentrations in the larger and smaller nestlings in each of the four forests. The results of the model selection are summarised in Table 2. The best model for MDA included only tarsus length as a predictor variable, which positively correlated with MDA level ($\chi^2 = 7.80$, $p = 0.005$; Fig. 2). The second-best model included tarsus length and weight, but this was significantly worse (ΔAIC >2). For GPX activity, the best model included only forest type as a predictor ($\chi^2_3 = 29.60$, $p < 0.0001$). The other two models were not significantly worse (ΔAIC <2), but these included forest type together with tarsus length, and tarsus length plus weight as predictors. In these models, however, only forest type significantly affected GPX activity. Specifically, the GPX activity was less in the mixed forest, higher in the Scots pine forest, and intermediate in holm oak and Pyrenean oak forests (Fig. 3). *Post-hoc* analyses indicated that the GPX activity in the mixed forest differed significantly from the other forests (in all cases: $p < 0.05$). For the other dependent variables (TEAC, CAT and GST), no model differed significantly from the null model (in all cases: ΔAIC < 2).

4. Discussion

Our results show that the glutathione peroxidase (GPX) activity differed among the forests, being the highest in the Scots pine forest, the lowest in the mixed forest, and intermediate in the dry zone forests, suggesting that habitat heterogeneity can shape the expression of some antioxidant enzymes, such as GPX (Norte et al., 2009a). Furthermore, the lipid peroxidation, a biomarker of oxidative damage, increased with nestling tarsus length. Larger nestlings suffered more oxidative damage, suggesting that this damage is a consequence of growth costs (see below). In contrast, although brood size, laying date, and ectoparasites have been shown to alter nestling oxidative status in other bird species (Costantini et al., 2006; Bourgeon et al., 2011; Hanssen et al., 2013; López-Arrabé et al., 2015), we did not find evidence that these factors can alter the oxidative status in blue tit nestlings.

Table 1
Lipid peroxidation level (MDA), total antioxidant capacity (TEAC), antioxidant enzymatic activity and haemoglobin concentration (Hb) for larger and smaller blue tit nestlings from the four forests. The table shows the mean values, standard error (±SE) and sample size (n) in brackets.

	Holm oak forest		Pyrenean oak forest		Scots pine forest		Mixed forest	
	Larger	Smaller	Larger	Smaller	Larger	Smaller	Larger	Smaller
MDA (μM)	9.38 ± 1.59 (6)	8.06 ± 0.86 (7)	8.20 ± 0.41 (15)	7.14 ± 0.44 (15)	7.52 ± 0.88 (7)	7.04 ± 0.60 (8)	8.62 ± 1.55 (5)	5.86 ± 0.79 (5)
TEAC (μM eq. Trolox)	99.63 ± 17.41 (6)	105.51 ± 27.02 (7)	82.26 ± 14.31 (13)	76.65 ± 21.52 (13)	105.43 ± 16.72 (8)	102.05 ± 19.92 (8)	142.00 ± 30.20 (5)	107.55 ± 67.46 (4)
CAT (U/mg Hb)	14.48 ± 1.29 (6)	17.14 ± 2.45 (7)	21.45 ± 2.71 (15)	19.75 ± 1.03 (15)	16.05 ± 2.41 (8)	18.46 ± 1.53 (8)	17.36 ± 3.53 (5)	14.49 ± 1.39 (5)
GPX (mU/mg Hb)	24.13 ± 5.85 (2)	21.44 ± 6.49 (5)	23.74 ± 2.51 (13)	23.25 ± 2.72 (14)	39.37 ± 8.62 (7)	39.96 ± 8.06 (6)	8.53 ± 2.11 (4)	6.98 ± 0.78 (4)
GST (mU/mg Hb)	13.78 ± 1.18 (6)	16.94 ± 3.38 (6)	16.01 ± 1.57 (15)	16.90 ± 1.45 (15)	13.22 ± 1.00 (8)	14.76 ± 1.39 (8)	17.20 ± 2.37 (5)	13.93 ± 0.58 (5)
Hb (mg/mL)	21.78 ± 1.04 (6)	20.81 ± 2.29 (7)	21.72 ± 1.25 (15)	21.85 ± 1.13 (15)	21.30 ± 3.22 (8)	16.71 ± 1.42 (8)	18.66 ± 2.34 (5)	21.85 ± 1.48 (5)

MDA = malondialdehyde, TEAC = trolox-equivalent antioxidant capacity, CAT = catalase, GPX = glutathione peroxidase, GST = glutathione S-transferase, Hb = haemoglobin.

