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The adaptive function of melanin-based coloration in the tawny owl (Strix aluco); the proximate role of the melanocortin system

Guillaume Emaresi

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Département d'Ecologie et d'Evolution

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Thèse de doctorat ès sciences de la vie (PhD)

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par

Guillaume Emaresi

Master en Biologie (BEC) de l'Université de Lausanne

Jury

Prof. Nicolas MERMOD, Président
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Prof. Frank CEZILLY, Expert
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UNIL | Université de Lausanne
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GENERAL ABTRACT

Colour polymorphism is common in wild population. One of the main questioning of evolutionary biologists is to understand how different colour variants could have evolved and be maintained in fluctuating environments, a selective process that forces individuals to constantly adapt their strategies in order to survive. This issue is particularly true for traits that are genetically inherited. Natural selection erodes genotypes with lowest fitness (less adapted), reducing in turn global genetic variation within population. In this context, the study of the evolution and maintenance of melanin-based coloration is relevant since inter-individual variation in the deposition of these pigments is common in animal and plant kingdoms and is under strong genetic control.

In this thesis, I focus on the specific case of the tawny owl (*Strix aluco*), a species displaying continuous variation in reddish pheomelanin-based coloration. Interestingly, empirical studies highlighted covariations between melanin-based coloration and important behavioural, physiological and life history traits. Recently, a genetic model pointed out the melanocortin system and their pleiotropic effects as a potential regulator of these covariations. Accordingly, this PhD thesis further investigates colour-specific behavioural, physiological, or life history strategies, while examining the proximate mechanisms underlying these reaction norms.

We found that differently coloured tawny owls differently resolve fundamental trade-off between offspring number and quality (***Chapter 1***), light melanic individuals producing many low-quality offspring and dark melanic ones producing few high-quality offspring. These reproductive strategies are likely to induce alternative physiological constraints. Indeed, we demonstrated that light melanic individuals produced higher levels of reactive oxygen species (ROS, ***Chapter 2***), but also expressed higher levels of antioxidant (GSH, ***Chapters 2 & 3***). Interestingly, we showed that light melanic breeding females could modulate their POMC prohormone levels according to the environmental conditions, while dark reddish ones produced constant levels of this prohormone (***Chapter 4***). Finally, we highlighted colour-specific patterns of prohormone convertase 1 (PC1) gene expression (***Chapter 5***), an enzyme responsible for POMC prohormone processing to ACTH and α–MSH, for instance.

Altogether, these results provide strong evidence of colour-specific strategies, light and melanic tawny owls better coping with stressful and relaxed environments, respectively. Variation in melanin-based coloration is likely to be maintained by the heterogeneity of our study area and strong environmental stochasticity within and between years, these process favouring differently coloured tawny owls at different periods of time. From a proximate point of view, this PhD thesis supports the hypothesis that covariations between phenotypic traits and melanin-based coloration stems from the melanocortin system, especially the fundamental role of *POMC* gene expression and its processing to melanocortin peptides.

RESUME

Le polymorphisme de couleur est une variation phénotypique très fréquente dans la nature. En biologie évolutive, une des problématiques clés est donc de comprendre comment différent morphes de couleur peuvent être apparus et maintenus au cours du temps dans des environnements aussi variables que les nôtres, surtout que ces fluctuations forcent ces morphes à s'adapter constamment pour assurer leur survie. Cette thématique est particulièrement réelle lorsque les variations phénotypiques sont héréditaires et donc sous forte influence génétique. La sélection naturelle a en effet le pouvoir d'éroder rapidement la variation génétique en éliminant les génotypes mal adaptés. Dans ce sens, l'étude de l'évolution et de la maintenance de la coloration mélanique est donc tout à fait pertinente car la variation de coloration entre individus est très répandue à travers les règnes animal et végétal et sous forte influence génétique.

Dans cette thèse, je me suis concentré sur le cas spécifique de la chouette hulotte (*Strix aluco*), une espèce présentant une variation continue dans la déposition de pigments pheomélaniques roux. De précédentes études ont déjà montré que cette variation de coloration était associée avec des variations de traits comportementaux, physiologiques ou d'histoire de vie. Récemment, une étude a souligné l'importance du système des mélanocortines et de leurs effets pléiotropes dans la régulation de ces covariations. En conséquence, cette thèse de doctorat a pour but d'étudier un peu plus les stratégies comportementales, physiologiques ou d'histoire de vie spécifiques à chaque morphe de couleur, tout en examinant un peu plus les mécanismes proximaux potentiellement à la base de ces normes de réactions.

Nous constatons tout d'abord que les morphes de couleurs étaient associés à différentes stratégies dans la résolution de compromis telle que la production de beaucoup de jeunes ou des jeunes de qualité (***Chapitre 1***). Les morphes gris (dit peu mélaniques) ont tendance à produire beaucoup de jeunes mains de moindre qualité, alors que les morphes roux (dit fortement mélaniques) produisent moins de jeunes mais de meilleure qualité. Ces stratégies sont susceptibles alors d'induire certaines contraintes physiologiques. Par exemple, nous montrons que les morphes gris produisent plus de dérivés réactifs de l'oxygène (ROS, ***Chapitre 2***), mais aussi plus d'antioxydants (GSH, ***Chapitres 2 & 3***). Nous montrons ensuite que les femelles grises ont une plus grande capacité à moduler leur niveau de POMC prohormone dans le sang en fonction des conditions environnementales, alors que les femelles rousses gardent un niveau constant (***Chapitre 4***). Finalement, nous démontrons que les patterns d'expression du gène codant pour la prohormone convertase 1 varient chez des jeunes issus de parents gris ou roux (***Chapitre 5***). Ceci est particulièrement intéressant car cette enzyme permet de scinder la POMC prohormone en plusieurs peptides importants tels que l'ACTH ou l'α–MSH.

En conclusion, ces résultats démontrent qu'il y a bel et bien des stratégies évolutives différentes entre les morphes de couleurs, les chouettes hulottes grises et rousses étant respectivement plus adaptés à des environnements stressants ou favorables. L'hétérogénéité de notre zone d'étude et

la stochasticité environnementale qui caractérise ses habitats pourraient donc agir comme une source de sélection temporelle, laquelle favoriserait les différents morphes de couleurs à diverses périodes. D'un point de vue plus proximale maintenant, cette thèse de doctorat soutient l'hypothèse que les covariations observées entre la coloration mélanique et des traits phénotypiques importants sont modulées par les effets pléiotropes du système des mélanocortines, et met en avant le rôle prépondérant que pourrait jouer l'expression du gène *POMC* et sa post traduction en mélanocortines.

INTRODUCTION

This introduction was published in *Ecology and Conservation of European forest-dwelling raptors* (2012), Zuberogoitia I., Martinez J.E. (eds.). Bizkaiko Foru Aldundia.

Phenotypic variation among individuals within the same population is common in most organisms, some individuals being, for example, larger, heavier or more colourful than others. The theory of evolution by natural selection provides a general background to understand such broad phenotypic variation in nature (Darwin 1872; 1930). However, fluctuating environments force individuals to constantly adapt their behavioural, physiological or life history strategy, a selective process that eliminates phenotypes with lowest fitness (Fisher 1930). Interestingly, high phenotypic variation can still persist in populations despite the eroding effects of natural selection and genetic drift (Lewontin 1974). The extent of variation in a population is crucial for current and future evolution, especially in genetically polymorphic phenotypes that are encoded by a limited number of alleles. Understanding how such variation is generated and maintained in natural populations is thus a central issue in evolutionary theory, but also in field ecology (Coleman *et al.* 1994; Hallgrimsson & Hall 2005).

