

Oxidative damage and plasma antioxidant capacity in relation to body size, age, male sexual traits and female reproductive performance in the collared flycatcher (*Ficedula albicollis*)

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Abstract

The study of oxidative stress is a potential tool for studying the functional interactions among life history traits, sexual traits and physiological status in animals. In this study, we investigated relationships between measures of plasma oxidative status and male sexual traits, female reproductive investment and three other life history traits, in a wild population of collared flycatchers (*Ficedula albicollis*). Flycatcher males with a larger white forehead patch had higher level of plasma antioxidant capacity. For females, clutch size was not associated with plasma oxidative status but egg size was positively correlated with antioxidant capacity. The relationship between age and levels of plasma oxidative damage remain controversial in this species: young female flycatchers showed higher levels of hydroperoxides compared to antioxidants, whereas age did not predict oxidative status of males. Males had higher levels of oxidative damage than females, although the concentration of antioxidant compounds was similar between the sexes. Females that mated with more ornamented males had higher plasma antioxidant capacity. Our results suggest that, for males and females, greater investment in sexual signal and reproduction, respectively, does not reduce the capacity for self-maintenance or avoidance of oxidative stress. Finally, our data support indirectly the occurrence of assortative mating in our species, since females with higher plasma antioxidant capacity mated with more ornamented males.

Keywords: Oxidative stress – Free radicals – Antioxidants – Life history – Trade off

Introduction

The cost of producing and/or maintaining sexual signals under limiting resource supply ensures that they ‘honestly’ reflect individual quality (Andersson and Iwasa 1996). Physiological correlates, such as metabolic rate, immune response and secretion of hormones, potentially provide an informative framework for quantifying the costs underlying the expression of sexual signals (Mougeot et al. 2004; Ricklefs and Wikelski 2002; Safran et al. 2008). Two alternative hypotheses purport to link the expression of sexually selected traits to the physiological state of individuals. On the one hand, trade offs among life history traits predict that more ornamented individuals have to cope with increased physiological costs, such as reduced immunocompetence, higher parasite load or oxidative stress, because resources are diverted from these functions to the production of ornamental colouration (Hamilton and Zuk 1982; Hartley and Kennedy 2004; Kokko 2001; Kotiaho 2001; Lozano 1994). On the other hand, secondary sexual traits might be expected to be highly integrated with physiological measures of quality such that trade offs may be unimportant (Badyaev 2004). This ‘having it all’ theory suggests that individuals can achieve high rates of fecundity, extended lifespan and sexual ornamentation while still meeting the energy demands of physiological processes (Johnston et al. 2006; Partridge et al. 2005). Nevertheless if the costs of increasing a particular trait are smaller for individuals in good condition compared to for those in poor condition, a positive correlation should also exist between individual quality and the degree of expression of the sexual signal (Grafen 1990). That is, the expression of the trait is expected to positively correlate with the physiological status of the individual.

Oxidative stress and antioxidant protection have been proposed to be informative traits to explain the physiological costs underlying the production of sexual colourations (Von Schantz et al. 1999). More recently, it has been suggested that oxidative stress could modulate life history strategies (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009).

An imbalance between the production of reactive chemical species (e.g., reactive oxygen and nitrogen species) and antioxidant defences (e.g., enzymes, dietary antioxidants and repair systems) in favour of the former increases the rate at which oxidative damage to lipids, proteins and nucleic acids occurs (Sies 1997; Halliwell and Gutteridge 2007; Costantini and Verhulst 2009). Oxidative stress refers to this condition of biochemical stress and measures of oxidative damage can represent proxy of such a condition. Oxidative stress is generally considered an important factor underlying reproductive performance, cellular senescence and aging: resistance to oxidative stress, therefore, potentially enhances fitness (Beckman and Ames 1998; Finkel and Holbrook 2000; Dröge 2002; Hulbert et al. 2007). However, experimentally increasing tissue antioxidants through dietary supplementation, pharmacological induction, or transgenic techniques moderately increases mean life expectancy but does not change maximum life span (Pamplona and Barja 2006; Sanz et al. 2006; Hulbert et al. 2007; Pérez et al. 2009). Moreover, animals in which genes coding for particular antioxidant enzymes are downregulated can show different pathologies although their rates of aging do not seem to be affected (Salmon et al. 2010). On the basis of such evidence, Salmon et al. (2010) suggested that oxidative stress generally has a mild effect on aging, but a strong effect on the progression of age-related pathologies (i.e., health span or health aging), and that oxidative stress can only have a significant effect on aging under stressful environmental conditions.

