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RESEARCH ARTICLE

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

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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 melanin: eumelanins yield blackish hues, whereas pheomelanins yield reddish hues. The production of eumelanins requires low levels of glutathione (GSH), which is the most important intracellular antioxidant, whereas 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 with brown males, indicating enhanced stress in black males. Foster offspring of black males had a 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 with those of brown males, and vice versa under relatively warm weather. Although differently colored males experienced changes in their oxidative status under different temperatures, the link between paternal melanin coloration and offspring oxidative stress appears to be environmentally induced.

KEY WORDS: Environmental heterogeneity, Genetic quality, Genotype-by-environment interaction, Oxidative stress, Phenotypic variation, Phenotypic quality, Sexual selection, Secondary sexual trait

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 (a phenomenon known as genotype-by-environment interaction; Qvarnström, 2001; Bell, 2010; Cornwallis and Uller, 2010). Many variable phenotypic traits are secondary sexual characteristics that have been evolved through sexual selection (Andersson, 1994). In many cases, males are showier than females as females are often the choosy sex (Glutton-Brock and Vincent, 1991; Andersson, 1994). Moreover, phenotypic variation among males is often higher than that of females (Glutton-Brock and Vincent, 1991; Andersson, 1994). Secondary sexual characteristics 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 Rodriguez, 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. Piault et al., 2009; Jacquin et 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 (Finkel 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 and Summers, 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, whereas pheomelanin is responsible for reddish and yellowish hues (McGraw, 2006). The production of pheomelanin needs high levels of glutathione (GSH), which is the most important intracellular antioxidant (Wu et al., 2004), whereas eumelanogenesis requires low GSH levels (Galván and Solano, 2009; Hõrak et al., 2010). It seems that eumelanic individuals are more adapted to high oxidative stress environments, whereas pheomelanic individuals are more adapted to low oxidative stress

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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 to produce 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 stress.

Alternatively, individuals with different melanin coloration are adapted to different environments because genes responsible for the expression of melanin coloration are also responsible for the expression of several physiological and/or behavioral traits (a phenomenon known as pleiotropy; Ducrest et al., 2008). The melanocortin system is highly pleiotropic, as one gene (the proopiomelanocortin gene) produces five different melanocortins that bind to five receptors (Ducrest et al., 2008; Roulin, 2015). These five melanocortins are responsible not only for melanin production but also for processes such as immune function, stress response, energy expenditure and thermoregulation, and behaviors such as aggressiveness and sexual activity (Ducrest et al., 2008). Thus, differently colored individuals might be adapted to different environmental conditions owing to such covariation among physiological, behavioral and color traits. However, the abovementioned possibilities (the effect of GSH on melanin coloration and oxidative stress and the pleiotropic system of melanin production) are not mutually exclusive (Galván and Solano, 2009).

The pied flycatcher 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 and 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 than pheomelanic individuals in stressful conditions, it is surprising that eumelanic pied flycatcher fathers have lower breeding success when the weather is relatively cold during the nestling period compared with 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 the coloration of foster, but not genetic, fathers (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%) were 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). However, in the present study, the main interest was 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 for 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, whereas the 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 owing 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.

MATERIALS AND METHODS

Study species and study site

The pied flycatcher [*Ficedula hypoleuca* (Pallas 1764)] 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 (60°35′N, 27°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 seven 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 3 days old (hatching day=day 0; see Järvistö et al., 2015). In addition to cross-fostering, we simultaneously performed a brood size manipulation (BSM). In the present study, the effect of BSM on oxidative status was not the main topic of interest as we concentrated on the effect of temperature based on our previous findings (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 was smaller than that of males (N=55) as in control nests of the BSM we did not perform 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 and placed in Eppendorf

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tubes, which were immediately put into liquid nitrogen (−196°C) and stored in the freezer after the field day (−80°C). All captured males were ringed (unless they already had a ring) when their offspring were 10 days old. 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 percentage of black in the dorsal plumage of males was estimated by eye from 0 to 100% (mean=57%, s.d.=32%, N=155; see Järvistö et al., 2015). 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 father 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 BSM to the day before offspring measurements (i.e. days 4 to 11). See Fig. 1 and Fig. S1 for the distribution of average daily temperatures across the study period (maximum 17.80°C, minimum 12.80°C). The temperature data were 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) in Artukainen, Turku, Finland.