The tight association between genotype and phenotype in polymorphic species allows researchers to carry out detailed studies on the effect of natural and sexual selection on evolutionary processes. Classical examples of genetic polymorphisms include blood group system or eye coloration in human as well as melanic *vs*. peppered adult Moths *Biston betularia* (Grant 2004). Colour polymorphic organisms provide a particularly promising study system because of the dramatic colour variation easily observed within and between species (Endler 1990) and its strong genetic control (e.g. insects: Majerus 1998; reptiles: Shine *et al.* 1998; anurans: Hoffman & Blouin 2000; birds: Kruger & Lindstrom 2001; plants: Warren & Mackenzie 2001; mammals: Majerus & Mundy 2003). For my thesis, I will focus on the most widespread and conserved pigmentation system in animal kingdom (Majerus 1998), namely melanin-based coloration.

THE ADAPTIVE FUNCTION OF MELANIN-BASED COLORATION

Melanin is the most abundant pigment in animal taxa and is found in all of the main types of integuments among vertebrates (Ito & Fujita 1985; Shiojiri *et al.* 1999; Lesser *et al.* 2001; Mundy & Kelly 2003; Hoekstra 2006). Melanin-based coloration is determined by the deposition of two distinct pigments: grey to black eumelanin and yellow to reddish-brown pheomelanin (Wakamatsu & Ito

2002). In birds, a comparative study revealed that 334 species owned colour polymorphisms (3.5% of all birds), with higher occurrence in Strigiformes, Ciconiformes, Cuculiformes and Galliformes (Galeotti *et al.* 2003). Well-known examples of melanin-based colour polymorphism include the Ruff *Philomachus pugnax* (Lank *et al.* 1995), the arctic skua *Stercorarius parasiticus* (Mundy *et al.* 2004), the snow goose *Anser caerulescens* (Mundy 2005), the feral pigeon (Johnston & Janiga 1995), the Bananaquit *Coereba flaveola* (Theron *et al.* 2001) and the common buzzard *Buteo buteo* (Kruger & Lindstrom 2001). Nowadays, the distinction between discrete colour morphs and continuous variation in genetically-based coloration fades away since several colour polymorphic species display continuous variation in melanin expression (see for example: McGraw *et al.* 2004; McGraw *et al.* 2005; Hofmann *et al.* 2007).

The adaptive function of melanin-based coloration has been a central evolutionary issue since Darwin and Wallace. A colour trait is defined as being adaptive when in some habitats or social conditions individuals displaying a particular coloration have a fitness benefit (in terms of growth, reproduction or survival) over other differently coloured individuals. Based on this definition, scepticism emerged among evolutionary biologists regarding the adaptive value of displaying different melanic attributes, mainly because the expression of these traits were frequently found to be condition-independent. Conversely to carotenoid pigments that cannot be endogenously synthesized in vertebrates, melanic pigments are synthesized *de novo* in specialized cells, i.e. the melanocytes. Thus, inter-individual variation in melanin-based coloration is often under tight genetic control (Mundy & Kelly 2003; Roulin & Dijkstra 2003; Bize *et al.* 2006; Hoekstra 2006; see Box 1). Accordingly, melanin-based pigmentation is often considered as a phenotypic marker of alternative genotypes (Hoekstra 2006).

Box 1 – Molecular mechanisms regulating melanin production

The molecular cascade leading to the production of both melanins have been intensively studied (Jackson 1994; Mishima 1994; Bennett & Lamoreux 2003; Hoekstra 2006; Mundy 2006; Hoekstra 2010). In epidermal tissues, binding of α- melanocyte-stimulating-hormone (α-MSH) to melanocortin-1-receptor (MC1R) promotes eumelanogenesis (Slominski *et al.* 2004; Pritchard & White 2007; Walker & Gunn 2010). This binding on the surface of melanocytes activates indeed the production of intracellular $_c$AMP, a second messenger that up-regulates the eumelanogenic activity of tyrosinase (TYR) within melanocyte (Kobayashi *et al.* 1995; Barsh *et al.* 2000; Ito *et al.* 2000; Slominski *et al.* 2004; Lin & Fisher 2007; Spencer & Schallreuter 2009). Rate limiting enzyme tyrosinase catalyses the first two-steps of melanin production (see Figure Int.1): the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) and the subsequent oxidation of 5,6-dihydroxyindole (DHI) to L-dopaquinone (Slominski *et al.* 1991; Ozeki *et al.* 1997; Ito *et al.* 2000; Land & Riley 2000; Park *et al.* 2009; Ito & Wakamatsu 2010; Schallreuter *et al.* 2011). However, binding of inverse agonist and antagonist Agouti-signalling-protein to MC1R can block α-MSH binding, leading to the production of pheomelanin at the expense of eumelanin (Suzuki *et al.* 1997; Gantz & Fong 2003; Lin & Fisher 2007). Note here that the switch between eu- and pheomelanogenesis depends also on the presence of thiol groups (Figure Int.1), resulting from cysteine depletion in melanosomes (del Marmol *et al.* 1996; Ito & Wakamatsu 2010). In absence of thiols, dopaquinone undergoes intramolecular cyclization, resulting in the formation of eumelanin, whereas thiol intervention promotes the transformation of dopaquinone in cysteinyldopas, which is further oxidized to produce pheomelanin (Ito *et al.* 2000; Wakamatsu & Ito 2002).

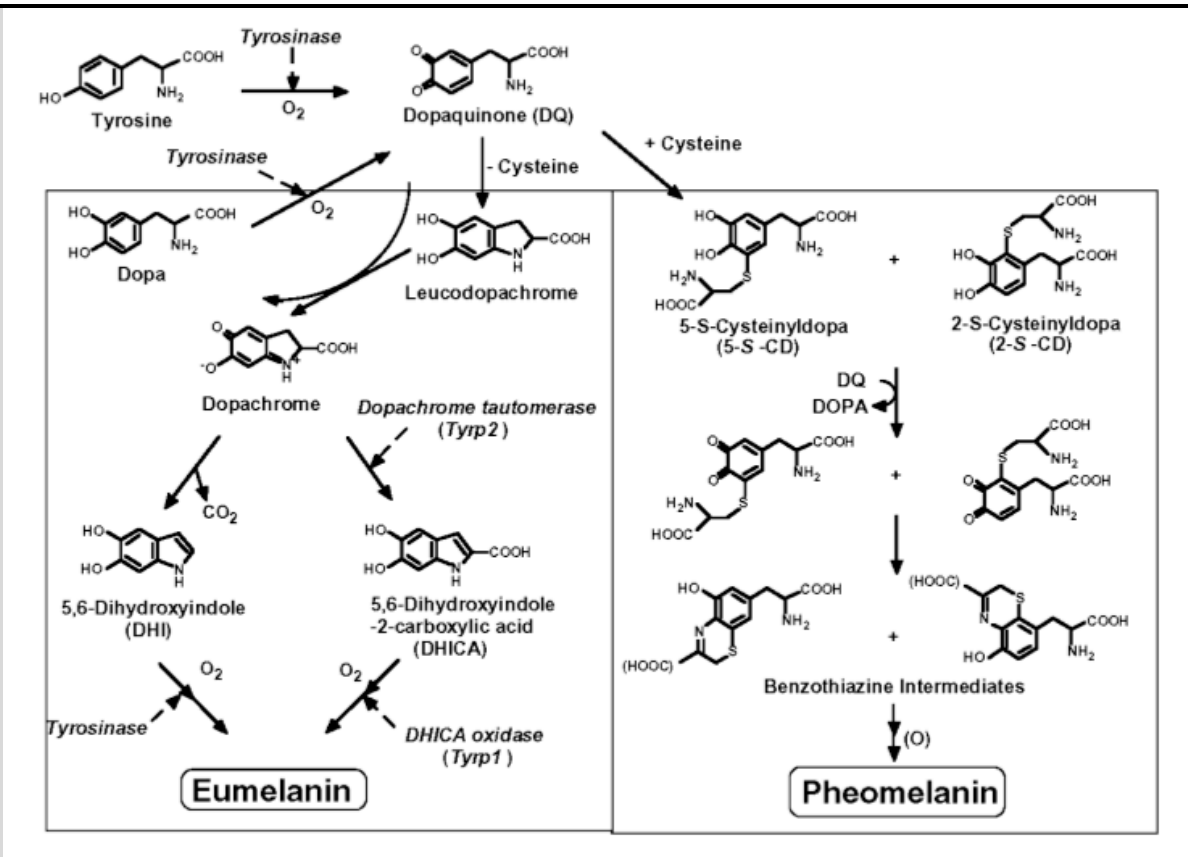


Figure Int.1: The biosynthetic pathways to eu- and pheomelanin. Redrawn from Wakamatsu and Ito (2002).