In this study, we analysed the relationships between plasma oxidative status and several fitness-related traits, including sexual ornamentation, reproductive output, age and body size, in the collared flycatcher (*Ficedula albicollis*). This species is a sexually dichromatic, socially monogamous, migratory passerine. The sexually selected plumage signals (i.e., a depigmented area on forehead and wings) have been thoroughly studied in several European populations across a wide geographical area (Saetre et al. 1997; Hegyi et al. 2002; Qvarnström et al. 2006). These studies have demonstrated the important role of white

plumage patches as a sexual selection mechanism for this species. Few data are available on the association between physiological status and phenotypic life history traits. *F. albicollis* represents an excellent model organism to investigate these questions.

In the present study, our aims were threefold. From the classical trade-off model of life history evolution, we predicted, first, negative correlations between sexual traits of males and reproductive performance of females (number of eggs and egg mass) and, respectively, two biomarkers of oxidative status (plasma hydroperoxides, which are early oxidative damage products; plasma antioxidant capacity). Second, we predicted that three life history components (body size, body condition, age) traded off with the measures of oxidative status. The mutually exclusive “having it all” hypothesis predicts opposite relationships between biomarkers of oxidative status and these fitness-related traits. Finally, we evaluated whether (i) sexes differ in oxidative damage or antioxidant capacity and (ii) biomarkers of oxidative status in one pair member correlate with those of mate or with other characteristics of the mate.

Materials and methods

Field methods

The study was carried out in Pilis Mountains (47°43'44'' N, 19°0'40'' E), located 30 km North of Budapest in Hungary. Our study plots are covered by oak woodland where the population dynamics of hole nesting birds has been studied since 1982.

At the breeding sites, male collared flycatchers immediately occupy nest boxes after arriving from long distance migration and establish small territories where they start singing and displaying. The pair formation period for males one of the most energetically demanding phases of the breeding season (Chastel et al. 2003). Females typically arrive at the breeding site a week later than males. After mate selection, females subsequently undertake the

energetically demanding processes of egg formation, laying and incubation (Nilsson and Raberg 2001).

Twenty-two established breeding pairs were randomly chosen after females had started nest building. Males from each pair were captured for sampling using a decoy male placed close to the occupied nest boxes to attract the resident male. Unfortunately, two nests were predated during the breeding season, reducing the sample size of females to 20. Females were caught by spring trap during the 12th-13th day of their incubation. Standard morphological traits were measured for all birds. Body mass was measured with a spring balance (to 0.1 g accuracy) and body size (tarsus length) was measured with callipers (to 0.1 mm accuracy). Forehead patch size was quantified as the product of patch height and width, and was measured with callipers (accuracy 0.1 mm). Wing patch size was estimated as the sum of the lengths of white bars on the outer vanes of the 4th-8th primaries measured from the tip of the coverts. Because the expression of wing patch size changes when males moult from yearling to adult plumage, wing patch size was standardized across age categories by bringing the age classes to a common mean of 0 and standard deviation of 1.

The exact age of recaptured birds was assessed, where possible, from our long term records of bird ringing within the study area. Where exact ages were not available, (for 9 out of the 42 birds studied) individuals were classified as yearlings or older based on plumage characteristics (Svensson 2002). We checked the boxes three times a week during the nesting period to determine the first egg date. Clutch size (number of eggs) and egg mass (with electronic balance near 0.01 g accuracy) were also recorded.

Blood samples

After morphological data were recorded for each bird, a blood sample was then taken from the individual within a maximum of 30 min after capturing. A sterilized needle was

inserted into the brachial wing vein to collect the blood sample (60–80 μ L) which was then placed into Na-heparinised micro-haematocrit tubes. The samples were kept cool (0–5 $^{\circ}$ C) until centrifugation, which occurred within a few hours, and the plasma was kept at –20 $^{\circ}$ C. These samples were shipped to the lab on ice and stored at –20 $^{\circ}$ C. Haemolysed plasma samples were not included in lab analyses.

Oxidative stress analyses

ROMs (reactive oxygen metabolites) and OXY (plasma antioxidant capacity) were measured by the d-ROMs test and the OXY-Adsorbent test (Diacron International, Grosseto, Italy), respectively, following the protocols developed in previous studies (e.g. Costantini et al. 2006, 2007; Costantini and Dell’Omo 2006). Briefly, hydroperoxides measured by the d-ROMs test are early peroxidation products of the exposure of biological macromolecules to reactive oxygen species. Hydroperoxides were determined by diluting the plasma (5 μ L) with 200 μ L of a solution containing 0.01 M acetic acid/sodium acetate buffer (pH-value 4.8) and N,N-diethyl-p-phenylenediamine as chromogen, then incubating it for 75 min at 37 $^{\circ}$ C. An acidic pH favours the release of iron and copper from plasma proteins that catalyse the cleavage of hydroperoxides in two different free radicals. When these compounds react with the alkyl-substituted aromatic amine of the chromogen, they produce a coloured complex, whose intensity is directly proportional to the concentration of ROMs. Hydroperoxides are expressed as mM of hydrogen peroxide equivalents.

