Elevated oxidative stress in pied flycatcher nestlings of eumelanic foster fathers under low rearing temperatures

Teerikorpi PE[1,2], Stauffer J[1], Ilmonen P[1], Calhim S[3], Schuett W[4,5], Laaksonen T[1,6]

[1] Department of Biology, University of Turku, Finland
[2] Institute for Evolutionary Life Sciences, University of Groningen, Netherlands
[3] Department of Biology and Environmental Sciences, University of Jyväskylä, Finland
[4] Insititute of Zoology, Universität Hamburg, Hamburg, Germany
[5] School of Life Sciences, University of Sussex, Falmer, Brighton, United Kingdom
[6] Natural Resources Institute Finland (Luke), Turku, Finland

* Corresponding author: Pauliina Elisabet Teerikorpi, Section of Ecology, Department of Biology, University of Turku, FI-20014 Turku, Finland. Tel.: +358 2 333 5243; Fax: + 358 2 333 6598; Email: peteer@utu.fi

Keywords: environmental heterogeneity, genetic quality, genotype-by-environment interaction, oxidative stress, phenotypic variation, phenotypic quality, sexual selection, secondary sexual trait

Summary statement

This study demonstrates a temperature-dependent association between oxidative stress of offspring and variation in the melanin coloration of their foster father in the pied flycatcher.

ABSTRACT

Striking variation in melanin coloration within natural populations is likely due to the different fitness outcomes of alternative phenotypes in varying environmental conditions. There are two types of melanins. Eumelanins yield blackish hues, while pheomelanins yield reddish hues. The production of eumelanins requires low levels of glutathione (GSH), which is the most important intracellular antioxidant, while the production of pheomelanins requires high levels of GSH. We investigated the oxidative status of male pied flycatchers *(Ficedula hypoleuca)* with different degrees of melanin coloration under different temperatures during the nestling period. Moreover, we assessed the oxidative status of offspring in relation to their biological or foster father's melanin coloration and ambient temperature. To separate offspring genotype effects and paternal effects in different temperatures, we used a partial cross-foster design. The temperature differently affected the oxidative status of differently colored male pied flycatchers and their foster offspring. When the weather was relatively cold, black males had higher glutathione S-transferase levels compared to brown males, indicating enhanced stress in black males. Foster offspring of black males had lower ratio between reduced and oxidized GSH followed by higher total amount of GSH than foster offspring of brown males. Thus, foster offspring of black males seem to suffer from oxidative stress under relatively cold weather compared to those of brown males, and vice versa under relatively warm weather. While differently colored males experienced changes in their oxidative status under different temperatures, the link between father melanin coloration and offspring oxidative stress appears to be environmentally induced.

INTRODUCTION

Phenotypic variation within wild populations is extremely wide (Dale 2006). One potential explanation for this variation is that alternative phenotypes are adapted to different environmental conditions (phenomenon known as genotype-by-environment interaction, Qvarnström, 2001; Bell, 2010; Cornwallis and Uller, 2010). Many variable phenotypic traits are secondary sexual characters that have been evolved through sexual selection (Andersson 1994). In several cases males are showier than females as females are often the choosy sex (Clutton-Brock and Vincent, 1991; Andersson, 1994). Moreover, phenotypic variation among males is often higher than that of females (Clutton-Brock and Vincent, 1991; Andersson, 1994). Secondary sexual characters reflect either genotypic or phenotypic quality (Zahavi, 1975; Hamilton and Zuk, 1982; Hoelzer, 1989). Nevertheless, contrary to what was long thought, the choosy sex does not always benefit by selecting a mate with the most conspicuous ornamental traits (Greenfield and Rodrigues, 2004). In fact, in certain conditions the choosy sex may benefit from selecting the least ornamented mate as selection acting on secondary sexual traits may not be constant, leading to fitness differences among alternative sexual phenotypes in varying environmental conditions (Qvarnström, 2001).

Fitness differences among alternative phenotypes in relation to genetic and phenotypic quality in varying environmental conditions have recently received quite some attention (e.g. Piaultet et al., 2009; Jacquinet al., 2012; Järvistö et al., 2015). Most studies have investigated such scenarios in terms of offspring body mass and survival. However, proximate physiological mechanisms associated with future survival or breeding success are far less studied. Oxidative stress (often highly damaging to biological tissues) has been suggested to act as one of the mechanisms underlying such fitness variations in life-history traits (see Roulin et al., 2011). Oxidative stress is caused when there is an imbalance between the production of damaging reactive oxygen species (ROS) and antioxidant machinery so that ROS production exceeds

antioxidant defenses (Finkel and Holbrook, 2000; Halliwell and Gutteridge, 2007; Costantini et al., 2010). ROS are produced by normal metabolic activities that require oxygen (Finle and Holbrook, 2000). Being unstable and highly reactive, ROS have a potential to damage DNA, proteins and lipids (Fang et al., 2002). However, there are several endogenous and exogenous antioxidant compounds that convert ROS into less damaging molecules (Felton, 1995; Surai, 2002).

When considering the abilities of varying phenotypes to deal with oxidative stress, melanin coloration is particularly intriguing. Two types of melanin pigments determine the color: eumelanin and pheomelanin (McGraw, 2006). Eumelanin is responsible for black and grey hues, while pheomelanin is responsible for reddish and yellowish hues (McGraw, 2006). The production of pheomelanin needs high levels of gluthatione (GSH), which is the most important intracellular antioxidant (Wu et al., 2004), while eumelanogenesis requires low GSH levels (Galván & Solano, 2009, Hõrak et al., 2010). It seems that eumelanic individuals are more adapted to high oxidative stress environments, while pheomelanic individuals to low oxidative stress environments (Galván and Solano, 2009; Galván et al., 2011; Roulin et al., 2011; Solano, 2014). Eumelanic individuals do not need to maintain high GSH levels for eumelanogenesis, but instead can freely use GSH as an antioxidant or/and can afford producing alternative antioxidants (Halliwell and Gutteridge, 2007; Galván and Solano, 2009; Galván et al., 2011). In turn, pheomelanic individuals need to maintain high levels of GSH for the expression of pheomelanic traits (Galván and Solano, 2009; Galván et al., 2011). Such maintenance of high GSH levels is costly, reducing the ability to maintain basal levels of other antioxidants or the ability to produce other antioxidants rapidly when faced with oxidative environment.

Alternatively, individuals with different melanin coloration are adapted to different environments because genes responsible for the expression of melanin coloration are responsible also for the expression of several physiological and/or behavioral traits

(phenomenon known as pleiotropy, Ducrest et al., 2008). The melanocortin system is highly pleiotropic as one gene (proopiomelanocortin gene) produces five different melanocortins that bind to five receptors (Ducrest et al., 2008; Roulin, 2016). These five melanocortins are responsible not only for melanin production but also for e.g. immune function, stress response, energy expenditure, thermoregulation and behaviors such as aggressiveness and sexual activity (Ducrest et al., 2008). Thus, differently colored individuals might be adapted to different environmental conditions due to such covariation among physiological, behavioral and color traits. However, the above-mentioned possibilities (the effect of glutathione on melanin coloration and oxidative stress and the pleiotropic system of melanin production) are not mutually exclusive (Galván and Solano, 2009).

The pied flycatcher (*Ficedula hypoleuca* Pallas) is a sexually dimorphic bi-parental migratory passerine. Dorsal melanin-based coloration of males varies from almost completely black to female-like brown (Drost, 1936; Lundberg & Alatalo, 1992; Calhim et al., 2014; Laaksonen et al., 2015). Melanin coloration is heritable and partly age-dependent as males become slightly darker (ca. 15-20%) between the ages of 1 and 2 years (Lundberg and Alatalo, 1992). Male melanin coloration has been shown to have a temperature-dependent association with breeding success measured as offspring mortality (Sirkiä et al., 2010) and body mass (Järvistö et al., 2015). As it has been suggested that eumelanic individuals cope better in stressful conditions compared to pheomelanic individuals, it is surprising that eumelanic pied flycatcher fathers have lower breeding success when the weather is relatively cold during the nestling period compared to pheomelanic males, and *vice versa* during relatively warm weather. The temperature-effect has been shown to arise through behavior of differently colored males in different temperatures, as the effects were only linked to foster, but not genetic, father's coloration (Järvistö et al., 2015).

Here, we investigated the oxidative status of male pied flycatchers with different degrees of melanin coloration under different temperatures during the nestling period. Moreover, we studied the oxidative status of offspring in relation to their biological or foster father's melanin coloration and temperature during the nestling period. In order to distinguish between offspring genotype effects (genetic father) from paternal effects (foster father) in different temperatures, we used a partial cross-foster design where a certain number of chicks (close to 50%) was swapped between breeding pairs. Brood sizes were simultaneously either reduced or enlarged for the purpose of other studies (see Järvistö et al., 2015; Schuett et al., 2017). In this study the main interest was however in the interaction between coloration and temperature, as we did not find any interactive effect between brood size manipulation and male melanin coloration on offspring body mass in our previous study (Järvistö et al., 2015). Nevertheless, we tested the possibility of an interaction between brood size manipulation and male melanin coloration on the oxidative status of both nestlings as well as the males themselves but did not find evidence for any interactions. Given our previous results our *a priori* prediction was that during the nestling period oxidative stress in black males is elevated by decreasing temperature, while oxidative status of brown males is less likely to be temperature dependent. Furthermore, we predicted that elevated stress under low temperatures in foster fathers lead to the poorer ability of the fathers to take care of their nestlings. Consequently, offspring of eumelanic fathers would also suffer increased oxidative stress under low temperatures due to the poor paternal care. Thus, we predict that the potential link between oxidative stress of the black fathers and their offspring is not genetically but rather environmentally induced.

METHODS

Study species and study site

The pied flycatcher is a migratory passerine that breeds in most of Europe and western Siberia, and winters in sub-Saharan Africa. Individuals breed in natural cavities and also willingly in nest boxes (Lundberg and Alatalo, 1992). The present study was conducted on the island of Ruissalo in Turku, Finland (60º 35'N, 27º 09'S), in summer 2012. In Finland, the species arrives at the breeding grounds in May and breeds from late May to early July. Nestlings grow quickly and fledge at 16-17 days of age (Lundberg and Alatalo, 1992).

In 2012, 216 wooden nest boxes (inner bottom area: 144 cm^2, entrance hole: 32 mm) out of 436 total boxes were successfully occupied by pied flycatchers in our study area. Nest boxes were monitored at minimum once every 4-5 days for detecting laying date (pied flycatchers lay one egg per day), clutch size (median clutch size of the population is 7 eggs), hatching date, and brood size at hatching. The hatching date was initially estimated to be the 14th day of incubation, and nests were daily checked from the 12th day of incubation to determine the actual hatching day.