Moreover, experimental studies manipulating brood size (Roulin *et al.* 1998), endoparasites (McGraw & Hill 2000), calorie (McGraw *et al.* 2002) and protein intake (Gonzalez *et al.* 1999) in birds revealed no or little effect on the extent of melanic plumage coloration (but see: Fargallo *et al.* 2007; Piault *et al. in press*). But this sceptic view is however challenged by the finding that melanin-based traits frequently covary with behavioural, morphological, life history or physiological traits (Galeotti *et al.* 2003; Jawor & Breitwisch 2003; Roulin 2004), raising the hypothesis that melanic colour traits can signal individual quality. The proximate basis for such covariations to evolve in natural populations lies in three distinct reasons. Melanin-based coloration may have an adaptive function if natural selection exerts its influence:

(i) on coloration, which can be the case when coloration plays a role in foraging (Galeotti *et al.* 2003; Roulin & Wink 2004) and anti-predatory strategies (Jones 1977; Endler 1988; Johannesson & Ekendahl 2002; Caro 2005; Bond 2007);

(ii) on the melanic pigments, because of their effective chemico-physical properties that operate in antibiotic activity, resistance to solar radiation, oxidative stress or thermoregulation (Mackintosh 2001; Slominski *et al.* 2004; McGraw 2005; Clusella Trullas *et al.* 2007; Galvan & Alonso-Alvarez 2008; Galvan & Alonso-Alvarez 2009; Galvan & Solano 2009);

(iii) on phenotypic traits that are genetically correlated with coloration (Strand 1999; Ducrest *et al.* 2008).

In the latter case, selection is acting on genes that are closely associated with genes coding for coloration (genetic disequilibrium) or on genes with pleiotropic effects on coloration and other attributes. Hence, melanin-based coloration may also evolve as indirect response to selection exerted on alternative physiological, morphological or behavioural attributes (Kittilsen *et al.* 2009), and thus may signal alternative life history or physiological strategies to cope with variation in habitats (for reviews: Galeotti *et al.* 2003; Roulin 2004; and see Roulin & Bize 2007) or social environments (Rohwer 1975, 1977). In this context, I review hereafter empirical studies carried out in the tawny owl *Strix aluco* (Box 2) with the aim of highlighting how the study of variation in melanin-based coloration can inform us about (i) the evolution and maintenance of genetic polymorphism in natural populations and (ii) individual adaptation to heterogeneous environments.

Box 2 – The Tawny Owl (*Strix aluco*)

The tawny owl is the commonest owl in central Europe, its distribution stretching across temperate Eurasia (Figure Int.2). The European breeding population is estimated between 480’000 to 1’000’000 breeding pairs (International 2012). In Switzerland, the breeding population size is estimated at about 6000 pairs (Vogelwarte 2004). This resident species is monogamous and particularly territorial all year round, but immatures may disperse up to 100km (König & Weick 2008). This medium-size owl (36-40cm) is sexually dimorphic in size (with females 20% bigger than males). Tawny owls live mainly in woodlands (e.g. semi-open deciduous and mixed forest, parks, large gardens and coniferous wooded patches where clearings and rides exist). This nocturnal perching hunter preys mainly upon

small rodents such as wood mice (*Apodemus sylvaticus*) and bank voles (Clethrionomys glareolus; Roulin *et al.* 2008a; Roulin *et al.* 2009), but also passerines, frogs, reptiles and even fish or large insects. Breeding starts early in the season, laying beginning at the end of January. Analyses of genetic parentage revealed that extra-pair copulation is low (Saladin *et al.* 2007). The breeding season is relatively synchronised between pairs and usually last until early June. The female incubates the clutch (i.e. one to nine eggs) during 28 days, and then remains in the nest to guard her hatchlings and to distribute among them prey items collected by the male. Once owlets are thermo-independent at 15-20 days of age, the female patrols around the nest to protect her offspring from potential predators, while helping her partner with provisioning food to the brood (Glutz von Blotzheim & Bauer 1980; Galeotti 2001).

Figure Int.2: Geographic distribution of tawny owls around the world with green areas indicating the repartition of the species. Map produced by www.oiseaux.net

Nestling growth rate and survival strongly depend on prey availability. Offspring leave the nest at 25–30 days of age but are still being fed and protected by the parents until 90-120 days of age (Sunde 2008). Sexual maturity is reached within a year (König & Weick 2008). Tawny owls may live to 18-19 years (König & Weick 2008). In our local population, however, the mean expected life span is approximately 3.5 years and the maximum life span was up to 15 years.

PLUMAGE COLORATION IN THE TAWNY OWL

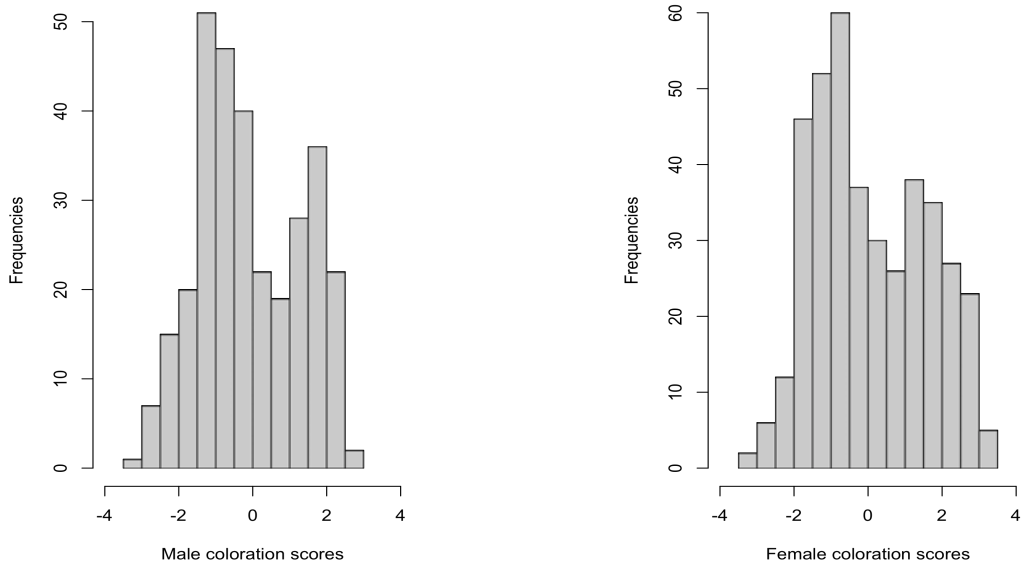
The tawny owl *Strix aluco* displays large inter-individual variation in the degree of melanin-based plumage coloration (from pale to dark reddish-brown, Figure Int.3), such variation being explained to a large extent by the level of pheomelanin pigments (68% of total variance) deposited in feathers and to a lesser extent by the level of eumelanin pigments (21% of total variance; Gasparini *et al.* 2009a).

A B C

Figure Int.3: Illustration of the variation in the degree of melanin-based coloration in the tawny owl *Strix aluco*. Pictures A and B show light and dark melanic mothers, respectively. Picture C illustrates two differently coloured chicks born from distinct parents.