The total plasma antioxidant capacity was quantified as the ability of the plasma antioxidant barrier to cope with the oxidant action of hypochlorous acid (HOCl; oxidant of pathologic relevance in biological systems). The plasma (10 μ L) was diluted 1:100 with distilled water. A 200 μ L aliquot of a titred HOCl solution was incubated with 5 μ L of the diluted plasma for 10 min at 37 $^{\circ}$ C. Then, 5 μ L of the same chromogen solution which was

used for the ROMs determination was added. An alkyl-substituted aromatic amine dissolved in the chromogen is oxidized by the residual HOCl and transformed into a pink derivative. The intensity of the coloured complex is inversely related to OXY. Measurements are expressed as mM of HOCl neutralised. Here it is important to stress that the contribution of uric acid to plasma antioxidant capacity as measured by the OXY-adsorbent test is low (Costantini 2010), hence, unlike other methods, the OXY-test does not overemphasize the antioxidant contribution of uric acid.

Index of plasma oxidative status

Hydroperoxides represent molecules derived from oxidative damage and are, therefore, a proxy of oxidative stress level. However, these molecules may also function as pro-oxidants because they can be cleaved in two different free radicals (see Costantini and Dell'Omo 2006). Due to this dual-function, an index relating the level of hydroperoxides and the plasma antioxidant capacity is informative. In this study we used the ratio between ROMs and OXY ($\times 1,000$) values as an index of plasma oxidative status (OS) according to previous studies (Costantini et al. 2006, 2007). With this approach, higher values of OS indicate that the plasma contains a greater concentration of oxidised molecules. We also calculated another previously used index from the difference between standardized values of ROMs and of OXY (Vassalle 2008). For these calculations, values were standardized as (individual value – mean value) / (standard deviation). The values calculated using these two different indices were highly correlated ($r = 0.94$). Correspondingly, for the analyses presented in this study only the first index, which has been previously applied to the study of OS in birds, is reported.

Statistical analyses

Values of OXY, ROMs and OS were normally distributed across samples. We used general linear models (GLM) to investigate the relationship between secondary sexual traits of males and measures of their oxidative status. Values for the two biomarkers of oxidative status (ROMs and OXY) and of our index of plasma OS, were dependent variables and forehead patch size and wing patch size (corrected for age) and body condition were included as covariates. Body condition was estimated from the standardized residuals of a regression of body mass on tarsus length. In addition, we investigated the relationship between reproductive performance of females (number of eggs laid and mean of egg masses per brood) and oxidative status of females. For this analysis, OXY, ROMs, and OS were dependent variables, egg number was a categorical factor and mean egg mass and age were covariates.

To detect the association between measures of oxidative status (dependent variables), and age, body condition and tarsus length (predictor variables) we performed analysis separately for sexes in GLM module.

In all models, we employed a stepwise analysis with a backward deletion procedure, removing insignificant effects from the model one by one in decreasing order of statistical significance and re-entering the removed variables to the final model to obtain relevant statistics. Degrees of freedom differ between analyses, partly because we did not have measurements for all birds, and partly because the number of other terms included in the model after the selection procedure differs between tests.

We used the Student's t-test to test for sex differences in oxidative status. In this analysis, a paired samples test was applied because the data from mated partners are not statistically independent. In addition, we performed a cross-correlation analysis between measures of oxidative status (OXY, ROMs, OS) for both sexes individually, and used the sequential Bonferroni corrections to adjust the statistical significance criterion for these analyses. From the assortative mating model, we hypothesised that females in good oxidative

status would mate with more ornamented males, so for this analysis we correlated oxidative measures of females with males' sexual plumage signals. All tests were two tailed. STATISTICA package (version 7.0, StatSoft, Inc. 2004, Tulsa, OK, USA) was used for all statistical analysis.

Results

More ornamented flycatcher males, with a larger forehead patch, also had stronger antioxidant defences (Fig 1, Table 1). Larger male flycatchers had lower OXY-levels (Fig 2), while their OS was higher, but this relationship was marginally non-significant. On the other hand, age, body condition, and age corrected for wing patch size did not explain the variation in oxidative measures (Table 2).

In flycatcher females, variation in morphological measurements did not explain variation in any biomarker of oxidative status. Similarly to males, ROMs or OXY did not correlate with age, however, older females showed significantly lower values of OS (Table 2). The proportion of the variance in OS accounted for by age was 25 %. Females that laid larger eggs tended to showed lower values of OS ($F_{1,16}=4.56$; $p=0.048$, partial $r=-0.538$). Nevertheless, OXY and ROMs of females were not related to average egg mass. There was no difference in the plasma oxidative status of females which laid six or seven eggs (Table 3).