Experimental design

We conducted a partial cross-fostering between pairs of nests matched for clutch size and hatching date when the chicks were at the age of three days (hatching day = day 0, see Järvistö et al., 2015). In addition to cross-fostering, we simultaneously did a brood size manipulation (BSM). In the current study the BSM effect on oxidative status was not main interest as we concentrate on temperature effect based on our previous finding (Järvistö et al., 2015). We measured the oxidative status of 55 males and one foster offspring of each male to separate the

phenotypic paternal and genetic effects on offspring oxidative status. This means that we analyzed the oxidative status of foster offspring that had been transferred into a new nest against the coloration of their foster father and their biological father. The sample size of foster (N = 38 out of 38 nests) offspring is smaller than that of males (N = 55) as in control nests of the brood size manipulation we did not do cross-fostering.

Sampling

At the age of 12 days body mass and wing length of the nestlings were measured (with an accuracy of 0.1 g and 0.5 mm, respectively), and blood samples were taken from a wing vein for oxidative status measurements, paternity analysis and sex determination. Blood was collected to Eppendorf tubes, which were immediately put into liquid nitrogen (-196 C^o), and stored in freezer after the field day (-80 C^o). All captured males were ringed (unless they already had a ring) when they were feeding 10-day-old nestlings. At this time, we measured body mass, wing length and tarsus length (with an accuracy of 0.1 g, 0.5 mm and 0.01 mm, respectively). Furthermore, blood samples were collected from a wing vein for paternal analysis and oxidative status measurements (see below). All males were aged as young (1-year-old) or old (≥2 years old) on the basis of feather wear (Svensson, 1992). The proportion of black in the dorsal plumage of males was estimated by eye from 0 to 100% (see Järvistö et al. 2015: mean = 57%, SD = 32%, N = 155). Repeatability of our estimation was ensured by assessing melanin-coloration percentages twice (by two different observers) for 34 males during the breeding season in 2012 ($r = 0.88$, $F_{1, 33} = 24.96$, $P < 0.001$). Moreover, there was no correlation between biological and foster fathers' plumage coloration ($r = -0.32$, $P = 0.12$, $N = 25$).

Temperature measures

Temperatures for the nestling phase were determined individually for each nest. We used an average daily temperature for the whole nestling period from the day after the brood size manipulation to the day before offspring measurements (i.e. days 4 to 11). See Fig1.S1. for the distribution of average daily temperatures across the study period (maximum 17.80 ^{o} C, minimum 12.80 ^{o} C). The temperature data was provided by the Finnish Meteorological Institute, and were recorded 2 km from the study area at the meteorological station (60°27′ N, 22°10′ E), Artukainen, Turku.

Paternity analyses and sexing

Extra-pair paternity occurs in our pied flycatcher population (Lehtonen et al., 2009). Thus, to verify that the right biological father was assigned to each offspring, we determined paternity through genetic parentage analysis from the blood samples collected in the field. Moreover, as males and female offspring may show different behaviors in different conditions we genetically determined the sex of the chicks (e.g. Ruuskanen and Laaksonen, 2010). All laboratory work and parentage analyses were done in the Center of Evolutionary Applications (University of Turku, Finland). See Järvistö et al., (2015) for more detailed information on sexing and parentage analyses.

Oxidative status measures

We measured multiple antioxidant biomarkers [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), glutathione S-transferase (GST), total amount of glutathione (GSHtot) and the ratio between reduced and oxidized glutathione (GSHratio)], and oxidative damage [protein carbonylation, (CARB)], to achieve a comprehensive assessment of oxidative

status. Oxidative status was determined by measuring several antioxidant biomarkers and oxidative damage (CARB). Sigma kits were used to measure CAT-, GP- and GST- activity (Sigma CAT100, CGP1 and CS0410, Sigma Chemicals, St. Louis, USA), a Fluka kit to measure SOD-activity (Fluka, Buchs, Germany) and protocol from Glutathione Fluorescent Detection Kit was used to measure GSHratio and GSHtot (K006-F1, Arbor Assays). All methods were adjusted to minimize sample volumes as described in Supplementary material of Stauffer et al. 2017. The CARB-measurement was done according to Rainio et al., (2015) with 1 mg/ml sample dilution. All oxidative status measures are expressed per protein content, which was determined with the BCA (bicinchoninic acid) protein assay (Pierce, IL, USA) (Smith et al. 1985). Measurements were done with EnVision and EnSpire microplate readers (PerkinElmer-Wallac, Finland). In all assays, samples were randomly placed on the plates and measured in a triplicate (intra-assay coefficient of variability [CV] < 15 % in all cases). In addition, three control samples were used on every plate to correct for interassay variation with the ratio specific to the particular plate (0.8-1.2).

Statistical analyses

All statistical analyses were conducted using R (v 3.5.1; R Core Development Team, 2018). We used linear models (lm function) to investigate possible interactive effects of melanin-based phenotype of males (proportion of black) and temperature on the oxidative status (GSHratio, GSHtot, SOD, CAT, GP, GR, GST, CARB) of males and nestlings. Being a ratio variable, GSHratio was transformed to logarithmic scale. The degree of male melanin-based coloration was on a continuous scale from 0 to 100%. We used original clutch size as a covariate in foster and biological father models to control for the possible effects of pre-manipulation clutch size. Age of males was used as a categorical variable to control for possible age effects. When analyzing offspring oxidative status, we ran different models for biological and foster father traits because models including both biological and foster father traits and

their interactions with environmental variables would have been strongly overparameterized (Grueberet al., 2011). In these models the sex of the chicks was used as a categorical variable to control for possible sex effects. In addition, we used offspring body mass as a covariate to control for variation in body condition caused by variation in body mass. BSM was added in all models to control for the increase/decrease in parental effort. The degrees of freedom in each model were estimated according to the Kenward-Roger's approximation (Littell et al., 2006). We used visual examination of the model residuals to assess their assumed gaussian distribution.

Ethical standards

The study was conducted with the authorization of the national board on animal experiments (animal experiment committee of Southern Finland).

RESULTS

Male oxidative status in relation to melanin coloration and temperature

Temperature had a different effect on GST levels of black and brown breeding males, as indicated by a significant interaction between temperature and male phenotype (Table 1). As illustrated in Fig. 1, this interaction means that black males had higher GST levels than brown males when it was relatively cold (temperature below 14.6 C^{o}) during the nestling period, while it was the opposite pattern when it was relatively warm (temperature above 14.6 C^{o}). There were no interactive effects between male melanin coloration and temperature on other measures of oxidative status in males (Table 1). It furthermore appears that old males had lower levels of GST than young ones (Table 1). Age did not affect any other measures of traits related to oxidative status levels (Table 1). Original clutch size, brood size manipulation, male melanin coloration or temperature alone did not influence any measures of oxidative status in males (Table 1).

Offspring oxidative status in relation to biological father's melanin coloration

There were no interactions between temperature and blackness of the biological father on the oxidative status of the offspring raised in foster nests (Table 2). However, there was a tendency of temperature to affect SOD levels of the offspring, so that under low temperature during the nestling period, the offspring had higher levels of SOD compared to high temperatures during the nestling period (Table 2). Moreover, offspring of dark biological fathers had lower GSHratio than offspring of brown biological fathers (Table 2). Additionally, offspring raised in enlarged broods had higher GSHratio than those raised in reduced broods in these models (Table 2). Otherwise, single variables (body mass, brood size manipulation, sex of the offspring, or temperature) did not affect the oxidative status of the offspring (Table 2).

Offspring oxidative status in relation to foster father's melanin coloration

The oxidative status of foster offspring was associated with their foster father melanin coloration differently in different temperatures. Foster offspring raised by black males had a lower GSHratio than those raised by brown males when it was relatively cold (temperature below 14.4 C^{o}, Table 3, Fig 2). In contrast, foster offspring raised by black males had higher GSHratio than those raised by brown males when it was relatively warm (temperature above 14.4 C^{o}, Table 3, Fig 2). Moreover, foster offspring of black males seemed to have higher GSHtot compared to those of brown males when it was relatively cold (temperature below 14.5 C^{o}, Table 3, Fig 3), while it was lower in foster offspring when the temperature was relatively high (above 14.5 C^{o}, Table 3, Fig 3). However, it seems that GSHtot levels of foster offspring of brown males were not as temperature-dependent as levels of foster offspring of black males (Fig 3). There were no other statistically significant interactions between temperature and male melanin coloration affecting oxidative measures of the foster offspring (Table 3). Nevertheless, male and female offspring differed in their SOD levels so that female offspring had lower levels

of SOD compared to male offspring (Table 3). Sex did not affect any other measures of oxidative status of the offspring (Table 3). Body mass, melanin coloration of the foster father, brood size manipulation or temperature alone did not influence oxidative status of offspring (Table 3).

DISCUSSION

Our results revealed that the temperature during the nestling period differently affected the GST levels of black and brown male pied flycatchers and their foster offspring. When the weather was relatively cold, black males had higher GST levels compared to brown males, and *vice versa*. This might indicate that black males under such environmental conditions are more stressed than brown males. However, there were no interactions between temperature and plumage coloration affecting other measures of oxidative status. Moreover, foster offspring of black males had lower GSHratio and higher GSHtotal than those of brown males under prevailing cold weather, and *vice versa* under relatively warm weather. As low ratio between reduced and oxidized glutathione followed by high sum of reduced and oxidized glutathione indicates high oxidative stress levels, our results indicate that foster offspring of black males suffer from higher oxidative stress under relatively cold weather compared to those of brown males. Under relatively warm weather the situation seems to be the opposite.

Oxidative status in male parents under different temperatures

Our results indicate that black males in cold weather might suffer from elevated oxidative stress as their GST levels in circulation were higher compared to that of brown males, and *vice versa* when it was warm. The function of the GST enzymes is to act against several damaging "foreign compounds" produced endogenously and by the surrounding environment (Halliwell and Gutteridge, 2007; Strange et al., 2001). Our result may first seem surprising, as other studies have shown in several avian species that dark melanic individuals are adapted to

stressful conditions, while pheomelanic individuals to less-stressful conditions (e.g. Roulin et al., 2011; Galván et al., 2014). Though, to our knowledge there are no studies that have shown temperature effects on oxidative status of individuals with different degrees of melanin. It has been shown in the pied flycatcher as in other species that melanin coloration covaries with a basal metabolic rate (BMR, Røskaft et al., 1986). A high BMR leads to elevated oxygen consumption, and therefore increases the amount of produced harmful oxidizing compounds (Finkel and Holbrook 2000). The produced damaging compounds are neutralized by GSH and other antioxidants, but sometimes such conjugation eventuates further in oxidizing compounds (Halliwell and Gutteridge, 2007). GST enzymes are needed to neutralize these generated compounds (Kampranis et al., 2000; Halliwell and Gutteridge, 2007). Moreover, BMR has been shown to negatively correlate with ambient temperatures (Williams and Tieleman, 2000; White et al., 2007). Therefore, the metabolic rate of black pied flycatcher males may reach an extreme in cold weather when feeding offspring, and thus lead to higher oxidative stress compared to brown males.