Paternity analyses and sexing

Extra-pair paternity occurs in our pied flycatcher population (Lehtonen et al., 2009). Thus, to verify that the correct biological father was assigned to each offspring, we determined paternity through genetic parentage analysis from the blood samples collected in the field. Moreover, as male 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 (total GSH) and the ratio between reduced and oxidized glutathione (GSH ratio)] 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 (CAT100, CGP1 and CS0410, Sigma Chemicals, St Louis, MO, USA), a Fluka kit was used to measure SOD activity (Fluka, Buchs, Germany) and the protocol from the Glutathione Fluorescent Detection Kit was used to measure GSH ratio and total GSH (K006-F1, Arbor Assays). All methods were adjusted to minimize sample volumes, as described in the supplementary material of Stauffer et al. (2017). The CARB measurement was performed according to Rainio et al. (2015) with 1 mg ml^{-1} sample dilution. All oxidative status measures are expressed per protein content, which was determined with the bicinchoninic acid (BCA) protein assay (Pierce, IL, USA) (Smith et al., 1985). Measurements were taken with EnVision and EnSpire microplate readers (PerkinElmer-Wallac, Finland). In all assays, samples were randomly placed on the plates and measured in triplicate [intra-assay coefficient of variability (CV) <15% in all cases]. In addition, three control samples were used on every plate to correct for inter-assay 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; https://www.r-project.org/). We used linear models (lm function) to investigate possible interactive effects of melanin-based

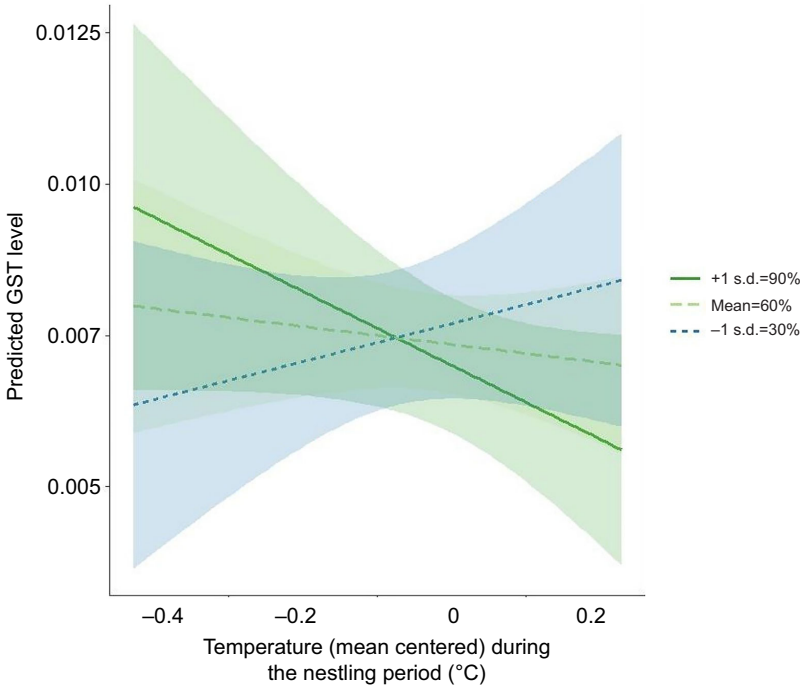

Fig. 1. Predicted glutathione S-transferase (GST) levels in males is relation to male dorsal melanin coloration and temperature during the nestling period. Model predictions derived from the linear model are given for males with different percentages of black in their plumage; the different lines represent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

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phenotype of males (percentage of black) and temperature on the oxidative status (GSH ratio, total GSH, SOD, CAT, GP, GR, GST and CARB) of males and nestlings. Being a ratio variable, GSH ratio was transformed to a 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 of 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 (Grueber et 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 (estimate=−0.00015±0.000064, $F_{1,39}$=5.69, P=0.022, N=55; Table S1). 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) during the nestling period, whereas the opposite pattern occurred when it was relatively warm (temperature above 14.6°C). There were no interactive effects between male melanin coloration and temperature on other measures of oxidative status in males (Table S1). Furthermore, it appears that old males had lower levels of GST than young males (Table S1). Age did not affect any other measures of traits related to oxidative status level (Table S1). Original clutch size, BSM, male melanin coloration or temperature alone did not influence any measures of oxidative status in males (Table S1).