Different methods have been used to assess the degree of melanin-based coloration in the tawny owl. Colour morphs can be directly scored in the field by human eyes (Galeotti & Pavan 1993; Roulin *et al.* 2003; Brommer *et al.* 2005). Another approach is to collect feathers on the back of individuals, a body part that displays substantial variation in reddishness, and to measure them either through pictures which are then analysed with the software ADOBE PHOTOSHOP, or by reflectance spectra collected with a spectrophotometer. Interestingly, a bimodal distribution of colour morphs can be observed (Figure Int.4, see also Brommer *et al.* 2005), suggesting the occurrence of two main morphs, so-called grey (i.e. light melanic) and reddish (i.e. dark melanic), with some variation in the degree of pheomelanin within each morph. Expression of plumage coloration is neither sexually dimorphic (individuals of one sex are as likely to display a given coloration as an individual of the other sex; Galeotti & Cesaris 1996; Roulin *et al.* 2003; Brommer *et al.* 2005; Figure Int.4), nor sensitive to sibling competition (Roulin & Dijkstra 2003; Roulin *et al.* 2008b), but is found to be highly heritable, h^2 ranging from 0.72 in Finland (Brommer *et al.* 2005; Karell *et al.* 2011b) to 0.93 in

Switzerland (Gasparini *et al.* 2009a; Table Int.1).



A B

Figure Int.4: Frequency distribution of melanin-based plumage coloration in male (A) and female (B) tawny owls. Data are from 177 males and 208 females from a Swiss population of tawny owl. Coloration scores were obtained by reflectance spectra. Low scores reflect light melanic feathers (i.e. grey morph) and high score dark melanic feathers (i.e. reddish morph).

POTENTIAL ADAPTIVE FUNCTIONS OF COLORATION IN THE TAWNY OWL

Table Int.1 provides a survey of the empirical studies that investigated the potential adaptive function of melanin-based coloration in the tawny owl. Because melanin-based coloration, as well as pigment density or distribution, are experimentally difficult to manipulate, evidence of direct selection on these traits is lacking in natural populations. By contrast, several studies established covariations between melanin-based coloration and physiological, morphological and behavioural phenotypic traits, suggesting that the degree of plumage reddishness reflects adaptations to different environmental conditions. Hereafter, we discuss five major selective forces to which plumage coloration of tawny owls may signal an adaptation.

Table Int.1: Summary of empirical studies (performed before or during my PhD thesis) highlighting covariation between melanin-based plumage coloration and other phenotypic traits in the tawny owl. Within the studied parameter (in bold italic), different phenotypic traits (e.g. morphological, behavioural, physiological or life history traits) are associated. For each trait, we provide a description of the main result. References of the empirical studies are also given.

Studied parameters	Description	Reference
Immune parameters		
1 Antibody production	Dark melanic breeding females maintain a stronger level of antibody for a longer period of time compared to light melanic females	Gasparini et al. 2009a
2	The same humoral challenge enhanced immune response to PHA in dark melanic nestlings while reducing it in light melanic nestlings	Gasparini et al. 2009b
3 Body mass after PHA vaccination	Nestlings born from dark melanic mothers suffered greater body mass loss than those born from lighter melanic females	Piault et al. 2009
4 Body mass after Tetravac vaccination	Dark melanic breeding females suffered greater body mass losses than light melanic ones	Gasparini et al. 2009a
5 Blood parasites intensity	Dark melanic adult owls hosted higher total parasite burden than pale melanic adult owls	Galeotti & Sacchi 2003; Karell et al. 2011a
Genetic parameters		
6 Heritability	Expression of plumage coloration shows high heritability (h^2= 0.72 and h^2= 0.93)	Brommer et al. 2005; Gasparini et al. 2009a; Karell et al. 2011b
Hormone levels		
7 POMC prohormone	Light melanic females exhibit more circulating POMC prohormone than darker females when the brood size was experimentally enlarged, but not when reduced.	Roulin et al. 2011c, ***Chapter 4***
Physiological parameter		
8 Oxygen consumption	Foster offspring raised by light melanic mothers showed not only a lower body mass than offspring raised by dark melanic mothers, but also consumed more oxygen under warm temperature	Roulin et al. 2005
Fitness components		
9 Body mass in relaxed environment	When fed *ad libitum* nestlings born from dark melanic mothers converted food more efficiently into body mass than offspring born from lighter melanic mothers	Piault et al. 2009
10	In some years, dark melanic owls produce heavier offspring than lighter melanic individuals, while the reverse is true in other years	Roulin et al. 2003

11		Nestlings reared by dark melanic parents grew more rapidly in body mass than offspring reared by light melanic parents when the size of their brood was experimentally reduced (but not enlarged)	Roulin et al. 2008b
12	Body mass in stressful environment	When food is restricted nestlings born from dark melanic mothers suffered greater body mass losses than those born from lighter melanic females	Piault et al. 2009
13	Adult survival	Dark melanic adults suffer a higher mortality in cool-dry years	Galeotti & Cesaris 1996
14		Light melanic adults suffer a higher mortality in warm-wet years	Galeotti & Cesaris 1996
15		Light melanic morphs surviving better than dark morphs	Brommer et al. 2005
16		In Finland, frequency of dark melanic individuals increased with global warming	Karell et al. 2011b
17		In a Swiss population, tawny owl survival was not associated with melanin-based coloration	Roulin et al. 2003
18		Dark melanic tawny owls are more frequent near than away from the equator	Roulin et al. 2011a
19	Fledgling production	Light melanic male and female owls had a higher lifetime production of fledglings	Brommer et al. 2005
20	Recruitment	Light melanic male owls produced more recruits during their lifetime than brown individuals	Brommer et al. 2005
21		In a Swiss population, light melanic individuals were less frequently recaptured than dark melanic individuals	Roulin et al. 2003
22	Probability of breeding	Proportion of dark melanic females that were breeding was greater in low breeding year. Although not breeding every year, light melanic females produce offspring of higher quality.	Roulin et al. 2003
Mating behaviour			
23	Assortative mating	Sexes did not mate assortatively with respect to their colour	Brommer et al. 2005; Roulin et al. 2003
Environmental parameters			
24	Habitat background	Dark melanic owls may be particularly cryptic in closed forest	Majerus 1998; Gehlbach & Gehlbach 2000; Galeotti & Sacchi 2003

Cryptism. It has been proposed that light and dark melanic morphs are more cryptic in opened and closed habitats, respectively (Majerus 1998; Gehlbach & Gehlbach 2000), suggesting an adaptive value according to environmental background (Table Int.1). Indeed, such characteristic can confer fitness benefits by lowering the vulnerability to predators, enhancing foraging success and minimizing the risks of mobbing from passerine birds (Negro *et al.* 2007). Although Galeotti & Sacchi (2003) observed that dark melanic tawny owls lived in more closed forest habitats than lighter individuals across a latitudinal gradient, data on larger populations and experimental studies are still needed to validate this hypothesis.

Adaptation to climate conditions. The tawny owl is a long-lived species (up to 18 years; Galeotti 2001; König & Weick 2008) distributed throughout Eurasia and thus experiencing a wide range of climatic condition. Several studies have reported a relationship between adult survival and colour morphs, the direction of this relationship changing among years and countries/environments. In Italy, dark melanic (i.e. so-called reddish morph) and light melanic (i.e. grey morph) tawny owls were found dead more often in cool-dry and warm-wet years, respectively (Galeotti *et al.* 1996). In Finland, a country characterized by cool-dry conditions, light melanic individuals have a better survival than darker conspecifics (Brommer *et al.* 2005). However, the frequency of the reddish morph increased rapidly as winter became milder in the last decades (Karell *et al.* 2011b). In line with this hypothesis, a comparative analysis of colour morph frequencies along a latitudinal gradient in four owl genera revealed that dark melanic species are more prevalent near the equator than polewards (Roulin *et al.* 2011b). In Switzerland, recapture rate was associated with tawny owl plumage coloration, light melanic individuals being less recaptured than dark melanic ones, suggesting that grey morphs skip more often reproduction (Roulin *et al.* 2003). In contrast, the authors failed to highlight colour-specific survival years in this population.