On the other hand, it needs to be noted that black males might actually have higher levels of GST compared to brown ones when it is relatively cold, because they might be in a better physical condition and can afford high levels of GST enzymes. In such a scenario it is more difficult to explain why black males have low quality (measured as body mass and oxidative stress) foster offspring during cold weather. Black males might invest in themselves even more when they are in a good condition to survive to the next breeding season. In turn, brown males experiencing large changes in their oxidative status during the cold weather might invest a lot in their current brood as their probability to survive to the next breeding season would be low. However, young males that are not usually in as good shape as old males (e.g. Mitrus, 2007) had higher levels of GST compared to those of the old males. This suggests that, indeed, high GST levels indicate high stress. Moreover, we found that during relatively warm nestling

period brown males have higher GST levels compared to black males. As temperature rises with increasing duration of sunshine the intensity of solar radiation escalates (Meza and Varas, 2000). UV-radiation is especially harmful for pheomelanic individuals causing elevated oxidative stress (Natarajan et al., 2014). Thus, brown pied flycatcher males might suffer higher levels of oxidative stress during high temperatures. Eumelanic individuals, on the other hand, have a better protection against UV-radiation because of the strong structure of eumelanin pigments (McGraw, 2006; Brenner and Hearing, 2007; Roulin 2014).

Oxidative status in offspring in different temperatures

As we previously showed that the interactive effect between the temperature during the nestling period and male melanin coloration on offspring body mass is due to paternal effects rather than genetic effects (Järvistö et al., 2015), it seems that males with different melanin colorations differ in their abilities to take care of offspring in varying environmental conditions. In this study we have shown that an interaction between the temperature and male melanin coloration most likely leads to differences in the oxidative status in differently colored males. Such an effect may possibly lead to differences in ability to feed offspring. This would cause differences in body mass and oxidative status in foster offspring of black and brown males under certain temperatures. Poor feeding capability of black/brown males under low/high temperatures may lead to increased oxidative stress in their nestlings and therefore low body mass. In contrast, underfeeding may first lead to low nestling body mass, which eventually causes oxidative stress in the nestlings. Indeed, it has been shown that, for example, within-brood competition elevates oxidative stress in passerine nestlings (e.g. Stier et al., 2015; Stauffer et al., 2017). On the contrary, there was no relationship between oxidative status of offspring in varying temperatures and the coloration of their biological fathers. Thus, the link between male coloration and offspring oxidative stress in varying temperatures appears to be environmentally induced. However, biological offspring of black males had lower GSHratio than biological

offspring of brown males. This is in accordance with previous knowledge that vertebrates with high eumelanin levels have lesser amounts of reduced glutathione compared to individuals with low eumelanin levels (Benedetto et al. 1982). Moreover, we found that female offspring had lower levels of SOD compared to the levels of male offspring, thus indicating lower levels of stress in female offspring. Testosterone might be a cause of such sex-specific difference in oxidative status of male and female offspring (see Alonso-Alvarezet al., 2007). In addition, SOD levels of offspring decreased with increasing temperature indicating that relatively warm weather is less stressful compared to cold weather. Furthermore, we found that GSHratio was higher in offspring of enlarged broods than in offspring of reduced broods. This was surprising as high GSHratio reflects low levels of oxidative stress. However, it seems that the breeding season 2012 was exceptionally good for pied flycatchers in terms of food availability as offspring mortality was very low (1.4 %, see Järvistö et al., 2015). Thus, sharing the nest with additional nestling might not have caused any additional stress in terms of elevated within-brood competition, but rather might have added some extra warmth to the nest.

Despite our findings on temperature-dependent differences in oxidative status of differently colored males and their foster offspring, males with different degrees of melanin coloration or their foster offspring did not suffer from oxidative damages (protein carbonylation). However, the sufficient antioxidant machinery prevents oxidative damages. Moreover, organisms are able to repair and remove proteins that are damaged by ROS. In order to have a more comprehensive picture of cell damages it would be valuable to measure also potential lipid and DNA damages.

Euemelanic and pheomelaninc phenotypes in stressful conditions

An intriguing link between melanin coloration and oxidative stress has been suggested already more than a decade ago (e.g. McGraw, 2005). As glutathione (the most crucial endogenous antioxidant) has an inhibitory effect on eumelanogenesis and an enhancing effect on pheomelanogenesis in melanocytes, it can be assumed that vulnerability to oxidative stress is linked with melanin coloration (Roulin et al., 2011). Thus, individuals with different levels of eu- and pheomelanins might cope differently with varying intensity of oxidative stress (Galván and Alonso-Alvarez, 2009; Roulin et al., 2011). It has been shown that dark eumelanic and light pheomelanic individuals signal their abilities to cope with oxidative stress differently along environmental gradient so that eumelanic individuals would cope better under stressful conditions (large brood size) than pheomelanic individuals (e.g. Emaresi et al., 2016). Our results, however, showed a different pattern. Eumelanic individuals coped worse under stressful environmental conditions (low temperature) compared to pheomelanic individuals as their GST levels were elevated. Nevertheless, stressful conditions created by relatively cold weather are more likely to be challenging to dark individuals in a different way than stressful conditions created by increased reproductive effort. Intriguingly, it has been shown that in barn owl (*Tyto alba*) nestlings the higher the number of eumelanic black spots, the higher the oxygen consumption and the lower the body temperature (at 24 ^{o}C, Dreiss et al., 2016). This suggests that highly eumelanic individuals have a lower ability to thermoregulate and thus, might experience elevated stress under low temperatures. Our results are the first ones to demonstrate how the physiological mechanisms associated with eumelanin coloration might be costly in terms of oxidative stress in varying temperatures. Our finding might be also related to temperature-dependent tyrosinase activity (Kim et al. 2003). Low temperatures reduce tyrosinase activity leading to increased glutathione levels and pheomelanogenesis, while high

temperatures result in the inactivation of glutathione reductase and thereafter in eumelanogenesis (Ito 1993, Kim et al. 2003). However, as we found a temperature-dependent effect only on GST levels in adult males, more studies on the effects of temperature on oxidative status of alternative melanic phenotypes are needed. In general, such potential difference of alternative color morphs in the ability to thermoregulate might be due to the pleiotropic effects of the melanocortin system instead of glutathione levels and oxidative stress. On the other hand, we found evidence that there is a temperature-related association between father phenotype and offspring oxidative stress. However, this association seems to be environmentally rather than genetically induced.

Conclusions

Our study demonstrates that varying temperatures during the nestling period can interact with male parent melanin coloration to affect oxidative status in males themselves and the offspring they are rearing. Our study is the first one to show that eumelanic individuals might suffer from oxidative stress under stressful conditions in terms of low temperature. Elevated oxidative stress in parents might lead to a poorer ability to take care of the offspring and thus, induce stress in offspring as well. Individuals are constantly affected by changes occurring in environmental conditions. Therefore, it is crucial to study the effects of environmental conditions on physiological as well as ecological processes to better understand population responses to ever-changing environment (e.g. such as human induced climate change).

ACKNOWLEDGEMENTS

We thank Jenna Ruohonen, Kirsi Ukkola, Matias Ukkola and William Velmala for great help in the field, and Meri Lindqvist and Katja Salminen for doing parentage and sexing analyses at the Center of Evolutionary Applications (University of Turku).

COMPETING INTERESTS

No competing interests declared.

FUNDING

Funding for the project was provided by the Academy of Finland (T.L.: project 263651, P.I.: project 135653 and 272713), the Ella and Georg Ehrnrooth Foundation (P.T.), the Turku University Foundation (P.T.).

DATA ACCESSIBILITY

Data will be available from the Dryad Digital Repository.

AUTHORS CONTRIBUTION

T.L., J.S., P.I, C.S., W.S., P.T. designed the study; P.T. C.S., W.S. collected the data; J.S. performed laboratory analyses; P.T. performed statistical analyses; P.T. wrote the first draft of the manuscript, and all other authors contributed substantially to revisions.

REFERENCES

Alonso-Alvarez, C., Bertrand, S., Faivre, B., Chastel, O. and Sorci, G. (2007). Testosterone and oxidative stress: the oxidation handicap hypothesis. Proc.R. Soc. Lond. B. 274, 819–825. https://doi.org/10.1098/rspb.2006.3764

Andersson, M. (1994) Sexual selection.Princeton: Princeton University Press.

Bell, G. (2010). Fluctuating selection: the perpetual renewal of adaptation in variable environments. Phil. Trans. R. Soc. B. 365, 87–97. https://doi.org/10.1098/rstb.2009.0150

Benedetto, J.-P., Ortonne, J.-P., Voulot, C., Khatchadourian, C., Prota, G. and Thivolet, J. (1982). Role of thiol compounds in mammalian melanin pigmentation. II. Glutathione and related enzymatic activities. J. Invest. Derma. 79, 422-424.

Brenner, M. and Hearing, V. J. (2008). The protective role of melanin against UV damage in human skin. Photochem. Photobiol. 84, 539–549. https://doi.org/10.1111/j.1751-1097.2007.00226.x

Byers, D. L. (2005). Evolution in heterogeneous environments and the potential of maintenance of genetic variation in traits of adaptive significance. Genetica 123, 107–124.

Calhim, S., Adamík, P., Järvistö, P., Leskinen, P., Török, J., Wakamatsu, K. and Laaksonen, T. (2014). Heterospecific female mimicry in *Ficedula* flycatchers. J. Evol. Biol. 27, 660–666. https://doi.org/10.1111/jeb.12328

Clutton-Brock, T. H. and Vincent, A. C. J. (1991). Sexual selection and the potential reproductive rates of males and females. Nature 351, 58–60. https://doi.org/10.1038/351058a0

Cornwallis, C. K. and Uller, T. (2010). Towards an evolutionary ecology of sexual traits. Trends Ecol. Evol. 25, 145–152. https://doi.org/10.1016/j.tree.2009.09.008

Costantini, D., Rowe, M., Butler, M. W. and McGraw, K. J. (2010). From molecules to living systems: historical and contemporary issues in oxidative stress and antioxidant ecology. Funct. Ecol. 24, 950–959. https://doi.org/10.1111/j.1365-2435.2010.01746.x

Dale, J. (2006). Intraspecific variation in coloration. In: Hill GE, McGraw KJ (eds) Bird coloration, Vol. II. Harvard University Press, Harvard, pp. 36–86.