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 S2). However, there was a tendency of temperature to affect SOD levels of the offspring, so that during the nestling period, the offspring had higher levels of SOD under low temperatures compared with high temperatures (Table S2). Moreover, offspring of dark biological fathers had lower GSH ratios than offspring of brown biological fathers (Table S2). Additionally, offspring raised in enlarged broods had higher GSH ratios than those raised in reduced broods in these models (Table S2). Otherwise, single variables (body mass, BSM, offspring sex, or temperature) did not affect the oxidative status of the offspring (Table S2).

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 under different temperatures. Foster offspring raised by black males had a lower GSH ratio than those raised by brown males when it was relatively cold (temperature below 14.4°C; estimate=0.029±0.010, $F_{1,18}$=8.00, P=0.0095, N=38; Table S3, Fig. 2). In contrast, foster offspring raised by black males had a higher GSH ratio than those raised by brown males when it was relatively warm (temperature above 14.4°C; Table S3, Fig. 2). Moreover, foster offspring of black males seemed to have higher total GSH compared with that of brown males when it was relatively cold (temperature below

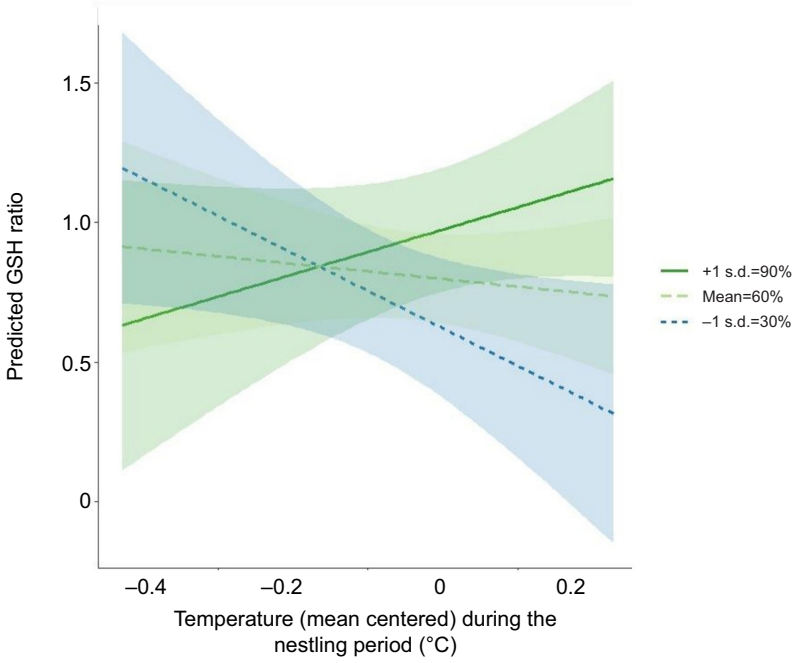

Fig. 2. Predicted ratio between reduced and oxidized glutathione (GSH ratio) of foster offspring as a function of temperature during the nestling period. Model predictions derived from the linear model are given for foster males with different percentages of black in their plumage; the different lines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

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14.5°C), whereas total GSH was lower in foster offspring when the temperature was relatively warm (temperature above 14.4°C; estimate=−0.033±0.016, $F_{1,19}$=4.50, P=0.044, N=38; Table S3, Fig. 3). However, it seems that total GSH 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 S3). Nevertheless, male and female offspring differed in their SOD levels so that female offspring had lower levels of SOD compared with male offspring (Table S3). Sex did not affect any other measures of oxidative status of the offspring (Table S3). Body mass, melanin coloration of the foster father, BSM or temperature alone did not influence oxidative status of offspring (Table S3).

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 with 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 GSH ratios and higher total GSH than those of brown males under prevailing cold weather, and vice versa under relatively warm weather. As a low ratio between reduced and oxidized GSH followed by a high sum of reduced and oxidized GSH indicates high oxidative stress levels, our results indicate that foster offspring of black males suffer from higher oxidative stress under relatively cold weather compared with 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 with those 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 eumelanic individuals are adapted to stressful conditions, and pheomelanic individuals to less-stressful conditions (e.g. Roulin et al., 2011; Galván et al., 2014). However, 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 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 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 with brown males.