Collared flycatcher males had significantly higher ROMs (paired Student t-test: $t=-6.59$; $p<0.0001$) and OS ($t=-3.37$; $p=0.007$) compared to females, while there was no difference between the sexes in OXY ($t=-0.045$; $p=0.96$) (Table 4). If the sexual traits measured in this study are honest signals of the condition (oxidative status) of individual flycatchers, we expected that, under mutual pair formation, there would be positive associations between measures of oxidative balance and the mate life history traits. Comparisons showed that females with higher OXY mated with males with larger forehead

patch size ($r=0.47$, $p=0.048$, $N=18$). There was also some evidence of a positive trend between males OS and the tarsus length of females, with this relationship being marginally significant ($r=-0.51$, $p=0.076$, $N=13$).

Direct correlations between oxidative measures of mates showed a positive, but not statistically significant, relationship between female and male OXY ($r=0.63$; $n=11$ $p=0.036$, Bonferroni-adjusted $\alpha=0.0062$) and female OXY and male ROMs ($r=0.66$; $n=13$; $p=0.013$, Bonferroni-adjusted $\alpha=0.0055$). None of the other comparisons were significant (all $p>0.314$).

Discussion

This study investigated the relationships between physiological quality (determined by plasma oxidative status) and several key phenotypic traits of a wild, free living population of collared flycatchers. We found evidence that attractive males, bearing large forehead patches, had higher plasma antioxidant capacity. In male collared flycatchers, the white forehead patch is a sexually selected trait. Previous studies have shown that males with larger forehead patches are more successful in gaining experimentally vacated territories (Pärt and Qvarnström 1997), in becoming polygynous (Gustafsson et al. 1995), and in obtaining extra-pair fertilizations (Sheldon and Ellegren 1999). Females prefer males with large forehead patches as social mate (Qvarnström et al. 2000) and are less-likely to cuckold males with a large white forehead patch (Michl et al. 2002). Despite the strong interest of ecologists in sexually selected traits, evidence for the physiological background underlying the expression of these traits remains scarce. A recently proposed hypothesis (Alonso-Alvarez et al. 2007) suggests that testosterone might mediate the expression of sexual ornamentation, and this should generate a physiological cost in terms of increase in oxidative stress. Behind this cost, there might also be a trade-off between ornament expression and the maintaining of the balance between oxidant and antioxidant systems. However, Garamszegi and co-workers

(2004) demonstrated experimentally that collared flycatcher males with larger forehead patches had less modification in their song, after confrontation with an immunological stress therefore showing better physiological status than males bearing smaller patches. Both Garamszegi et al.'s (2004) and our findings do not support the trade-off hypothesis, rather they suggest that higher quality males might be able to invest at the same time in the expression of sexual signals and maintaining of a balanced redox state. Therefore, the forehead patch seems to be a good indicator of the plasma capacity of the male to cope with oxidative challenges. Conversely, the wing patch size was not a good predictor of oxidative status in either sex.

Earlier studies indicated that oxidative stress may underlie physiological trade-offs in reproduction, whereby higher investment in reproductive activity might reduce resources available for basic maintenance costs (Ilmonen et al. 2000; Alonso-Alvarez et al. 2004; Wiersma et al. 2004; Bertrand et al. 2006). In our study, we did not find a significant correlation between clutch size (number of eggs) and any of the oxidative status measures. However, females that produced heavier eggs had a better plasma redox balance, with relatively lower levels of hydroperoxides compared to antioxidant compounds. These findings suggest that a higher investment in eggs does not come at the cost of decreased capacity for regulating oxidative balance. Consequently, these results also do not support the trade-off hypothesis. Congruent with our result, Bize et al. (2008) showed that wild Alpine swift (*Apus melba*) females that invested more into reproduction had also higher resistance of red blood cells to resist to an *in vitro* free radical attack.

The age of females was negatively related to the index of plasma oxidative status, meaning that older females had relatively lower levels of hydroperoxides compared to antioxidant compounds. The connection between aging, survival and oxidative status is poorly understood in bird species. Bize and colleagues (2008) found that male Alpine swifts

that survived to the next breeding season tended to have red blood cells that were more resistant to free radical attack, and that females with similar red blood cell characteristics laid larger clutches. These data suggest that the relationship between life history traits and resistance to oxidative stress is sex specific in this species: high resistance is related to fecundity in females and to survival in males. In captive flamingos (*Phoenicopterus ruber roseus*) red blood resistance to free radical attack was higher in young adult birds (12–20 years old) than in immature (5 months old) or old flamingos (30–45 years old, Devevey et al. 2010). Similarly, Alonso-Alvarez et al. (2010) found that older partridges (*Alectoris rufa*) had higher levels of oxidized glutathione and end-products of lipid peroxidation (TBARS) in erythrocytes than did middle-aged individuals. In agreement with these studies, our study shows that 4-year-old female collared flycatchers compared to younger ones had a balance of oxidant and antioxidant plasma systems shifted in favour of antioxidants. Collared flycatchers can live up to 10 years in the wild, so our future work on this species will investigate whether oxidative status changes in older females and, if so, identify the modulators of such a change.