Dreiss, A.N., Séchaud, R., Béziers, P., Villain, N., Genoud, M., Almasi, B., Jenni, L. and Roulin, A. (2016). Social huddling and physiological thermoregulation are related to melanism in the nocturnal barn owl. Oecologia 180, 371–381.**Drost, R.** (1936). Über das Brutkleid männlicher Trauerfliegenfänger, *Muscicapa hypoleuca*. Vogelzug 6, 179–186.

Ducrest, A.L., Keller, L. and Roulin, A. (2008). Pleiotropy in the melanocortin system, coloration and behavioural syndromes. Trends Ecol. and Evol. 23, 502–510. https://doi.org/10.1016/j.tree.2008.06.001

Emaresi, G., Henry, I., Gonzales, E., Roulin, A. and Bize, P. (2016). Sex- and melanism-specific variations in the oxidative status of adult tawny owls in response to manipulated reproductive effort. J. Exp. Biol. 219, 73–79. https://doi.org/10.1242/jeb.128959

Fang, Y. Z., Yang, S. and Wu, G. (2002). Free radicals, antioxidants, and nutrition. Nutrition 18, 872–879.

Felton, G. W. and Summers, C. B. (1995) Antioxidant systems in insects. Arch. Insect. Biochem. Physiol. 29, 187–197. https://doi.org/10.1002/arch.940290208

Finkel, T. and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247. https://doi.org/10.1038/35041687

Galván, I. and Alonso-Alvarez, C. (2008). An intracellular antioxidant determines the expression of a melanin-based signal in a bird. PLoS ONE 3, e3335. https://doi.org/10.1371/journal.pone.0003335

Galván, I. and Alonso-Alvarez, C. (2009). The expression of melanin-based plumage is separately modulated by exogenous oxidative stress and a melanocortin. Proc.R. Soc. Lond. B. 276, 3089–3097. https://doi.org/10.1098/rspb.2009.0774

Galván, I. and Solano, F. (2009). The evolution of eu- and pheomelanic traits may respond to an economy of piegments related to environmental oxidative stress. Pigment Cell Melanoma R. 22, :339–342. https://doi.org/10.1111/j.1755-148X.2009.00559.x

Galván, I., Mousseau, T. A. and Møller, A. P. (2011). Bird population declines due to radiation exposure at Chernobyl are stronger in species with pheomelanin-based coloration. Oecologia 16, 827–835. https://doi.org/10.1007/s00442-010-1860-5

Galván, I., Bonisoli-Alquati, A., Jenkinson, S., Ghanem, G., Wakamatsu, K., Mousseau, T. A. and Møller, A. P. (2014). Chronic exposure to low-dose radiation at Chernobyl favours adaptation to oxidative stress in birds. Funct. Ecol. 28, 1387–1403. https://doi.org/10.1111/1365-2435.12283

Goldberg, A. L. and Rock, K. L. (1992). Proteolysis, proteasomes and antigen presentation. Nature 357, 375–378. https://doi.org/10.1038/357375a0

Greenfield, M. D. and Rodriguez, R. L. (2004). Genotype-environment interactions and the reliability of mating signals. Animal Behav. 68, 1461–1468. https://doi.org/10.1111/j.1420-9101.2010.02080.x

Grueber, C. E, Nakagawa, S., Laws R. J. and Jamieson I. G. (2011) Multimodel inference in ecology and evolution: challenges and solutions. J. Evol. Biol. 24, 699–711. https://doi.org/10.1111/j.1420-9101.2010.02210.x

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

Hamilton, W. D. and Zuk, M. (1982). Heritable true fitness and bright birds: A role for parasites? Science 218, 384–386.

Hedrick, P. W. (2006). Genetic polymorphism in heterogeneous environments: The age of genomics. Annu. Rev. Ecol. Evol. Syst. 37, 67–93. https://doi.org/10.1146/annurev.ecolsys.37.091305.110132

Hoelzer, G. A. (1989). The good parent process of sexual selection. Animal Behav. 38, 1067–1078. https://doi.org/10.1016/S0003-3472(89)80146-0

Hõrak, P., Sild, E., Soomets, U., Sepp, T. and Kilk, K. (2010). Oxidative stress and information content of black and yellow plumage coloration: an experiment with greenfinches. J. Exp. Biol. 213, 2225–2233. https://doi.org/10.1242/jeb.042085

Ito, S. (1993). High-performance liquid chromatography (HPLC) analysis of eu- and pheomelanin in melanogenesis control. J. Invest. Depart. 100, 166–171. https://doi.org/10.1038/jid.1993.71.

Jacquin, L., Récapet, C., Bouche, P., Leboucher, G. and Gasparini, J. (2012). Melanin-based coloration reflects alternative strategies to cope with food limitation in pigeons. Behav. Ecol. 23, 907–915. https://doi.org/10.1093/beheco/ars055

Järvistö, P. E., Calhim, S., Schuett, W., Velmala, W. and Laaksonen, T. (2015). Foster, but not genetic, father plumage coloration has a temperature-dependent effect on offspring quality. Behav. Ecol. Sociobiol.69, 335–346. https://doi.org/10.1007/s00265-014-1846-0

Kampranis, S. C., Damianova, R., Atallah, M., Toby, G., Kondi, G., Tsichlis, P. N. and Makris, A. M. (2000). A novel plant glutathione s-transferase/peroxidase suppresses bax lethality in yeast. J. of Biol.Chem. 275, 29207–29216. https://doi.org/10.1074/jbc.M002359200

Kaplowitz, N. (1981). The importance and regulation of hepatic gluthatione. Yale J. Biol. Med.54, 497–502.

Kassen, R. (2002). The experimental evolution of specialists, generalists, and the maintenance of diversity. J. Evol. Biol. 15, 173–190. https://doi.org/10.1046/j.1420-9101.2002.00377.x

Kim, D.-S., Park, S.-H., Kwon, S.-B., Joo, Y.-H., Youn, S.-W., Sohn, U.-D. (2003). Temperature regulates melanin synthesis in melanocytes. Arch. Pharm. Res. 26, 840. https://doi.org/10.1007/BF02980030

Laaksonen, T., Sirkiä, P. M., Calhim, S., Brommer, J. E., Leskinen, P. K., Primmer, C. R. et al. (2015). Sympatric divergence and clinal variation in multiple coloration traits of Ficedula flycatchers. J. Evol. Biol. 28, 779–790. https://doi.org/10.1111/jeb.12604.

Lehtonen, P. K., Primmer, C. R. and Laaksonen, T. (2009). Different traits affect gain of extrapair paternity and loss of paternity in the pied flycatcher, *Ficedula hypoleuca*. Animal Behav. 77, 1103–1110. https://doi.org/10.1016/j.anbehav.2009.01.014

Lundberg, A. and Alatalo, R. V. (1992). The pied flycatcher. T and AD Poyser, London

McGraw, K. J. (2005). The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants? Animal Behav. 69, 757–764. https://doi.org/10.1016/j.anbehav.2004.06.022

McGraw, K. J. (2006). Mechanics of melanin-based coloration. In: Hill, G. E., and McGraw, K. J. (eds) Bird coloration, Vol. I. Harvard University Press, Harvard, pp. 243–294.

Meza, F. and Varas, E. (2000). Estimation of mean monthly solar global radiation as a function of temperature. Agric For Meteorol. 100, 231–241. https://doi.org/10.1016/S0168-1923(99)00090-8

Mitrus, C. (2007). Is the later arrival of young male red-breasted flycatchers (Ficedula parva) related to their physical condition? J.Ornithol. 148, 53–58.

Moreno, J., Velando, A., González-Braojos, S., Ruiz-de-Castañeda R.,and Cantarero, A. (2013). Femaled paired with more attractive males show reduced oxidative damage: possible direct benefits of mate choice in pied flycatchers. Ethology 119, 727–737. https://doi.org/10.1111/eth.12112

Natarajan, V. T., Ganju, P., Ramkumar, A., Grover, R. and Gokhale, R. S. (2014). Multifaceted pathways protect human skin from UV radiation. Nature Chem. Biol. 10, 542–551. https://doi.org/10.1038/nchembio.1548

Piault, R., Gasparini, J., Bize, P., Jenni-Eiermann, S. and Roulin, A. (2009). Pheomelanin-based coloration and the ability to cope with variation in food supply and parasitism. Am. Nat. 174, 548–556. https://doi.org/10.1086/605374

Pomiankowski, A. and Møller, A. P. (1995). A resolution of the lek paradox. Proc. R. Soc. Lond. B. 260, 21–29 https://doi.org/10.1098/rspb.1995.0054

Qvarnström, A. (2001). Context-dependent genetic benefits from mate choice. Trends Ecol. Evol. 16, 5–7. https://doi.org/10.1016/S0169-5347(00)02030-9

Rainio, M. J., Eeva, T., Lilley, T., Stauffer, J. and Ruuskanen, S. (2015). Effects of early-life lead exposure on oxidative status and phagocytosis activity in great tits (*Parus major*). Comp. Biochem. Physiol. C. 167, 24–34. https://doi.org/10.1016/j.cbpc.2014.08.004

R Core Development Team (2018). R: *A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Rodríguez, R. L., Rebar, D. and Fowler-Finn, K. D. (2013). The evolution and evolutionary consequences of social plasticity in mate preferences. Animal Behav. 85,1041–1047. https://doi.org/10.1016/j.anbehav.2013.01.006

Røskaft, E., Järvi, T., Bakken M., Bech, C. and Reinersten R. E. (1986) The relationship between social status and resting metabolic rate in great tits (*Parus major*) and pied flycatchers (*Ficedula hypoleuca*). Animal Behav. 34, 838–842. https://doi.org/10.1016/S0003-3472(86)80069-0

Roulin, A., Almasi, B., Meichtry-Stier, K. S. and Jenni, L. (2011). Eumelanin- and pheomelanin-based colour advertise resistance to oxidative stress in opposite ways. J.Evol. Biol. 24, 2241–2247. https://doi.org/10.1111/j.1420-9101.2011.02353.x

Roulin, A. (2014). Melanin-based colour polymorphism responding to climate change. Global Change Biol. 20, 3344–3350. https://doi.org/10.1111/gcb.12594

Roulin, A. (2015). Condition-dependence, pleiotropy and the handicap principle of sexual selection in melanin-based colouration. Biol. Rev. 91, 328–348. https://doi.org/10.1111/brv.12171

Ruuskanen, S. and Laaksonen, T. (2010). Yolk androgens have sex-specific effects on behavior in the pied flycatcher *Ficedula hypoleuca*. Hormones and Behavior 57, 119–127. https://doi.org/10.1016/j.yhbeh.2009.09.017

Schuett, W., Teerikorpi, P. E., Calhim, S., Velmala, W. and Laaksonen, T. (2017). Nosy neighbours: large broods attract more visitors. A field experiment in the pied flycatcher, *Ficedula hypoleuca*. Oecologia 184, 1-12. https://doi.org/10.1007/s00442-0173849-9.