Table 2
AIC values and AIC increment of the linear mixed models for oxidative status biomarkers, indicating the model predictors (nest identity was the random factor in all models). Null models include only the intercept. The significant predictors ($p < 0.05$) are marked in bold.

Variable	AIC	ΔAIC
MDA		
Log(tarsus)	25.952	0.00
TEAC		
Log(tarsus)	144.051	0.00
Log(tarsus), log(weight)	144.518	0.47
Log(tarsus), blowflies	145.799	1.75
Null model	146.011	1.96
CAT		
Log(tarsus)	57.533	0.00
Null model	57.963	0.43
GST		
Null model	40.926	0.00
GPX		
Forest	87.561	0.00
Forest, log(tarsus)	87.635	0.07
Forest, log(tarsus), log(weight)	88.873	1.31

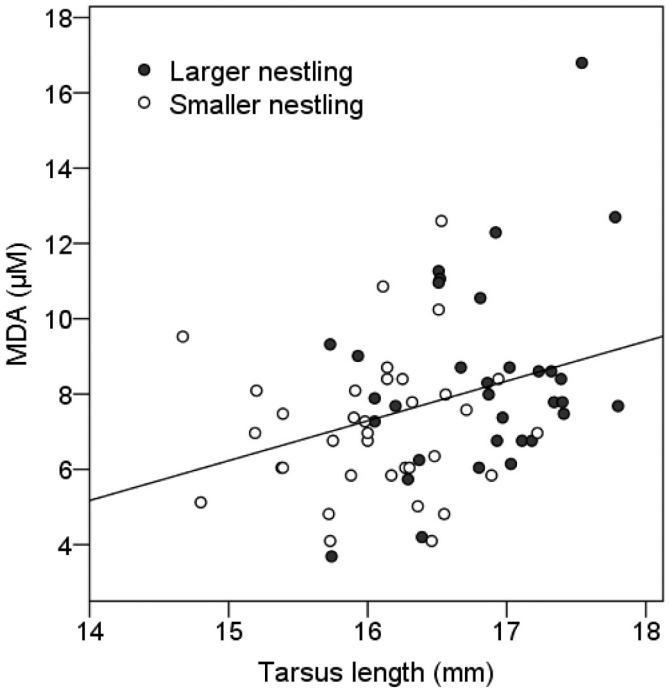

Fig. 2. Relationship between tarsus length and lipid peroxidation level (MDA) in blue tit nestlings. Larger nestlings: filled circles; smaller nestlings: open circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.1. Forest type modulates the GPX activity

The GPX activity varied between the forest types, being the lowest in the mixed forest and highest in the Scots pine forest, both located in the humid area, while intermediate values were found in the dry zone forests. Glutathione peroxidase is one of the main components of the cell enzymatic antioxidant system, which converts hydrogen peroxide to water using reduced glutathione (Costantini, 2014). It has previously been shown that the activity of this enzyme varies relative to nest rearing conditions in great tit nestlings (Norte et al., 2009b). In the same way, adult great tits show greater concentration of glutathione (an endogenously synthetized metabolite that intervene in the antioxidant function of the GPX) in pine and larch forests than in oak forests (Isaksson, 2013). In poor-quality habitats, with low food availability, the intake of dietary antioxidants is limited and birds are expected to respond by upregulating the production of endogenously derived antioxidants, such as GPX (Monaghan et al., 2009). This upregulation of GPX could explain the among-forest variation in its activity, as pine forests are typically poorer in antioxidants than oak forests (Tálos-Nebehaj et al., 2017).