Body condition of flycatchers did not predict the oxidative status in either males or females investigated in this study. However, the body size of males, expressed as tarsus length, was negatively correlated with plasma antioxidant capacity. Our experimental design does not allow us to identify the mechanisms driving this result. One potential explanation is that bigger individuals might be expected to have a higher muscle workload, which may generate higher metabolic needs and production of free radicals. However, a study on homing pigeons showed that heavier individuals depleted less serum antioxidants when experiencing a bout of flight activity (Costantini et al. 2008). A recent review on the associations among body size, energy metabolism, oxidative stress and lifespan, showed that results from intra- and interspecific comparisons are difficult to generalize, and in fact shed light on the complex linkages among these traits (Speakman 2005). Experimental studies will, therefore, be needed

to evaluate the conditions under which body size increases the metabolic needs of individual flycatchers, potentially exposing them to oxidative stress.

The assortative mating hypothesis predicts that individuals in similar physiological quality prefer each other. Alternatively, mating pairs might have similar physiological status because they rely on the same environmental resources and are exposed to similar stressors. Cross-correlations between measures of oxidative status of male versus female birds identified in this study are inconsistent with both of these paradigms. The correlations between biomarkers of oxidative status and a ‘quality’ trait of the other sex, showed that (i) females with higher OXY mated with males with larger forehead patch and (ii) males with higher values of OS mated with smaller females. Although after Bonferroni corrections the effects turned slightly insignificant, we think that both findings support the assortative mating in collared flycatchers, i.e. higher quality females mate with higher quality males. Alternatively, it could be that higher quality males (i.e., those with larger forehead patch) may provide the female more resources, so allowing her to have more resources for both egg production and self-maintaining. More studies will be therefore needed to test these assumptions in collared flycatchers.

Our results show that males had higher plasma hydroperoxides than females. There are two likely explanations for these results. First, males and females were sampled in two different phases of the breeding season. Specifically, males were sampled during the pair formation period, just after their arrival at the breeding site from a long distance migration. At this time, females could not be caught as they had not chosen nest boxes and/or because females tend easily to abandon the breeding area if disturbed. After arrival at the breeding plot, males are immediately occupied in energetically expensive behaviours - such as singing, sexual advertisement, and nest-box defence -, which may increase metabolic costs and expose individuals to higher oxidative attack. Sampled males also had only a short period to

recovering from the migration effort. Females, on the other hand, were sampled approximately 20 days later than males, and this longer time for physiological recovery could explain their better oxidative status. Nevertheless, females were sampled during incubation, which is an energy demanding activity. The availability of prey rich in antioxidants (e.g. caterpillars), increases from pair formation to the incubation period (Hargitai et al. 2008), and this factor may have assisted females to rebalance the redox state. Further work is required to confirm whether differences between sexes observed in the present study reflect strategies for managing oxidative status, or result from differences in the sampling periods.

Finally, our study was limited to one breeding season and so we cannot generalize that the lack of a trade-off among expression of sexual traits, reproductive activity and self-maintenance is a rule in our study region. It could be in fact that trade-offs could arise under harsh environmental conditions or when looking at different traits. Also, we cannot rule out the possibility that a trade-off can be mirrored in other molecular components of redox balance, as well as in other tissues. Further studies will be needed to clarify these points.

Conclusions

The key signal in mating attractiveness, the white forehead patch of collared flycatcher males was associated with higher plasma antioxidant capacity. Moreover, females that mated with more ornamented males had higher plasma antioxidant capacity. On the other hand, flycatcher females that laid larger clutches (an important intra-generational fitness component) did not suffer from higher oxidative stress. Also, females that laid heavier eggs had a smaller value of oxidative status. All these findings suggest that a higher investment in expression of sexual traits or reproductive activity was not accompanied by an increase in the physiological costs as expressed by oxidative stress. The life history trade-offs paradigm was challenged in a number of recent empirical studies. For example, in captive short-tailed field

voles (Selman et al. 2008), in outbreed lab mice (Johnston et al. 2006) and in free-ranging kittiwakes (Welcker et al. 2010), animals were able to combine phenotypic life history traits in a manner contrary to the traditional trade off model. Nevertheless, it should be noted that the connection between physiological state and different life history traits might be more complex to model in a simply unidirectional relationship. Safran and colleagues (2008) demonstrated that the manipulation of plumage ornaments in barn swallows (*Hirundo rustica*) alters the temporal circulating androgens. This dynamic feedback between phenotype and physiology suggests a more complicated view of the physiological causes and consequences of the trait expression.

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TABLES

Table 1. Association between measures of plasma oxidative status, plumage sexual traits and body condition of male and female collared flycatchers.