Sirkiä, P. M., Virolainen, M. and Laaksonen, T. (2010). Melanin coloration has temperature-dependent effects on breeding performance that may maintain phenotypic variation in a passerine bird. J.Evol. Biol. 23, 2385–2396. https://doi.org/10.1111/j.1420-9101.2010.02100.x

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. Analytical Biochem. 150, 76–85.

Solano, F. (2014). Melanins: skin pigments and much more-types, structural models, biological functions, and formation routes. New J. Science ID 498276. http://dx.doi.org/10.1155/2014/498276

Surai, P. F. (2002). Natural Antioxidants in Avian Nutrition and Reproduction. Nottingham University Press, Nottingham, UK.

Stauffer, J., Panda, B., Eeva, T., Rainio, M. and Ilmonen, P. (2017). Telomere damage and redox status alterations in free-living passerines exposed to metals. Science Total Env. 575, 841–848. https://doi.org/10.1016/j.scitotenv.2016.09.131

Stier, A., Massemin, S., Zahn, S., Tissier, M. L. and Criscuolo, F. (2015). Starting with a handicap: effects of asynchronous hatching on growth rate, oxidative stress and telomere dynamics in free-living great tits. Oecologia 179, 999–1010. https://doi.org/10.1007/s00442-015-3429-9

Strange, R. C., Spiteri, M. A., Ramachandran, S. and Fryer, A. A. (2001). Glutathione-S-transferase family of enzymes. Mut. Res. 482, 21–26.

Svensson, L. (1992). Identification Guide to European Passerines. Märsta Press, Stockholm.

Walsh, B. and Blows, M. W. (2009) Abundant genetic variation + strong selection = multivariate genetic constraints: a geometric view of adaptation. Annu. Rev. Ecol. Evol. Sys. 40, 41–59. https://doi.org/10.1146/annurev.ecolsys.110308.120232

White, C. R., Blackburn, T. M., Martin, G. R. and Butler, P. J. (2007) Basal metabolic rate of birds is associated with habitat temperature and precipitation, not primary productivity. Proc. R. Soc. Lond. B. 274, 287–293. https://doi.org/10.1098/rspb.2006.3727

Williams, J. B. and Tieleman, B. I. (2000). Flexibility in basal metabolic rate and evaporative water loss among hoopoe larks exposed to different environmental temperatures. J. Exp. Biol. 203, 3153–3159.

Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R. and Turner, N. D. (2004). Glutathione metabolism and its implications for health. J. Nutr. 134, 489–492. https://doi.org/10.1093/jn/134.3.489

Zahavi, A. (1975). Mate selection – A selection for a handicap. J. Theor. Biol. 53, 205–214. https://doi.org/10.1016/0022-5193(75)90111-3

Figures

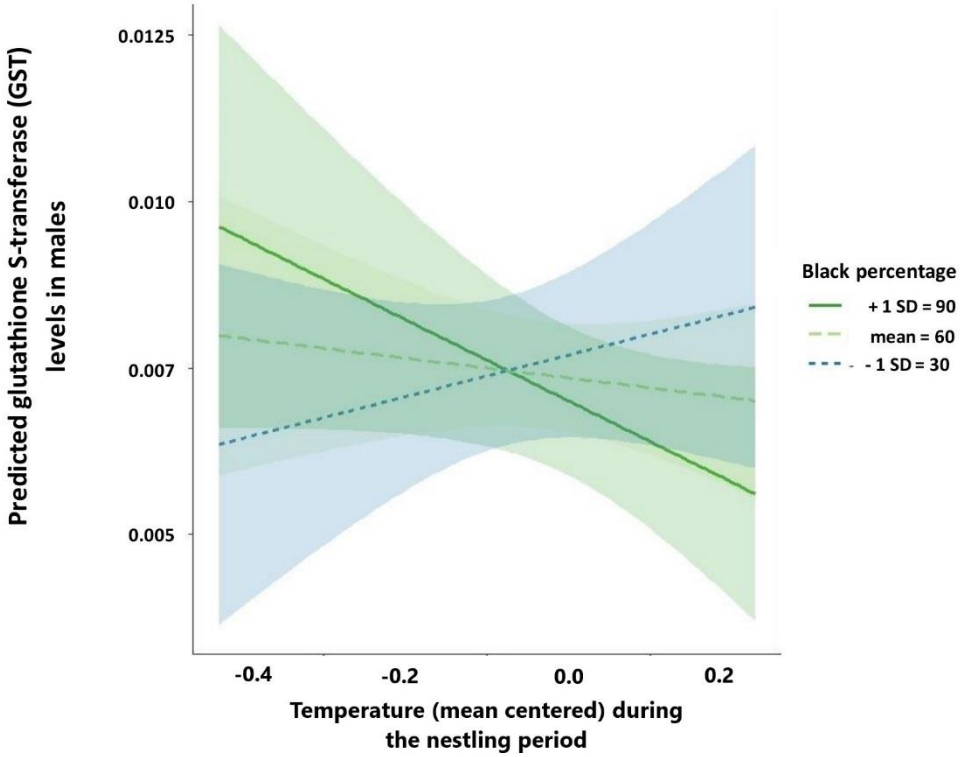

Fig 1. Predicted glutathione S-transferase (GST) levels in males is relation to male dorsal melanin colouration and temperature during the nestling period. Model predictions derived from the linear model are given for males with different proportion of black in their plumage; the different lines represent (····) 30 %, (- - -) 60 % and (—) 90 % of black feathers on the dorsal side, which was treated as a continuous variable in the analyses. P = 0.022, N=55

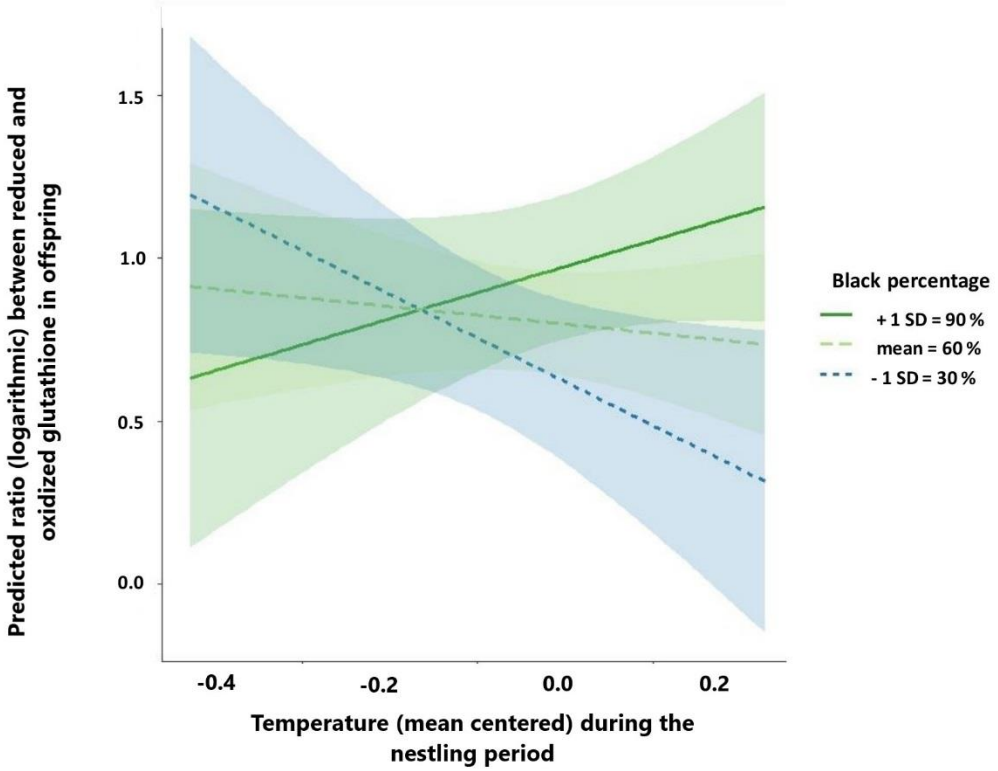

Fig 2. Predicted ratio between reduced and oxidized glutathione (GSHratio) of foster-offspring as a function of temperature during the nestling period. Model predictions derived from the linear model are given for foster males with different proportion of black in their plumage; the different lines represent (····) 30 %, (- - -) 60 % and (—) 90 % of black feathers on the dorsal side, which was treated as a continuous variable in the analyses. P=0.0095, N=38

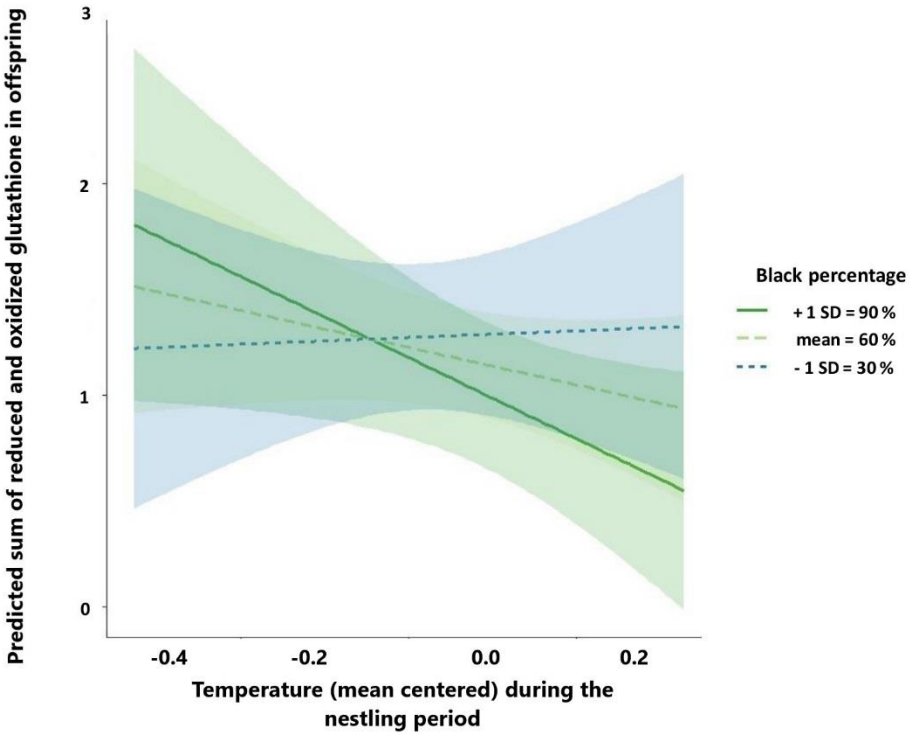

Fig 3. Predicted total amount of glutathione (GSHtot) of foster-offspring as a function of temperature during the nestling period. Model predictions derived from the linear model are given for foster males with different proportion of black in their plumage; the different lines represent (····) 30 %, (- - -) 60 % and (—) 9 0 % of black feathers on the dorsal side, which was treated as a continuous variable in the analyses. P=0.044, N=38