Adaptation to parasites. In Italy, dark reddish tawny owls hosted more endoparasites (*Haemoproteus*) than grey owls (Galeotti & Sacchi 2003; and see Karell *et al.* 2011a), especially during the breeding season. However, it remained unclear whether these observations were due to non-random habitat distribution of the different colour morphs. To clarify this issue, experimental immune challenges were performed in Switzerland and revealed that dark melanic females maintained higher

concentration of antibodies for a longer period of time than light melanic ones (Gasparini *et al.* 2009a). Similarly, another experiment done on the same population reported that the same humoral challenge enhanced T-cell mediated immunity in nestlings born from dark melanic mothers while reducing it in nestlings born from light melanic mothers (Gasparini *et al.* 2009b). These results suggest that offspring of dark melanic owls mount stronger immune responses either because (i) dark melanic individuals inhabit environments where parasites are more abundant or virulent than in habitats occupied by lighter melanic individuals (ii) and/or because dark melanic individuals have a less efficient immune response and are thereby longer exposed to parasites than pale melanic owls. Interestingly, resistance to parasitism by dark melanic individuals comes at a cost in terms of greater loss of body mass in both nestling and adult owls (Gasparini *et al.* 2009a; Gasparini *et al.* 2009b; Piault *et al.* 2009). This outcome indicates that the cost/benefit trade-off of immunocompetence is differentially resolved by dark and light reddish tawny owls.

Adaptation to variation in food resources. The tawny owl preys mainly upon small rodents, such as the wood mice *Apodemus* sp. and bank vole *Clethrionomys glareolus* (Roulin *et al.* 2009), which are known to show high density fluctuation over space and time (Karell *et al.* 2009). Although nestling appetite did not correlate significantly with plumage coloration of the biological mother, an experimental manipulation of food supply to chicks demonstrated that, when food supply was restricted, offspring born from light melanic mothers grow faster in body mass than those born from dark melanic mothers (Piault *et al.* 2009). But when preys were provided *ad libitum*, the opposite pattern was found (i.e. offspring of dark mothers grow faster). Moreover, a brood size manipulation experiment performed in the same Swiss population, but using other individuals, pointed out that offspring from dark melanic mothers grow quicker than offspring from light melanic mothers when brood size was experimentally reduced, but not when enlarged (Roulin *et al.* 2003; Roulin *et al.* 2008b). To determine whether these colour-specific growth patterns were associated with alternative metabolic rates between offspring of reddish and grey mothers, Roulin *et al.* (2005) analysed nestling oxygen consumption and found that oxygen consumption was greater in offspring raised by grey foster mothers under warm temperature. Altogether, these outcomes suggested colour-specific reaction norms in the tawny owl, raising the hypothesis that differently coloured individuals better cope with

different stress levels. During poor breeding seasons with harsh environmental conditions (i.e. as simulated by food depletion (Piault *et al.* 2009)), light melanic individuals outperform darker reddish conspecifics. Once environmental conditions are restored (i.e. as simulated by *ad libitum* food supply; Piault *et al.* 2009) or experimentally reduced broods (Roulin *et al.* 2008b), the opposite pattern was observed with dark reddish individuals performing better than light coloured conspecifics. This hypothesis could explain why dark melanic female tawny owls were found to produce heavier offspring than light melanic ones in some years, and inversely in other years (Roulin *et al.* 2003; Roulin *et al.* 2004; Roulin *et al.* 2005).

Adaptation to breeding conditions. Because physiological, behavioural and life-history traits are often closely associated, the four latter points can induce variation in reproductive parameters between light and dark melanic tawny owls. In Switzerland for instance, dark melanic females keep a constant resolution by breeding every year, whereas light melanic females are inclined to skip reproduction in poor years. This observation suggests a flexible decision rule according to environmental conditions (Roulin *et al.* 2003). Note also that, in Finland, light melanic females produced more fledglings and recruits than darker melanic ones, potentially because of longer life span (Brommer *et al.* 2005).

EVOLUTION OF POLYMORPHISM IN MELANIN-BASED COLORATION

The study of evolution and maintenance of polymorphism in melanin-based coloration is a tremendous work, while empirical studies raised important questioning on the adaptive potential of colour morphs. Melanin-based coloration can be non-neutral with respect to natural selection and is often associated with other phenotypic traits. Thus, selection acting directly on coloration or indirectly via genetically correlated traits should induce changes in the frequency of colour morphs as recently observed in Scops owl *Otus scops* (Galeotti *et al.* 2009) and several other birds (Roulin 2004). Colour polymorphism can therefore emerge and be maintained because of specific evolutionary processes, some of them being briefly discussed in Box 3.

Box 3 – Maintenance of genetic colour polymorphism

First, heterogeneity in the environment can act as divergent selective force that promotes the emergence and maintenance of alternative genetic colour morphs. This '*local adaptation*' hypothesis states that alternative morphs are locally adapted to particular habitats (Kassen 2002; Galeotti *et al.* 2003; Roulin 2004; Sgro & Hoffmann 2004; Byers 2005; Chunco *et al.* 2007). In the particular case of disruptive selection (Ford 1945; Huxley 1955; Lank 2002), individuals at both edges of colour distribution are favoured because of local adaptation, leading to monomorphism or dimorphism over the long term (Brommer *et al.* 2005). Second, without invoking frequency-dependent benefits of displaying a particular morph, one colour morph is likely to become slightly fitter over the long run, due to particular environmental conditions for instance. Under frequency-dependent selection, morphs perform less well when their frequency increase above the equilibrium frequency while their fitness increases when their frequency decreases. Classical example is the apostatic selection (Clarke 1962), for which individuals displaying a new coloration enjoy the advantage of being less rapidly detected by preys or predators compared to other colour morphs (Bond 2007). Thus, under this mechanism, a rare morph confers fitness advantages. Finally, heterozygous individuals can also have a fitness advantage over homozygous ones, a phenomenon called '*heterosis*'. This hypothesis has been proposed to occur in the Common Buzzard (Kruger *et al.* 2001), in which intermediate (i.e. heterozygous) breeding adults produce more offspring than light and dark conspecifics (i.e. homozygous).

From an ultimate point of view, research on tawny owls showed that the degree of melanism covaries linearly with physiological or life-history traits. Although we cannot firmly exclude scenarios of disruptive selection or heterosis in the maintenance of colour polymorphism in the tawny owl, empirical studies failed to demonstrate that selection favours either extreme colour morphs (Roulin *et al.* 2003; Brommer *et al.* 2005) or intermediately-coloured individuals (Piault *et al.* 2009), respectively. One plausible, albeit speculative, scenario is that colour polymorphism of adult tawny

owls is maintained if spatial and temporal heterogeneity of the environment generate balancing selection favouring locally adapted individuals at different time scale (i.e. generations).

From a proximate point of view, reported covariations between melanin-based coloration and fitness components (Table Int.1) are likely to be genetically linked (see for instance: Roulin *et al.* 2011c). However, comprehensive knowledge on the genetic basis of melanin-based coloration is restricted to a limited number of birds (Theron *et al.* 2001; Mundy *et al.* 2004; Mundy 2005, 2006; Nadeau *et al.* 2007; Bottje *et al.* 2008; Hiragaki *et al.* 2008; Nadeau *et al.* 2008), limiting, in turn, our understanding of the proximate mechanisms underlying the adaptive function of melanin-based coloration (Hoekstra 2006; Parker 2006). Recently, a review of the literature pointed out candidate genes, namely the melanocortin system (Box 4), that can pleiotropically affect melanin-based coloration and other important traits (Strand 1999; Gantz & Fong 2003; Ducrest *et al.* 2008).