We found that ectoparasites are not an important cause of the among-forest variation in the GPX activity of the blue tit nestlings in our population, since their antioxidant systems did not change in the presence of blowflies and fleas. Nevertheless, a greater infection by other parasites not examined in this study, such as haemosporidians, may to have increased the GPX activity in nestlings from the Scots pine forest (De Angeli Dutra et al., 2017). Also, during an immune challenge, an organism can downregulate certain antioxidant enzymes, such as GPX, in order to keep the production of some ROS (e.g. hypochlorous acid) up, thereby increasing the effectiveness of the immune response (Costantini, 2014). In other unpublished studies, we found that the blue tit nestlings from the humid zone were more probable to be parasitised by the haemosporidian *Leucocytozoon* and had more leucocytes than those from the dry area; therefore, it is plausible that GPX activity was downregulated in the mixed forest as a consequence of a greater immune challenge.

4.2. Being larger results in higher oxidative damage

We found that the larger the tarsus length, the higher the lipid peroxidation suffered by blue tit nestlings. The greater oxidative damage suffered by larger nestlings could be attributed to accelerated growth. A

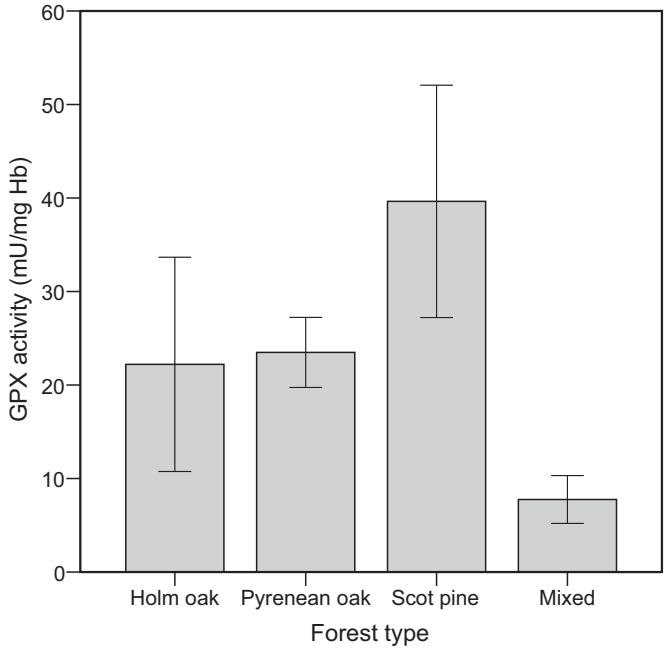

Fig. 3. Mean values (showing the CI 95%) of the glutathione peroxidase (GPX) activity in the blue tit nestlings from the four forests. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

faster growth rate results in greater oxidative stress in terms of increased oxidative damage, but not in terms of reduced antioxidant levels (Smith et al., 2016). Concretely, in nestlings, an accelerated growth rate may provoke an increment in oxidative damage (Alonso-Alvarez et al., 2007; Costantini, 2010; Moreno-Rueda et al., 2012; Stier et al., 2014). In asynchronously hatched blue tits, parents typically feed larger nestlings to the detriment of smaller nestlings (Dickens and Hartley, 2007; García-Navas et al., 2014), meaning early-hatched nestlings grow faster than last-hatched nestlings (Björklund, 1997). Furthermore, the extra resources provided by parents to core nestlings are invested in growth, rather than in reducing oxidative damage (Hall et al., 2010). Therefore, our findings suggest that an accelerated growth comes at the expense of an oxidative cost. Still, final tarsus length may determine survival prospects in blue tits (Charmantier et al., 2004), and so nestlings are probably selected for faster growth to attain a structurally large body size. Moreover, a reduction in the duration of the nestling period by an accelerated growth in birds is selected to escape both from parasites and predators (Møller, 2005; Cheng and Martin, 2012).