Tables

Table 1

Output of linear models on the oxidative status of males in relation to male melanin colouration and average temperature of the nestling period and their interactions. Oxidative status is measured as levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), glutathione S-transferase (GST), oxidative damage in proteins (CARB), total amount of glutathione (GSHtot) and ratio between reduced and oxidized glutathione (GSHratio). N=55

SOD		Estimate	SE	DF	F	P
Intercept		80.77	6.56			
Age				1,39	0.63	0.54
	old-young	-1.76	2.67			
Clutch size		-0.50	1.00	1,39	0.25	0.62
Blackness		0.041	0.029	1,39	1.96	0.17
BSM				2,39	1.14	0.33
	enlarged-control	-1.06	1.11			
	reduced-control	-1.01	1.24			
Temperature		-3.57	3.95	1,39	1.72	0.38
Removed:						
Temp X blackness		0.058	0.13	1,38	0.18	0.67

CAT		Estimate	SE	DF	F	P
Intercept		11.88	8.08			
Age				1,41	0.50	0.61
	old-young	-1.71	2.16			
Clutch size		-0.64	1.25	1,41	0.26	0.61
Blackness (social male)		0.054	0.037	1,41	2.18	0.15
BSM				2,41	0.99	0.38
	enlarged-control	-1.90	1.40			
	reduced-control	0.64	1.53			
Temperature		-4.19	4.87	1,41	0.74	0.39
Removed:						
Temp X blackness				1,40	0.12	0.73

GP		Estimate	SE	DF	F	P
Intercept		0.02	0.0074			
Age						
	old-young	0.0035	0.0031	1,40	0.38	0.69
Clutch size		-0.00049	0.0011	1,40	0.19	0.67
Blackness (social male)		0.0000083	0.000034	1,40	0.06	0.81
BSM				2,40	0.031	0.97
	enlarged-control	-0.00025	0.0013			
	reduced-control	0.00033	0.0011			

Temperature		-0.0058	0.0015	1,40	1.68	0.20
Removed:						
Temp X blackness				1,39	0.27	0.60
GST		Estimate	SE	DF	F	P
Intercept		0.011	0.0034			
Age				1,39	4278	**0.074**
	old-young	0.0020	0.00084			
Clutch size		-0.00047	0.00051	1,39	0.85	0.36
Blackness		0.0000040	0.000014	1,39	0.078	0.78
BSM				2,39	0,88	0.42
	enlarged-control	0.00042	0.00056			
	reduced-control	0.00080	0.00060			
Temperature		0.0078	0.0043	1,39	3.26	0.078
Temp X blackness		-0.00015	0.000064	1,39	5.69	**0.022**
GSHtot		Estimate	SE	DF	F	P
Intercept		0.81	0.84			
Age				1,40	0.29	0.75
	old-young	-0.21	0.35			
Clutch size		0.012	0.13	1,40	0.0085	0.93
Blackness		-0.0012	0.0038	1,40	0.10	0.75
BSM				2,40	1.56	0.22
	enlarged-control	-0.17	0.15			
	reduced-control	-0.27	0.16			
Temperature		-0.29	0.51	1,40	0.33	0.57
Removed:						
Temp X blackness				1,39	0.51	0.48
GSHratio		Estimate	SE	DF	F	P
Intercept		0124	0.30			
Age				1,40	0.32	0.73
	old-young	-0.052	0.079			
Clutch size		-0.056	0.047	1,40	1.46	0.23
Blackness		0.00051	0.0014	1,40	0.14	0.71
BSM				2,40	0.90	0.42
	enlarged-control	0.063	0.053			
	reduced-control	-0.065	0.057			
Temperature		0.056	0.18	1,40	0.09	0.76
Removed:						
Temp X blackness				1,39	2.09	0.16
CARB		Estimate	SE	DF	F	P
Intercept		0.12	0.11			
Age						
	old-young	0.018	0.029	1,39	0.01	0.91

Clutch size		0.0038	0.017	1,39	0.04	0.84
Blackness		-0.00040	0.00050	1,39	0.62	0.44
BSM				2,39	0.68	0.51
	enlarged-control	0.021	0.019			
	reduced-control	-0.021	0.021			
Temperature		-0.11	-0.069	1,39	2.59	0.12
Removed:						
Temp X blackness				1,38	0.12	0.73

Table 2

Output of linear models on the oxidative status of nestlings in relation to biological father melanin coloration, temperature during the nestling period and their interactions. Oxidative status is measured as levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), glutathione S-transferase (GST), oxidative damage in proteins (CARB), total amount of glutathione (GSHtot) and ratio between reduced and oxidized glutathione (GSHratio). N=34

SOD	Estimate	SE	DF	F	P
Intercept	39.53	22.75			
Sex			1,20	2.30	0.15
Female-male	8.73	4.89			
Body mass	2152	1.65	1,20	2.09	0.16
Blackness (biol father)	0.042	0.048	1,20	0.79	0.38
BSM			1,20	0.059	0.81
enlarged-reduced	-0.78	3.18			
Temperature	-15.22	7.64	1,20	3.97	**0.059**
Removed:					
Temp X blackness			1,19	0.58	0.46

CAT	Estimate	SE	DF	F	P
Intercept	9.49	31.15			
Sex			1,20	0.33	0.73
Female-male	5.18	6.63			
Body mass	-0.11	2.25	1,20	0.0022	0.96
Blackness (biol father)	-0.046	0.064	1,20	0.52	0.48
BSM			1,20	0.024	0.88
enlarged-reduced	-0.66	4.30			
Temperature	11.04	10.38	1,20	1.13	0.30
Removed:					
Temp X blackness			1,19	0.0003	0.99

GP	Estimate	SE	DF	F	P
Intercept	-0.0029	0.011			
Sex			1,20	2.28	0.13
Female-male	-0.0024	0.0024			
Body mass	0.00072	0.00082	1,20	0.78	0.39
Blackness (biol father)	0.000016	0.000022	1,20	0.52	0.48
BSM					
enlarged-reduced	-0.00077	0.0015	1,20	0.25	0.62
Temperature	0.00026	0.0037	1,20	0.0053	0.94
Removed:					
Temp X blackness			1,19	0.33	0.57

GST	Estimate	SE	DF	F	P
Intercept	0.013	0.0082			

Sex			1,21	0.76	0.48
Female-male	0.0019	0.0018			
Body mass	0.0012	0.00059	1,21	4.24	0.078
Blackness (biol father)	0.0000019	0.000016	1,21	0.014	0.91
BSM			1,21	0.51	0.48
enlarged-reduced	0.00078	0.0011			
Temperature	-0.0036	0.0027	1,21	1.78	0.20
Removed:					
Temp X blackness			1,20	0.14	0.89

GSHtot	Estimate	SE	DF	F	P
Intercept	-0.13	2.7300			
Sex			1,21	1.16	0.33
Female-male	0.76	0.58			
Body mass	0.019	0.20	1,21	0.009	0.93
Blackness (biol father)	0.0046	0.0054	1,21	0.71	0.41
BSM			1,21	0.93	0.35
enlarged-reduced	-0.36	0.37			
Temperature	-0.94	0.89	1,21	1-12	0.30
Removed:					
Temp X blackness			1,20	1.18.00	0.29

GSHratio	Estimate	SE	DF	F	P
Intercept	-0.45	1.41			
Sex			1,18	2.82	0.084
Female-male	0.46	0.30			
Body mass	0.12	0.10	1,18	1.46	0.24
Blackness (biol father)	-0.0094	0.0028	1,18	11.12	0.0033
BSM			1,18	5.76	**0.026**
enlarged-reduced	0.48	0.20			
Temperature	0.35	0.	1,18	0.75	0.46
Removed:					
Temp X blackness	-0.023	0.012	1,18	3.82	0.077

CARB	Estimate	SE	DF	F	P
Intercept	0.021	0.26			
Sex			1,19	1.095	0.35
Female-male	0.020	0.055			
Body mass	0.0047	0.019	1,19	0.061	0.81
Blackness (biol father)	-0.000053	0.00055	1,19	0.0093	0.92
BSM			1,19	0.015	0.90
enlarged-reduced	-0.0045	0.038			
Temperature	-0.050	0.088	1,19	0.32	0.58
Removed:					
Temp X blackness			1,18	1.43	0.25

Table 3.

Output of linear models on the oxidative status of nestlings in relation to foster father melanin coloration, temperature during the nestling period and their interactions. Oxidative status is measured as levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), glutathione S-transferase (GST), oxidative damage in proteins (CARB), total amount of glutathione (GSHtot) and ratio between reduced and oxidized glutathione (GSHratio). N=38

SOD		Estimate	SE	DF	F	P
Intercept		46.95	20.97			
Sex				1,20	4.41	**0.023**
	Female-male	6.84	2.97			
Body mass		1.28	1.49	1,20	0.74	0.40
Blackness (foster father)		0.0091	0.035	1,20	0.069	0.79
BSM				1,20	0.03	0.86
	enlarged-reduced	-0.42	2.41			
Temperature		-11.26	5.71	1,20	3.60	0.069
Removed:						
Temp X blackness				1,19	0.018	0.89
CAT		Estimate	SE	DF	F	P
Intercept		8.90	27.25			
Sex				1,20	2.0400	0.15
	Female-male	7.28	3.77			
Body mass		-0.62	1.94	1,20	0.10	0.75
Blackness (foster father)		0.059	0.044	1,20	1.74	0.20
BSM				1,20	0.076	0.79
	enlarged-reduced	-0.85	3.11			
Temperature		4.42	7.41	1,20	0.36	0.56
Removed:						
Temp X blackness				1,19	1.035	0.32
GP		Estimate	SE	DF	F	P
Intercept		-0.0021	0.011			
Sex				1,20	2.023	0.15
	Female-male	0.0017	0.0015			
Body mass		0.00047	0.00077	1,20	0.38	0.54
Blackness (foster father)		0.00000082	0.000019	1,20	0.0019	0.97
BSM				1,20	0.79	0.38
	enlarged-reduced	0.00011	0.0013			
Temperature		0.00049	0.0030	1,20	0.027	0.87
Removed:						
Temp X blackness				1,19	0.94	0.34
GST		Estimate	SE	DF	F	P
Intercept		-0.012	0.0081			

Sex				1,21	0.80	0.46
	Female-male	0.0015	0.0011			
Body mass		0.0012	0.00058	1,21	3.96	0.074
Blackness (foster father)		0.0000078	0.000013	1,21	0.34	0.56
BSM				1,21	0.54	0.47
	enlarged-reduced	0.00068	0.00093			
Temperature		-0.0025	0.0022	1,21	1.40	0.25
Removed:						
Temp X blackness				1,20	0.57	0.46