Box 4 – The melanocortin system

This molecular system is present in most vertebrates and is functionally equivalent between species (Schioth *et al.* 2005; Hoekstra 2006). It consists in 1) α-, β-, γ-melanocyte-stimulating-hormone (MSH) and adrenocorticotropic hormone (ACTH), posttranslational bioactive peptides derived from the cleavage of the proopiomelanocortin gene (POMC; Gantz & Fong 2003; Slominski *et al.* 2004; Millington 2006), 2) a family of five seven-transmembrane G protein-couple melanocortin receptors (MC1-5R; Schioth 2001; Butler & Cone 2002), well conserved among vertebrates (Schioth *et al.* 2005), and 3) endogenous melanocortin antagonist Agouti-signalling- and related-proteins (ASIP and AGRP), two proteins encoded by pheomelanogenesis-related *Agouti* genes (Ito 1993; Barsh *et al.* 2000; Abdel-Malek *et al.* 2001; Bonilla *et al.* 2005; Mundy & Kelly 2006; Lin & Fisher 2007; Lightner 2009). In epidermal tissues, binding of α-MSH to MC1R, promotes eumelanogenesis (Slominski *et al.* 2004; Pritchard & White 2007; Walker & Gunn 2010), while binding of inverse agonist and antagonist ASIP to MC1R can block α-MSH binding, leading to the production of pheomelanin at the expense of eumelanin (Suzuki *et al.* 1997; Gantz & Fong 2003; Lin & Fisher 2007). Of particular interest, bindings of the melanocortins and their antagonists to the five melanocortin-receptors can modulate numerous

physiological and behavioural functions (Figure Int.5), such as stress response, energy homeostasis, anti-inflammatory response, sexual activity, resistance to oxidative stress and aggressiveness (Cone 1999; Fan *et al.* 2000; Schioth 2001; Tatro & Sinha 2003; Slominski *et al.* 2004; Boswell & Takeuchi 2005; da Silva *et al.* 2005; Fan *et al.* 2005; Bertile & Raclot 2006; Butler 2006; Cone 2006; Hillebrand *et al.* 2006; Maaser *et al.* 2006; Millington 2006; Lin & Fisher 2007; Garruti *et al.* 2008; Page *et al.* 2011).

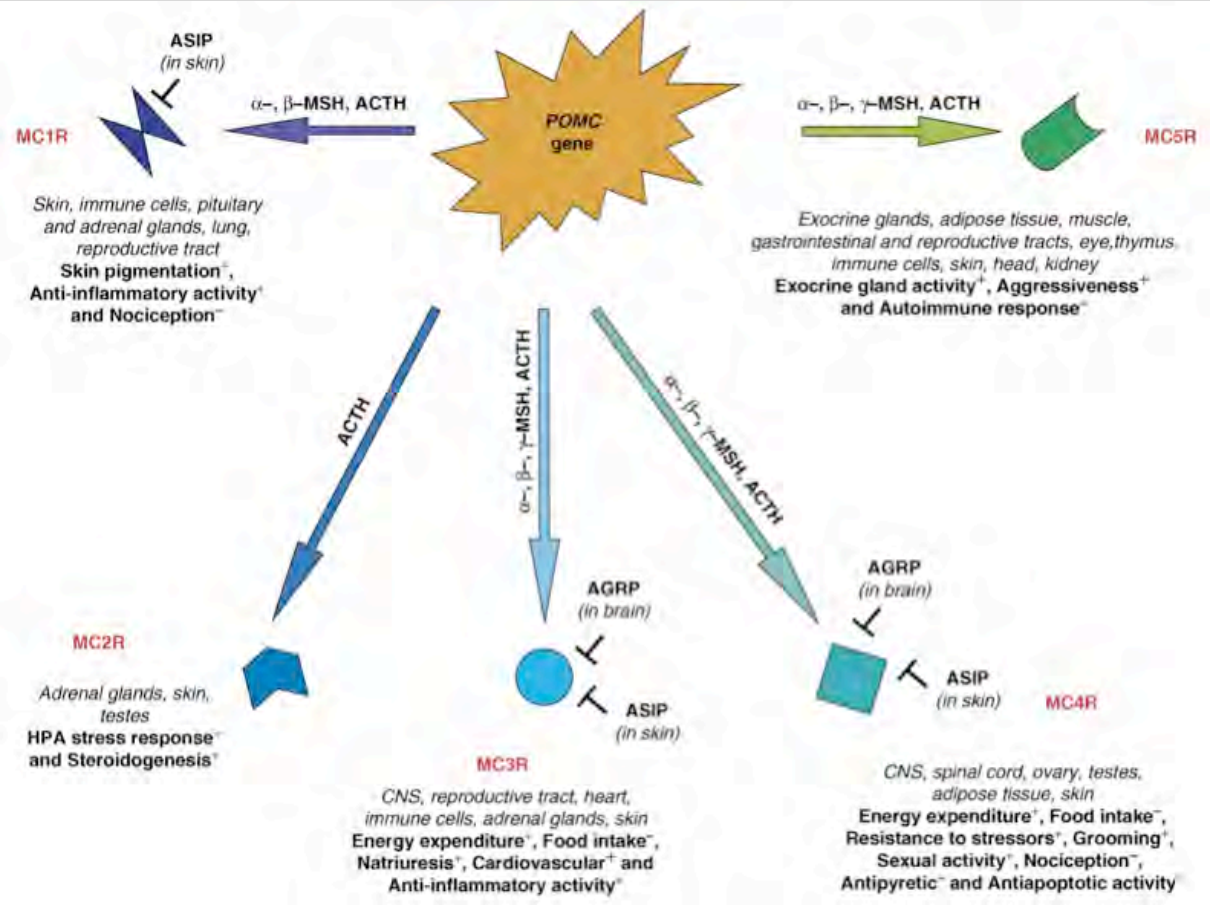


Figure Int.5: Bioactive melanocortin peptides (α-, β-, γ- MSH or ACTH), resulting from the processing of *POMC* gene, or their inverse agonist and antagonist (i.e. ASIP or AGRP) can bind to five distinct melanocortin-receptors (MC1-5Rs) expressed in different tissues (indicated in brackets). These bindings induce different physiological responses (indicated in bold). An increase in function is represented by a '+' sign, a decrease by a '-' sign.

Based the assumption that activities of melanocortins and their antagonists are correlated among tissues, as for instance between epidermal (i.e. where melanic pigments are produced) and brain tissues (i.e. where several physiological responses are regulated), the melanocortin system may account for the observed covariations between plumage melanin-based coloration and other phenotypic traits. If true, this proximate mechanism represents a good candidate system to study and

predict relationships between different traits. Indeed, the expression of melanic colour traits can be used as an indicator of individual quality, such as such as resistance to stress, strong immunity response or high sexual activity (see for example: Roulin & Ducrest 2011). In this context, a fundamental pursuit in the field of evolutionary genetics is to infer the exact genetic mechanisms responsible for variation in specific melanin-based coloration (e.g. nucleotide diversity, patterns of gene expression, posttranslational modification; Hoekstra 2006; Mundy 2006; Hoekstra & Coyne 2007).

THESIS OUTLINE

The present PhD thesis has two main objectives. First, I tackle further the hypothesis that pleiotropic effects of the melanocortin system account for covariations between melanin-based coloration and physiological responses to environmental stressors. To this end, I tested whether the degree of tawny owl melanism is associated with life history or physiological strategies, which may confer fitness-related benefits in different environments. Second, I investigate the underlying proximate mechanisms of these covariations through a candidate gene approach, exploring the genetic architecture of the melanocortin system in the tawny owl.

Chapter 1 tests the prediction that melanin-based coloration is associated with alternative life history strategy in the tawny owl. Based on brood size manipulation experiments that induced different levels of parental workload, I demonstrate that the trade-off resolution between fledgling production and quality covaries with plumage melanin-based coloration in male tawny owl.