Sex	Factors	OXY		ROMs		OS	
		F (d.f.)	p	F (d.f.)	p	F (d.f.)	p
Male	Forehead patch size	4.81 (1, 12)	0.048	0.48 (1, 13)	0.501	1.25 (1, 10)	0.289
	Wing patch size (age corrected)	0.22 (1, 11)	0.648	0.01 (1, 13)	0.941	0.16 (1, 10)	0.698
	Body condition	0.69 (1, 11)	0.423	1.90 (1, 13)	0.192	1.25 (1, 10)	0.289
Female	Wing patch size (age corrected)	0.57 (1, 14)	0.462	1.38 (1, 15)	0.259	1.06 (1, 14)	0.321
	Body condition	0.00 (1, 14)	0.997	0.30 (1, 15)	0.595	0.06 (1, 14)	0.816

F-statistics of GLM analyses are shown. Separate analyses were run for both sexes and models included age-corrected wing patch size, body condition and forehead patch size (in males) as factors. ROMs = reactive oxygen metabolites (plasma hydroperoxides); OXY = plasma antioxidant capacity; OS = oxidative status defined as the ratio between ROMs and OXY.

Table 2. Association between measures of plasma oxidative status and three life history traits of male and female collared flycatchers.

Sex	Factors	OXY		ROMs		OS	
		F (d.f.)	p	F (d.f.)	p	F (d.f.)	p
Male	Age	0.89 (1, 11)	0.364	0.00 (1, 13)	0.981	0.04 (1, 11)	0.836
	Tarsus length	6.49 (1, 12)	0.025	0.43 (1, 13)	0.524	4.50 (1, 12)	0.055
	Body condition	2.42 (1, 11)	0.148	1.51 (1, 13)	0.241	2.98 (1, 11)	0.112
Female	Age	0.94 (1, 13)	0.350	1.81 (1, 14)	0.200	4.96 (1, 15)	0.041
	Tarsus length	0.44 (1, 13)	0.517	0.00 (1, 14)	0.972	0.00 (1, 14)	0.948
	Body condition	0.00 (1, 13)	0.999	0.47 (1, 14)	0.502	0.03 (1, 14)	0.863

F-statistics of GLM analyses are shown. Separate analyses were run on male and female birds including age, tarsus length and body condition as factors. ROMs = reactive oxygen metabolites (plasma hydroperoxides); OXY = plasma antioxidant capacity; OS = oxidative status defined as the ratio between ROMs and OXY.

Table 3. Results of F-statistics in GLM analyses for the association between measures of plasma oxidative status and reproduction parameters when age was covariant in female collared flycatchers.

Factors	OXY		ROMs		OS	
	F (d.f.)	p	F (d.f.)	p	F (d.f.)	p
Clutch size	0.11 (1, 14)	0.741	0.08 (1, 16)	0.783	0.00 (1, 15)	0.952
Egg mass	2.51 (1, 14)	0.135	0.30 (1, 16)	0.590	4.56 (1, 16)	0.048
Age	0.64 (1, 14)	0.439	0.42 (1, 16)	0.528	3.01 (1, 15)	0.103

ROMs = reactive oxygen metabolites (plasma hydroperoxides); OXY = plasma antioxidant capacity; OS = oxidative status defined as the ratio between ROMs and OXY.

Table 4. Levels (mean $\pm$ SE, n) of plasma hydroperoxides (ROMs), plasma antioxidant capacity (OXY) and oxidative status (OS) of free living female and male collared flycatchers.

Sex	OXY		ROMs		OS	
	Mean $\pm$ SE	n	Mean $\pm$ SE	n	Mean $\pm$ SE	n
Female	212.59 $\pm$ 17.05	18	1.41 $\pm$ 0.11	20	7.51 $\pm$ 0.92	18
Male	207.87 $\pm$ 25.02	14	2.55 $\pm$ 0.16	17	14.03 $\pm$ 1.38	14