GSHtot		Estimate	SE	DF	F	
Intercept		-0.28	2.19			
Sex				1,19	2.42	0.11
	Female-male	0.66	0.30			
Body mass		0.070	0.16	1,19	0.20	0.66
Blackness (foster father)		-0.0046	0.0038	1,19	1.50	0.23
BSM				1,19	2.96	0.098
	enlarged-reduced	-0.43	0.25			
Temperature		0.80	1.10	1,19	0.53	0.47
Temp X blackness		-0.033	0.016	1,19	4.50	**0.044**

GSHratio		Estimate	SE	DF	F	P
Intercept		-0.48	1.42			
Sex				1,18	1.17	0.33
	Female-male	0.29	0.20			
Body mass		0.070	0.10	1,18	0.47	0.50
Blackness (foster father)		0.0055	0.0026	1,18	4.65	0.042
BSM						
	enlarged-reduced	0.36	0.17	1,18	4.78	0.075
Temperature		-1.65	0.72	1,18	6.99	0.017
Temp X blackness		0.029	0.010	1,18	8.00	**0.0095**

CARB		Estimate	SE	DF	F	P
Intercept		0.024	0.24			
Sex				1,19	1.38	0.27
	Female-male	0.037	0.035			
Body mass		0.0041	0.018	1,19	0.010	0.92
Blackness (foster father)		0.0017	0.0017	1,19	0.10	0.75
BSM				1,19	0.25	0.63
	enlarged-reduced	-0.026	0.027			
Temperature		-0.062	0.063	1,19	0.96	0.34
Removed:						
Temp X blackness				1,18	1.27	0.27

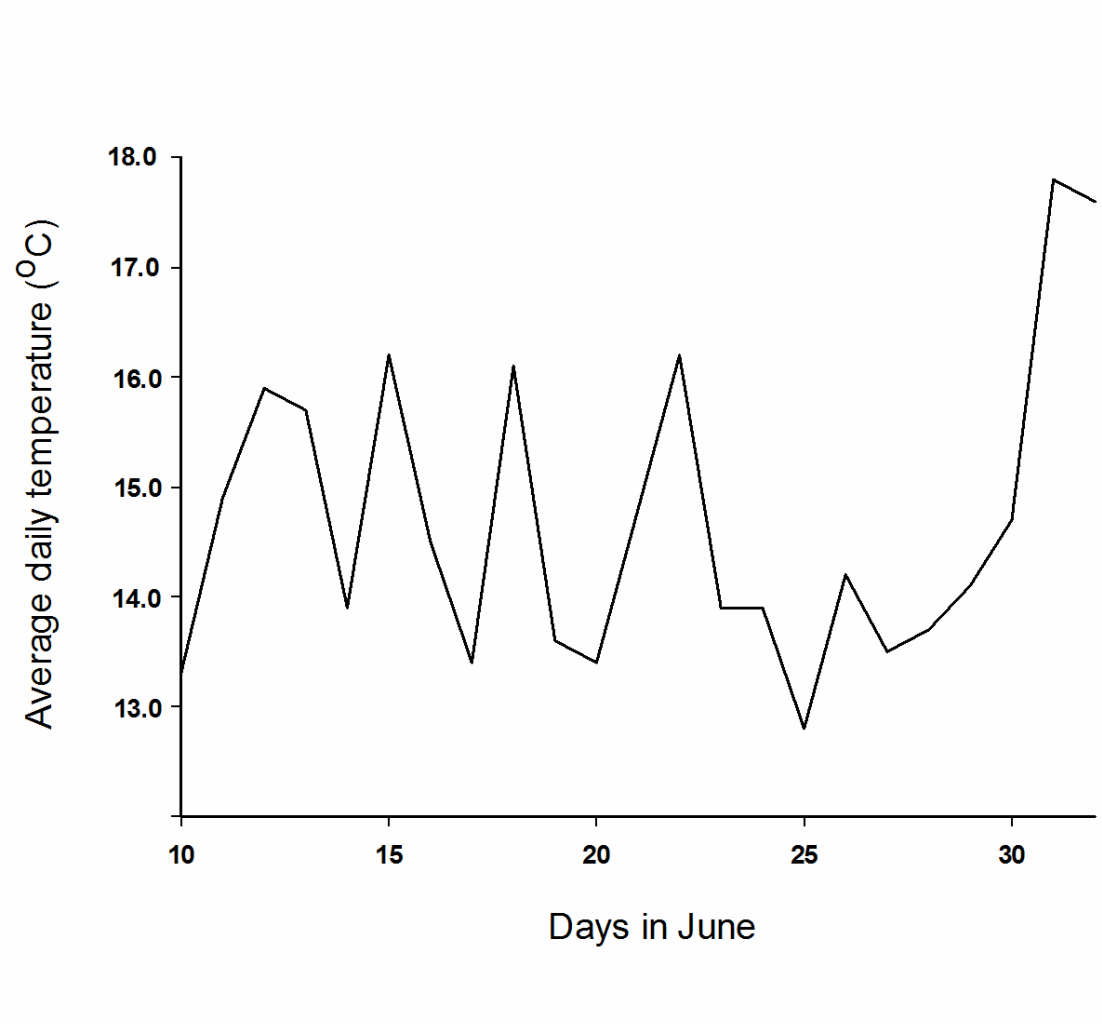

Fig. S1.

The distribution of average daily temperatures across the study period. Date range is based on the date when the first experimental nest had 4 day-old-nestlings (10 of June) to the date when the last experimental nest had 11 day-old-nestlings (2 of July)

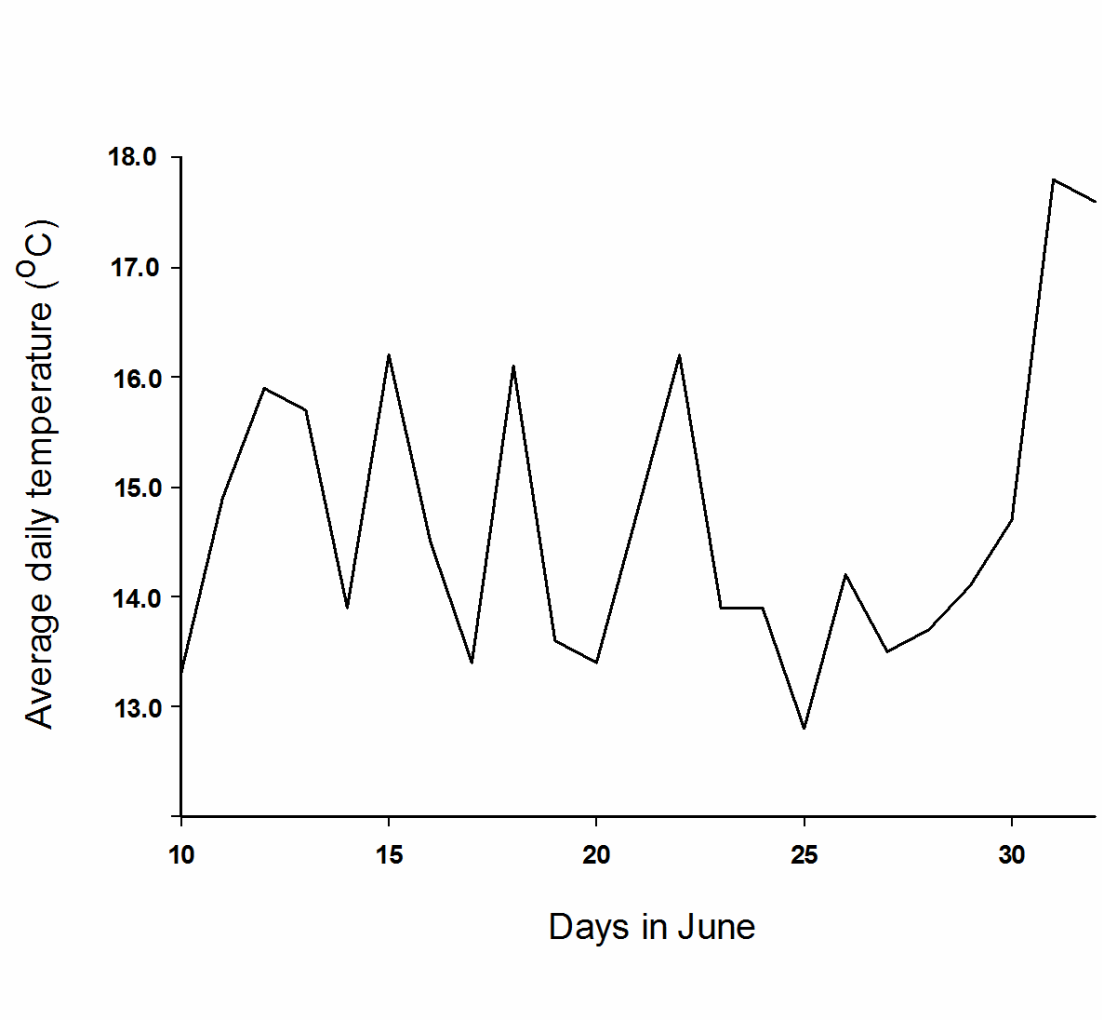

Fig. S1.