Based on these colour-specific reproductive strategies, we might expect hence different strategies regarding physiological oxidative balance, such as alternative ratio in the production of reactive oxygen species and important antioxidant response (e.g. glutathione, hereafter GSH). Yet, it is poorly understood whether individuals from the same population vary in their oxidative status under different reproductive conditions. In ***Chapter 2,*** I reveal that differently coloured adult tawny owls show distinct oxidative balance, especially in their production of reactive oxygen species (ROS) and total GSH.

Tyrosinase activity is a key enzyme controlling the switch between eu- and pheomelanogenesis (Barsh 1996; Ito *et al.* 2000). But its activity can be modulated by the concentration of sulfhydryl compounds (Benedetto *et al.* 1981; 1982; Land & Riley 2000), in particular glutathione (i.e. GSH, characterized by an important antioxidant activity). Thus, melanin-based coloration may be associated with different GSH expression. In ***Chapter 3***, I specifically investigate the link between tawny owl melanin-based coloration (i.e. in adults and nestlings) and GSH expression. I show that the expression of GSH and its consumption in adults covaries with plumage coloration. However, I also demonstrate that GSH levels do not necessarily influence melanogenesis activity and, in turn, the expression of melanic colour traits in nestlings.

POMC gene plays a central function in the melanocortin system (Gantz & Fong 2003; Cone 2005; Millington 2006; Rousseau *et al.* 2007; Ducrest *et al.* 2008). Hence, this candidate gene is likely to also play a key role in generating covariations between melanin-based coloration and other phenotypic traits. In ***Chapter 4***, I reveal that differential regulation of fitness components in relation to environmental factors by pale and dark melanic female tawny owls may be due to colour-specific regulation of the POMC prohormone. These findings support the hypothesis that the widespread links between melanin-based coloration and fitness components may be mediated, at least in part, by the melanocortin system. Finally, ***Chapter 5*** provides insights in the genetic architecture of the melanocortin system in the tawny owl. Although I could not detect non-synonymous mutations in the coding sequence of candidate melanogenic genes, I show interesting colour-specific patterns in gene expression of prohormone convertase 1 (PC1) in nestling skin tissues, a key prohormone in POMC processing to melanocortin peptides. This points out potential proximate mechanism underlying colour-specific reaction norms observed in this species.

In the discussion, I summarize the main outcomes of my research and, based on these evidences, I raise a global outline of colour-specific strategies to cope with stressful environments. To better understand these colour-specific strategies, especially in a context of trade-off resolution between offspring number and quality, I address, in the last chapter (***Annexe 1***), the hypothesis that differently coloured tawny owl own alternative nest defence behaviours. Accordingly, I show that nest defence towards conspecifics and human intruders is indeed related to dark reddish coloration. To

conclude, I discuss proximate mechanism potentially leading to alternative strategies in the tawny owl and identify some future lines of research that could help us improve our knowledge of the evolutionary origins, functions and mechanisms of melanin-based coloration.

CHAPTER 1

Melanin colour polymorphism reveals alternative pace of life in the tawny owl

Guillaume Emaresi[1], Pierre Bize[1], Res Altwegg[2,3], Isabelle Henry[1], Valentijn van den Brink[1], Julien Gasparini[4] & Alexandre Roulin[1]

[1]*Department of Ecology and Evolution, Biophore building, University of Lausanne, 1015 Lausanne, Switzerland*

[2]*South African National Biodiversity Institute, P/Bag X7, Claremont 7735, South Africa*

[3]*Animal Demography Unit, Department of Zoology, University of Cape Town, Rondebosch 7701, South Africa*

[4] *Laboratoire Ecologie et Evolution, Université Pierre et Marie Curie, CNRS UMR 7625, 7 quai St-Bernard, 75252 Paris, France*

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ABSTRACT

The maintenance of genetic variation is a long-standing debate. Although a role of ecological factors including environmental heterogeneity, predator-prey and host-parasite interactions has been acknowledged, the adaptive strategy associated with each genetic variant is usually unknown. Life-history theory explores the evolution of trade-off resolution between reproductive and maintenance traits along the demographic *r-K* selection and "fast-slow" physiological pace of life continuums. Long- and short-lived species typically produce few high-quality and many low-quality offspring, respectively. Evidences for the coexistence of evolutionary fixed life-history strategies at the population level remain scarce. Since heritable melanin-based colour morphs show strikingly different physiological and behavioural norms of reaction in the tawny owl (*Strix aluco*), we investigated whether these colour morphs have a different pace of life. We found that light melanic owls have lower survival compared to dark melanic conspecifics. Thus, morphs may differentially resolve the trade-off between offspring number and quality. To test this hypothesis, we increased offspring food demand by manipulating brood size. When brood size was experimentally enlarged, light melanic males produced more fledglings, but in poorer condition than dark melanic conspecifics. We conclude that dark melanic males have a slower pace of life than lighter reddish male conspecifics.

INTRODUCTION

Trade-off resolutions imposed by resource limitation and biotic interactions with predators and competitors are constrained by intrinsic individual-specific genetic (Lynch 2007) and physiological properties (Lancaster *et al.* 2008). In such circumstances, individuals cannot simultaneously maximize all life-history traits (MacArthur & Wilson 1967; Stearns 1989), but have to resolve trade-offs in an adaptive way depending on prevailing environmental and social conditions (Pianka 1970). One classical example is the effect of seasonality in temperate zones, causing fluctuations in the availability of resources and occurrence of stressful factors such as low temperatures during winter. In contrast, in the tropical zone, resources are less abundant but more homogeneously available through time and space. These contrasting ecological conditions promote the evolution of whole series of physiological traits (e.g. metabolic, hormonal, immunity), resulting in fast pace of life (*r*-strategists) in the temperate zone and slow pace of life (*K*-strategists) in the tropical zone (Dobzhansky 1950; Saether 1988; Promislow & Harvey 1990; Wiersma *et al.* 2007; Reale *et al.* 2010). These modes of adaptation have been mainly studied across species in comparative analyses (Saether 1988; Rushton 2004), while the issue of adaptive genotype-specific pace of life in the same population remains to be tested (Gross 1985; Lank *et al.* 1995; Sinervo & Lively 1996). Genotypes showing slower pace of life are predicted to be longer-lived and favour offspring quality at the expanse of offspring number.

In this context, the long-lived colour polymorphic tawny owl (*Strix aluco*) is a prime model species to investigate whether melanin-based coloration is associated with alternative strategies along the fast-*r* to slow-*K* continuum. This species exhibits a continuous inter-individual variation in melanin-based coloration (i.e. from light to dark reddish melanic), for which the expression is under strong genetic control and not or weakly sensitive to environmental conditions (Brommer *et al.* 2005; Gasparini *et al.* 2009a). Variation in melanin-based coloration can hence be used as a phenotypic marker of alternative genotypes (Hoekstra 2006). Previous studies in different countries already highlighted colour-specific norms of reactions to reproductive (Roulin *et al.* 2011c) and rearing conditions (Roulin *et al.* 2008b), food supply (Piault *et al.* 2009), pathogens (Gasparini *et al.* 2009a; Karell *et al.* 2011a) and climatic conditions (Karell *et al.* 2011b), leading to differences in lifetime recruit production (Brommer *et al.* 2005) and probability of skipping reproduction (Roulin *et al.*

2003). Melanin-based coloration in females covaries with offspring growth rate, dark melanic foster and genetic mothers producing nestlings in better conditions than light melanic mothers (Roulin *et al.* 2004). Given their aptitude to cope with stressful reproductive conditions (Roulin *et al.* 2003; Roulin *et al.* 2008b; Roulin *et al.* 2011c), light melanic owls are expected to adopt a faster pace of life strategy, characterized by the production of many low-quality offspring (i.e. *r*-selected traits), potentially explaining the observed lower parental care per nestling (i.e. higher rate of nest predation (Da Silva *et al.* in prep., ***Annexe 1***)). In contrast, dark melanic owls breed more regularly (Roulin *et al.* 2003) and their offspring grow particularly well in experimentally relaxed rearing conditions (Roulin *et al.* 2008b; Piault *et al.* 2009). These characteristics should lead them to adopt a slower pace of life, characterized by the production of limited number of high-quality offspring (i.e. *K*-selected traits), leading to higher parental care per nestling (lower rate of nest predation (Da Silva *et al.* in prep., ***Annexe 1***)), while maintaining sufficient resources for maintenance traits (e.g. survival (Karell *et al.* 2011b) and immunocompetence (Gasparini *et al.* 2009a)).