4.3. Nestling oxidative status unaffected by ectoparasites, brood size and laying date

We also found that nestling oxidative status did not vary with the prevalence of blowflies and hen fleas in the nest. These ectoparasites alter the expression of stress proteins and negatively impact haematocrit, body condition, immune response and growth rate of blue tit nestling (Hurtrez-Boussès et al., 1997; Tripet and Richner, 1997; Arriero et al., 2008; Pitala et al., 2009; Brommer et al., 2011), hence oxidative stress was predicted to be higher in parasitised nestlings. However, studies examining the relationships between nest-dwelling ectoparasites and the oxidative status of nestlings suggest complex relationships, with ectoparasites altering the oxidative status of nestlings in various ways (Hanssen et al., 2013; López-Arrabé et al., 2015), although these do not always cause observable effects (De Coster et al., 2012; Maronde et al., 2018). Removing ectoparasites from raptor nests reduced plasma oxidants and led to a higher total antioxidant capacity in the chicks (Hanssen et al., 2013). In contrast, removing nest-dwelling ectoparasites provoked no change in MDA levels or total antioxidant capacity in pied flycatcher (*Ficedula hypoleuca*) nestlings, although the concentration of glutathione, an important non-enzymatic intracellular antioxidant, was higher when ectoparasites were absent (López-Arrabé et al., 2015). However, and in line with our results, experimental manipulation of flea infestations had no influence on oxidative status in great tit nestlings (Maronde et al., 2018). Highlighting the complexity of the interrelationships between oxidative status and ectoparasites, De Coster et al. (2012) found that maternal effects may reduce oxidative stress in great tit nestlings parasitised with fleas, but the effect was only found in female nestlings. The results of our study, together with those of the aforementioned studies, suggest that exposure to ectoparasites does not necessarily affect the oxidative status of nestlings, probably due to the complex interactions between parasite pressure, immune function, and the oxidative challenge.

Some studies examining the oxidative stress of nestlings in broods of different sizes, or which were experimentally enlarged or reduced, have found that nestling oxidative stress (i.e. higher oxidative damage or lower antioxidant defence) is greater in larger broods (Alonso-Alvarez et al., 2006; Costantini et al., 2006; Bourgeon et al., 2011). However, other studies have not found this pattern (Hall et al., 2010; Losdat et al., 2010; López-Arrabé et al., 2016; this study). The discrepancies between these studies may be because it is difficult to determine to what degree the different behaviours and factors within the nests, including begging, scramble competition between nestlings, parental care, nestling growth rates, and quantity of food resources, contribute to modulate the oxidative status of nestlings. Moreover, most of these studies examined only one component of the redox system, which may not be sufficient to detect a significant increase in oxidative stress. Furthermore, inter-year variations in environmental quality can confound brood-size effects on nestling oxidative stress (Bourgeon et al., 2011). The oxidative costs of larger broods can be also masked by, for instance, the quality of the food provided by the parents, given that, with a high-quality diet, nestlings can allocate resources to both growth and antioxidant defences (Hall et al., 2010).

In our study population, we found no relationship between the laying date and nestling oxidative status. As the environmental temperature increases and food abundance normally decreases throughout the breeding season, it was predicted that the condition and physiology of the nestlings would be impaired. In Eurasian kestrel (*Falco tinnunculus*) nestlings, no relationship was found between oxidative stress and hatching date (Costantini et al., 2006). However, great tit nestlings can show a negative correlation between GPX activity and the hatching date (Norte et al., 2009b). In contrast, in other great tit populations, nestling resistance to oxidative stress (measured as enzymatic and non-enzymatic antioxidants combined) was found to increase with laying date in some years, although no relationship was evident in others (Losdat et al., 2010, 2011). Similarly, a positive association between hatching date and non-enzymatic antioxidants was found in pied flycatcher nestlings (López-Arrabé et al., 2016). These results suggest that local environmental factors that fluctuate during the breeding season, including food abundance and temperature, influence the relationship between laying date and oxidative status in nestlings, in different ways, making it difficult to draw a general conclusion.

5. Conclusions

Although we are aware that determining the natural causes of variation in oxidative stress is complex, our findings suggest that the local environmental conditions where the nestlings develop, such as habitat type, shape some components of their oxidative status. Specifically, environmental conditions are responsible for the variation in the expression of certain components of the antioxidant system of nestlings, such as GPX, which varies among forests. GPX activity was lowest in the mixed forest and highest in the Scots pine forest. Moreover, larger nestlings showed more oxidative damage than smaller nestlings, since

reaching a larger tarsus length implied suffer more from lipid peroxidation, suggesting that faster growth involves an oxidative cost. This study, therefore, helps to improve our knowledge of the environmental causes of variation concerning some components of the oxidative status of birds, highlighting the importance of habitat heterogeneity in the physiology of organisms.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2021.110986.

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