However, it needs to be noted that black males might actually have higher levels of GST compared with brown males 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 more in their current brood as their probability to survive to the next breeding season would be low. However, young males that are not

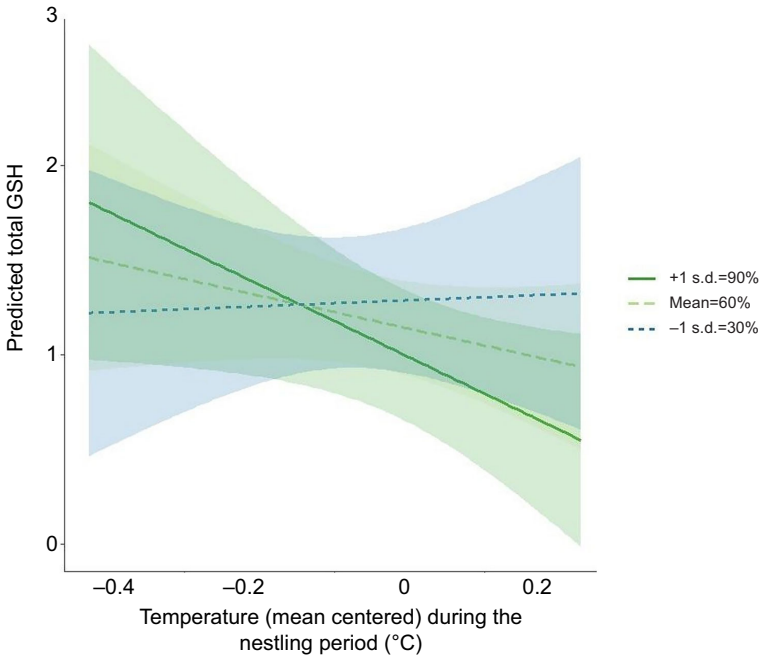

Fig. 3. Predicted total amount of glutathione (total GSH) of foster offspring as a function of temperature during the nestling period. Model predictions derived from the linear model are given for foster males with different percentages of black in their plumage; the different lines represent 30%, 60% and 90% black feathers on the dorsal side, which was treated as a continuous variable in the analyses. P=0.044, N=38.

usually in as good shape as old males (e.g. Mitrus, 2007) had higher levels of GST compared with those of the old males. This suggests that, indeed, high GST levels indicate high stress. Moreover, we found that during relatively warm nestling periods, brown males have higher GST levels than 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 periods of high temperatures. Eumelanic individuals, in contrast, have a better protection against UV radiation because of the strong structure of eumelanin pigments (McGraw, 2006; Brenner and Hearing, 2008; 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 temperature and male melanin coloration most likely leads to differences in the oxidative status of differently colored males. Such an effect may lead to differences in the 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 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 GSH ratios than biological offspring of brown males. This is in accordance with previous evidence that vertebrates with high eumelanin levels have lesser amounts of reduced GSH compared with individuals with low eumelanin levels (Benedetto et al., 1982). Moreover, we found that female offspring had lower levels of SOD compared with the levels of male offspring, thus indicating lower levels of stress in female offspring. Testosterone might be a cause of such sex-specific differences in oxidative status of male and female offspring (see Alonso-Alvarez et al., 2007). In addition, SOD levels of offspring decreased with increasing temperature, indicating that relatively warm weather is less stressful compared with cold weather. Furthermore, we found that GSH ratios were higher in offspring of enlarged broods than in offspring of reduced broods. This was surprising, as high GSH ratios reflect 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 nestlings 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 pheomelanic phenotypes in stressful conditions

An intriguing link between melanin coloration and oxidative stress was suggested more than a decade ago (e.g. McGraw, 2005). As GSH (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 eumelanins 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 gradients 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 with 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°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 to demonstrate how the physiological mechanisms associated with eumelanin coloration might be costly in terms of oxidative stress in varying temperatures. Our findings might be also related to temperature-dependent tyrosinase activity (Kim et al., 2003). Low temperatures reduce tyrosinase activity, leading to increased GSH levels and pheomelanogenesis, whereas 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 a potential difference in the ability of alternative color morphs to thermoregulate might be due to the pleiotropic effects of the melanocortin system instead of GSH levels and oxidative stress. In contrast, we found evidence that there is a temperature-related association between paternal 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 paternal melanin coloration to affect oxidative status in males themselves and the offspring they are rearing. Our study is the first 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 the ever-changing environment (such as human-induced climate change).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: P.E.T., J.S., P.I., S.C., W.S., T.L.; Methodology: P.E.T., J.S., S.C., W.S.; Data curation: P.E.T., J.S.; Writing - original draft: P.E.T.; Writing - review & editing: P.E.T., J.S., P.I., S.C., W.S., T.L.

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Data availability
Data are available from the Dryad Digital Repository (Teerikorpi et al., 2019): https://doi.org/10.5061/dryad.kc57603.

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