The distribution of average daily temperatures across the study period. Date range is based on the date when the first experimental nest had 4 day-old-nestlings (10 of June) to the date when the last experimental nest had 11 day-old-nestlings (2 of July)

Table S1. Output of linear models on the oxidative status of males in relation to male melanin colouration and average temperature of the nestling period and their interactions

	Estimate	s.e.	d.f.	F	P
SOD					
Intercept	80.77	6.56			
Age			1,39	0.63	0.54
Old-young	−1.76	2.67			
Clutch size	−0.50	1.00	1,39	0.25	0.62
Blackness	0.041	0.029	1,39	1.96	0.17
BSM			2,39	1.14	0.33
Enlarged-control	−1.06	1.11			
Reduced-control	−1.01	1.24			
Temperature	−3.57	3.95	1,39	1.72	0.38
Removed:					
Temp × blackness	0.058	0.13	1,38	0.18	0.67
CAT					
Intercept	11.88	8.08			
Age			1,41	0.50	0.61
Old-young	−1.71	2.16			
Clutch size	−0.64	1.25	1,41	0.26	0.61
Blackness (social male)	0.054	0.037	1,41	2.18	0.15
BSM			2,41	0.99	0.38
Enlarged-control	−1.90	1.40			
Reduced-control	0.64	1.53			
Temperature	−4.19	4.87	1,41	0.74	0.39
Removed:					
Temp × blackness			1,40	0.12	0.73

	Estimate	s.e.	d.f.	F	P
GP					
Intercept	0.02	0.0074			
Age					
Old-young	0.0035	0.0031	1,40	0.38	0.69
Clutch size	−0.00049	0.0011	1,40	0.19	0.67
Blackness (social male)	0.0000083	0.000034	1,40	0.06	0.81
BSM			2,40	0.031	0.97
Enlarged-control	−0.00025	0.0013			
Reduced-control	0.00033	0.0011			
Temperature	−0.0058	0.0015	1,40	1.68	0.20
Removed:					
Temp × blackness			1,39	0.27	0.60
GST					
Intercept	0.011	0.0034			
Age			1,39	4278	0.074
Old-young	0.0020	0.00084			
Clutch size	−0.00047	0.00051	1,39	0.85	0.36
Blackness	0.0000040	0.000014	1,39	0.078	0.78
BSM			2,39	0,88	0.42
Enlarged-control	0.00042	0.00056			
Reduced-control	0.00080	0.00060			
Temperature	0.0078	0.0043	1,39	3.26	0.078
Temp × blackness	−0.00015	0.000064	1,39	5.69	0.022
GSHtot					
Intercept	0.81	0.84			
Age			1,40	0.29	0.75

	Estimate	s.e.	d.f.	F	P
Old-young	−0.21	0.35			
Clutch size	0.012	0.13	1,40	0.0085	0.93
Blackness	−0.0012	0.0038	1,40	0.10	0.75
BSM			2,40	1.56	0.22
Enlarged-control	−0.17	0.15			
Reduced-control	−0.27	0.16			
Temperature	−0.29	0.51	1,40	0.33	0.57
Removed:					
Temp × blackness			1,39	0.51	0.48
GSHratio					
Intercept	0124	0.30			
Age			1,40	0.32	0.73
Old-young	−0.052	0.079			
Clutch size	−0.056	0.047	1,40	1.46	0.23
Blackness	0.00051	0.0014	1,40	0.14	0.71
BSM			2,40	0.90	0.42
Enlarged-control	0.063	0.053			
Reduced-control	−0.065	0.057			
Temperature	0.056	0.18	1,40	0.09	0.76
Removed:					
Temp × blackness			1,39	2.09	0.16
CARB					
Intercept	0.12	0.11			
Age					
Old-young	0.018	0.029	1,39	0.01	0.91
Clutch size	0.0038	0.017	1,39	0.04	0.84

	Estimate	s.e.	d.f.	F	P
Blackness	−0.00040	0.00050	1,39	0.62	0.44
BSM			2,39	0.68	0.51
Enlarged-control	0.021	0.019			
Reduced-control	−0.021	0.021			
Temperature	−0.11	−0.069	1,39	2.59	0.12
Removed:					
Temp × blackness			1,38	0.12	0.73

Oxidative status is measured as levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), glutathione S-transferase (GST), oxidative damage in proteins (CARB), total amount of glutathione (GSHtot) and ratio between reduced and oxidized glutathione (GSHratio). N=55.

Table S2. Output of linear models on the oxidative status of nestlings in relation to biological father melanin coloration, temperature during the nestling period and their interactions

	Estimate	s.e.	d.f.	F	P
SOD					
Intercept	39.53	22.75			
Sex			1,20	2.30	0.15
Female-male	8.73	4.89			
Body mass	2152	1.65	1,20	2.09	0.16
Blackness (biol father)	0.042	0.048	1,20	0.79	0.38
BSM			1,20	0.059	0.81
Enlarged-reduced	−0.78	3.18			
Temperature	−15.22	7.64	1,20	3.97	0.059
Removed:					
Temp × blackness			1,19	0.58	0.46
CAT					
Intercept	9.49	31.15			
Sex			1,20	0.33	0.73
Female-male	5.18	6.63			
Body mass	−0.11	2.25	1,20	0.0022	0.96
Blackness (biol father)	−0.046	0.064	1,20	0.52	0.48
BSM			1,20	0.024	0.88
Enlarged-reduced	−0.66	4.30			
Temperature	11.04	10.38	1,20	1.13	0.30
Removed:					
Temp × blackness			1,19	0.0003	0.99
GP					
Intercept	−0.0029	0.011			

	Estimate	s.e.	d.f.	F	P
Sex			1,20	2.28	0.13
Female-male	−0.0024	0.0024			
Body mass	0.00072	0.00082	1,20	0.78	0.39
Blackness (biol father)	0.000016	0.000022	1,20	0.52	0.48
BSM					
Enlarged-reduced	−0.00077	0.0015	1,20	0.25	0.62
Temperature	0.00026	0.0037	1,20	0.0053	0.94
Removed:					
Temp × blackness			1,19	0.33	0.57
GST					
Intercept	0.013	0.0082			
Sex			1,21	0.76	0.48
Female-male	0.0019	0.0018			
Body mass	0.0012	0.00059	1,21	4.24	0.078
Blackness (biol father)	0.0000019	0.000016	1,21	0.014	0.91
BSM			1,21	0.51	0.48
Enlarged-reduced	0.00078	0.0011			
Temperature	−0.0036	0.0027	1,21	1.78	0.20
Removed:					
Temp × blackness			1,20	0.14	0.89
GSHtot					
Intercept	−0.13	2.7300			
Sex			1,21	1.16	0.33
Female-male	0.76	0.58			
Body mass	0.019	0.20	1,21	0.009	0.93
Blackness (biol father)	0.0046	0.0054	1,21	0.71	0.41

	Estimate	s.e.	d.f.	F	P
BSM			1,21	0.93	0.35
Enlarged-reduced	−0.36	0.37			
Temperature	−0.94	0.89	1,21	1-12	0.30
Removed:					
Temp × blackness			1,20	1.18.00	0.29
GSHratio					
Intercept	-0.45	1.41			
Sex			1,18	2.82	0.084
Female-male	0.46	0.30			
Body mass	0.12	0.10	1,18	1.46	0.24
Blackness (biol father)	-0.0094	0.0028	1,18	11.12	0.0033
BSM			1,18	5.76	0.026
Enlarged-reduced	0.48	0.20			
Temperature	0.35	0.	1,18	0.75	0.46
Removed:					
Temp × blackness	-0.023	0.012	1,18	3.82	0.077
CARB					
Intercept	0.021	0.26			
Sex			1,19	1.095	0.35
Female-male	0.020	0.055			
Body mass	0.0047	0.019	1,19	0.061	0.81
Blackness (biol father)	-0.000053	0.00055	1,19	0.0093	0.92
BSM			1,19	0.015	0.90
Enlarged-reduced	-0.0045	0.038			
Temperature	-0.050	0.088	1,19	0.32	0.58
Removed:					

	Estimate	s.e.	d.f.	F	P
Temp × blackness			1,18	1.43	0.25

Oxidative status is measured as levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), glutathione S-transferase (GST), oxidative damage in proteins (CARB), total amount of glutathione (GSHtot) and ratio between reduced and oxidized glutathione (GSHratio). N=34.

Table S3. Output of linear models on the oxidative status of nestlings in relation to foster father melanin coloration, temperature during the nestling period and their interactions

	Estimate	s.e.	d.f.	F	P
SOD					
Intercept	46.95	20.97			
Sex			1,20	4.41	0.023
Female-male	6.84	2.97			
Body mass	1.28	1.49	1,20	0.74	0.40
Blackness (foster father)	0.0091	0.035	1,20	0.069	0.79
BSM			1,20	0.03	0.86
Enlarged-reduced	−0.42	2.41			
Temperature	−11.26	5.71	1,20	3.60	0.069
Removed:					
Temp × blackness			1,19	0.018	0.89
CAT					
Intercept	8.90	27.25			
Sex			1,20	2.0400	0.15
Female-male	7.28	3.77			
Body mass	−0.62	1.94	1,20	0.10	0.75
Blackness (foster father)	0.059	0.044	1,20	1.74	0.20
BSM			1,20	0.076	0.79
Enlarged-reduced	−0.85	3.11			
Temperature	4.42	7.41	1,20	0.36	0.56
Removed:					
Temp × blackness			1,19	1.035	0.32
GP					
Intercept	−0.0021	0.011			

	Estimate	s.e.	d.f.	F	P
Sex			1,20	2.023	0.15
Female-male	0.0017	0.0015			
Body mass	0.00047	0.00077	1,20	0.38	0.54
Blackness (foster father)	0.00000082	0.000019	1,20	0.0019	0.97
BSM			1,20	0.79	0.38
Enlarged-reduced	0.00011	0.0013			
Temperature	0.00049	0.0030	1,20	0.027	0.87
Removed:					
Temp × blackness			1,19	0.94	0.34
GST					
Intercept	−0.012	0.0081			
Sex			1,21	0.80	0.46
Female-male	0.0015	0.0011			
Body mass	0.0012	0.00058	1,21	3.96	0.074
Blackness (foster father)	0.0000078	0.000013	1,21	0.34	0.56
BSM			1,21	0.54	0.47
Enlarged-reduced	0.00068	0.00093			
Temperature	−0.0025	0.0022	1,21	1.40	0.25
Removed:					
Temp × blackness			1,20	0.57	0.46
GSHtot					
Intercept	−0.28	2.19			
Sex			1,19	2.42	0.11
Female-male	0.66	0.30			
Body mass	0.070	0.16	1,19	0.20	0.66
Blackness (foster father)	−0.0046	0.0038	1,19	1.50	0.23

	Estimate	s.e.	d.f.	F	P
BSM			1,19	2.96	0.098
Enlarged-reduced	−0.43	0.25			
Temperature	0.80	1.10	1,19	0.53	0.47
Temp × blackness	−0.033	0.016	1,19	4.50	0.044
GSHratio					
Intercept	−0.48	1.42			
Sex			1,18	1.17	0.33
Female-male	0.29	0.20			
Body mass	0.070	0.10	1,18	0.47	0.50
Blackness (foster father)	0.0055	0.0026	1,18	4.65	0.042
BSM					
Enlarged-reduced	0.36	0.17	1,18	4.78	0.075
Temperature	−1.65	0.72	1,18	6.99	0.017
Temp × blackness	0.029	0.010	1,18	8.00	0.0095
CARB					
Intercept	0.024	0.24			
Sex			1,19	1.38	0.27
Female-male	0.037	0.035			
Body mass	0.0041	0.018	1,19	0.010	0.92
Blackness (foster father)	0.0017	0.0017	1,19	0.10	0.75
BSM			1,19	0.25	0.63
Enlarged-reduced	−0.026	0.027			
Temperature	−0.062	0.063	1,19	0.96	0.34
Removed:					
Temp × blackness			1,18	1.27	0.27

Oxidative status is measured as levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), glutathione S-transferase (GST), oxidative damage in proteins (CARB), total amount of glutathione (GSHtot) and ratio between reduced and oxidized glutathione (GSHratio). N=38.