FIGURES

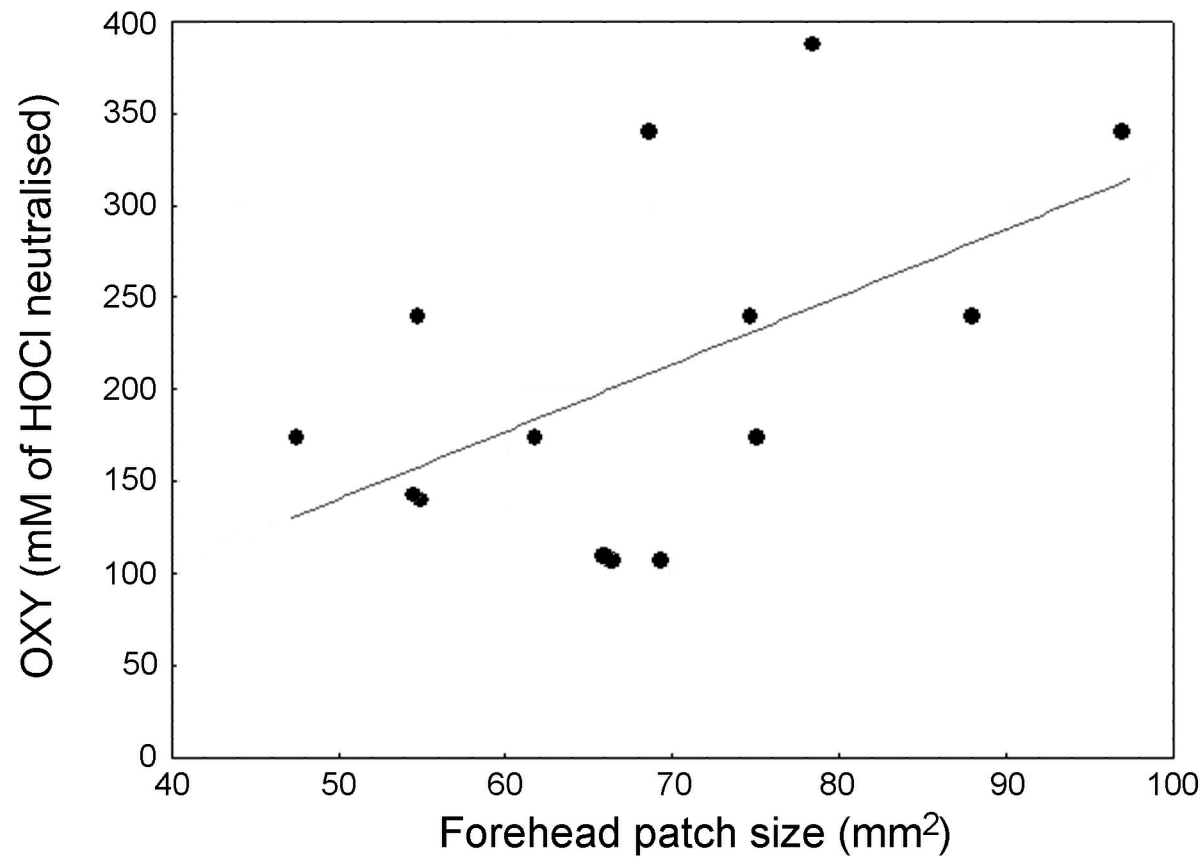


Figure 1. The relationship between forehead patch size and plasma antioxidant capacity (OXY) in male collared flycatchers.

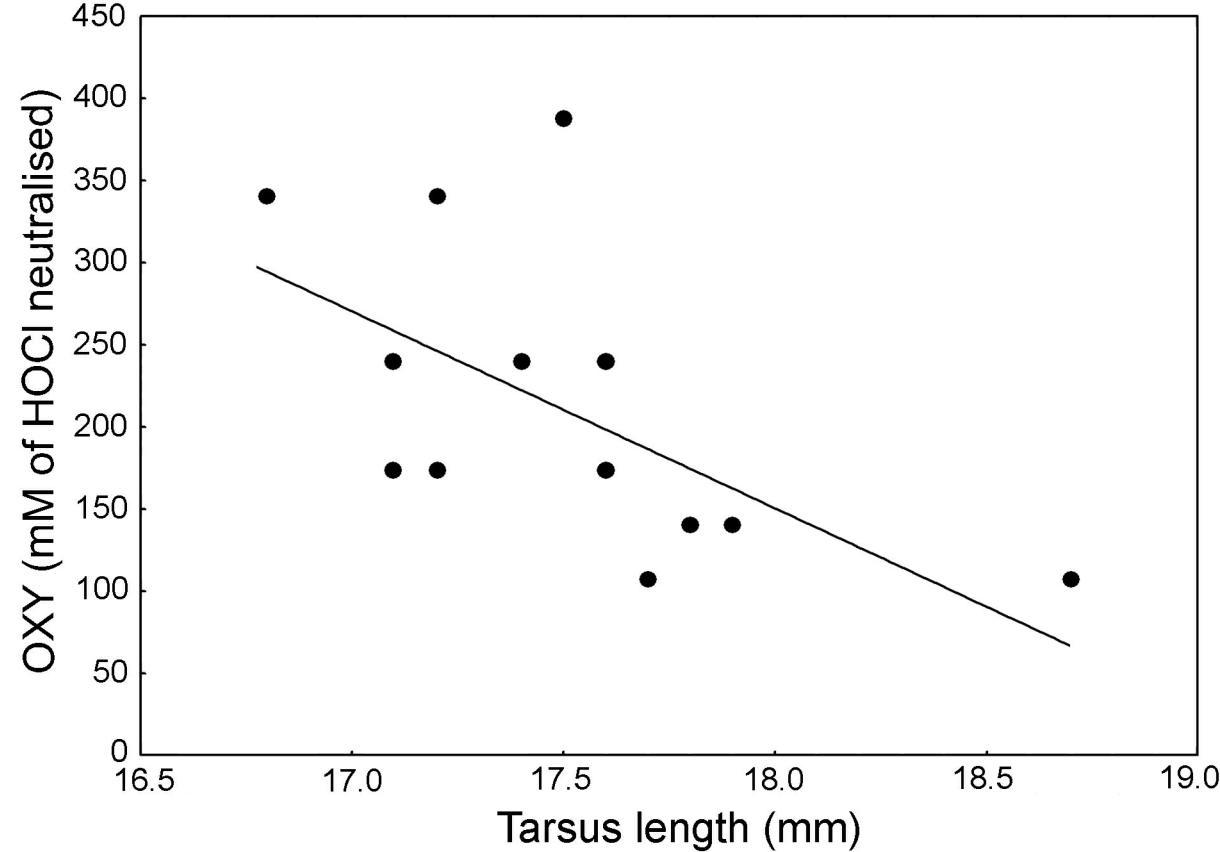
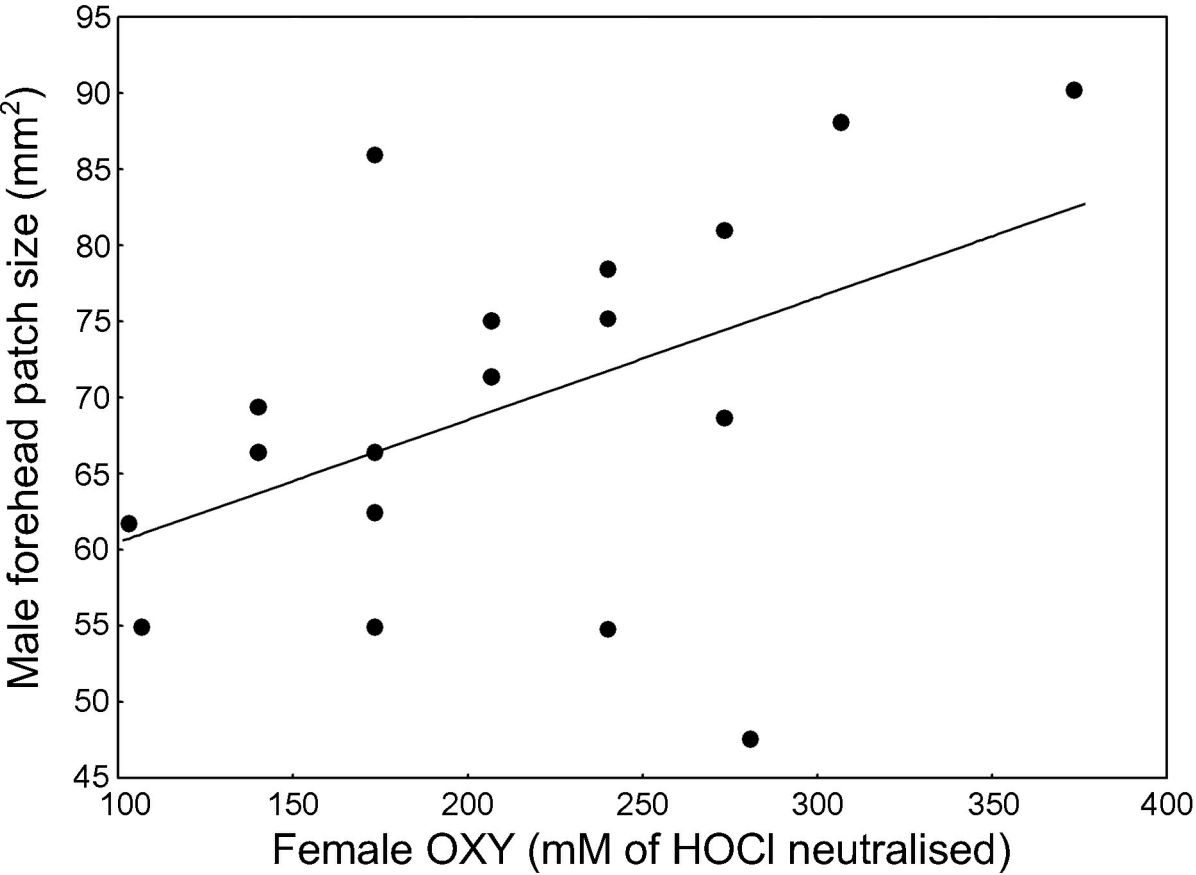


Figure 2. The associations between body size expressed by tarsus length and plasma antioxidant capacity (OXY) in male collared flycatchers.

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86 **Figure 3. Relationship between female plasma antioxidants and forehead patch size of males ($r= 0.47$, $p=0.048$, $n=18$).**

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