To test these predictions, we monitored a Swiss population of tawny owls during eight consecutive years (2005-2011), while experimentally manipulating brood sizes of differently coloured individuals to modify the level of parental workload (Roulin *et al.* 2011c). This experiment was repeated during four consecutive years (2007-2010) to investigate whether the impact of rearing experimentally enlarged (or reduced) broods over consecutive years increases the likelihood to detect morph-specific life history strategies. These predictions should be particularly pronounced in males since most prey items brought to offspring are hunted by males (Galeotti 2001).

METHODS

Study area and reproductive success

The present monitoring was conducted between 2004 and 2011 within a 911km^2 study area in western Switzerland, where we installed 366 nest boxes in forest patches of at least 4'000m^2; the minimal distance between two nest boxes was 627m. These managed forest patches are located at a mean altitude of 672m (range: [458 – 947m]) and are composed mainly of beeches (*Fagus sylvatica*), oaks (*Quercus* spp.) and pure spruce (*Picea abies*; Roulin *et al.* 2011c). Between 2005 and 2011, 694

distinct clutches were recorded (138 breeding pairs in 2005, 57 in 2006, 128 in 2007, 91 in 2008, 72 in 2009, 149 in 2010 and 59 in 2011). Overall, 548 of these 694 broods generated at least one hatchling (79.0%), while 436 broods produced at least one fledgling (62.8%). Clutch size varied between one and eight eggs (mean ± sd = 3.78 ± 1.51 eggs), laid between February 18 and May 29 (mean ± sd = April 6 ± 16 days). When considering nests where at least one egg hatched, 71% of the eggs hatched. The mean number of fledglings per brood was 2.1 ± 2.04.

Measurement of plumage coloration

The tawny owl has heterogeneous plumage colour patterns that vary continuously in the degree of reddishness (Roulin *et al.* 2005; Gasparini *et al.* 2009a). This species is nevertheless considered as colour polymorphic in the literature and textbooks (Glutz von Blotzheim & Bauer 1980; Galeotti 2001; Brommer *et al.* 2005), leading us to employ the same terminology. Scores of adult plumage coloration were visually determined in the field on the basis of five distinct colour morphs (1 = reddish, 2 = reddish-brown, 3 = brown, 4 = brown-grey, 5 = grey; Roulin *et al.* 2005). Because we recently found that a so-called grey coloration reflects an absence of melanin pigments stored in feathers, the scale we used in previous papers is a bit counter-intuitive (a larger score indicates a lower degree of melanism). To obtain a common sense scale from light to dark melanic, we therefore multiplied the above colour scores by -1. This scoring method is highly reliable, as evidenced by high inter-annual repeatability of colour scores visually assigned to the same individuals between 2005 and 2010 (r = 0.89 ± 0.02, $F_{174,383}$ = 13.76, P < 0.0001; Lessells & Boag 1987). Moreover, visual scores were strongly correlated with coloration measurements performed with a spectrophotometer (Pearson's correlation: r = -0.8, n = 302, P < 0.0001). Nevertheless, visual scoring into discrete numbers of morphs takes into consideration the whole plumage coloration and is therefore more representative of bird coloration than measuring coloration on the basis of three back feathers (Brommer *et al.* 2005). Consequently, we considered only visual colour scores in statistical analyses.

Experimental procedure

Out of the 694 clutches monitored between 2005 and 2011, 388 broods were matched into pairs according to similar hatching date (Pearson's correlation, P < 0.0001). In 2005, 2006 and 2011, we exchanged on average 2.84 (± 0.97) eggs from a nest E (enlarged) to nest R (reduced), while 4.11

(± 1.0) eggs were exchanged from nest R to nest E. Between 2007 and 2010, 274 broods were matched in pairs to experience partial cross-fostering experiment coupled with a brood size manipulation treatment. In this case, we exchanged on average 1.75 (± 0.86) nestlings from a nest E to nest R, while 2.75 (± 0.86) nestlings on average underwent the opposite exchange. In both experimental procedures, half of the nests were thus experimentally enlarged by one nestling (i.e. nests E), while the other half of nests was experimentally reduced by one nestling (i.e. nests R). Clutch sizes of enlarged and reduced nests were initially similar (Student's *t*-tests; $t_{1,272}$ = 1.43, P = 0.15). We successfully created broods with a different number of nestlings, as more nestlings fledged from enlarged than reduced treatment (mean ± sd, 3.95 ± 1.92 vs. 2.55 ± 1.61, respectively; Student's *t*-test: $t_{1,270}$ = -6.44, $P < 0.0001$). When nestlings were 10 days of age (mean ± sd: 9.98 ± 10.5), we captured both parents. Females were captured in the nest box during daylight hours (8am – 6pm, n = 274 captures), while males were captured at night when provisioning their brood (10pm – 6am, n = 252 captures). Note that the probability of capturing males was independent of the brood size manipulation treatment (logistic regression: χ^2 = 0.79, df = 1, P = 0.51). To estimate nestling growth, nestlings were recaptured every five days until they fledged at approximately 25 days of age (mean number of captures per individual ± sd = 5.0 ± 1.2). Upon capture, individuals were weighed to the nearest g, their left wing length measured to the nearest 1mm and left tarsus to the nearest 0.1mm. Note here that adult wing and tarsus lengths were neither associated with their plumage coloration (Student's *t*-tests, *P*-values > 0.47).

Plumage coloration, wing and tarsus lengths of breeding adults (197 males and 193 females) did not differ between the two brood size treatments (Student's *t*-tests, *P*-values > 0.08). Within each pair of nests, plumage coloration of foster and biological parents did not significantly resemble each other (female: r = -0.02, P = 0.84; male: r = -0.16, P = 0.09), while intra-nest pairing with respect to coloration was not assortative in both treatments (Pearson's correlations; nests R: r = 0.01, n = 116, P = 0.95, nests E: r = 0.01, n = 126, P = 0.94).

Statistical procedure

Using our long-term dataset of population monitoring, we examined whether tawny owls showed morph-specific survival rates, based on capture-mark-recapture techniques (Lebreton *et al.* 1992). This approach accounts for the fact that individuals may not have been captured in years where they were in fact alive. Even though the experiment concretely started in 2007, we included data from 2005 to 2011 to increase the precision of the base-line survival estimates of non-manipulated owls. The results did not qualitatively change if we excluded the data collected in 2005, 2006 and 2011. Our starting model was the classical Cormack-Jolly-Seber model with time dependent survival and recapture probabilities for male and female tawny owls. We first simplified the basic structure of this model and selected the most parsimonious structure based on the sample-size adjusted Akaike's Information Criterion (AIC_c). We then included colour score as a time-constant individual covariate, and treatment (enlarged, reduced, or not manipulated) as a time-varying individual covariate in the model. We also examined possible interactions between tawny owl coloration and treatment, and the three-way interaction between sex, coloration and treatment. Finally, we examined whether treatment in the previous year had an effect on present survival, and whether this interacted with the present brood size treatment to see whether sustained exposure to one treatment had particularly large effects. All models were fitted in MARK 6.0 (White & Burnham 1999).

To examine colour-specific trade-off resolution between offspring number and quality, we considered a subset of 247 successful (producing at least one fledgling, $n = 235$) or predated broods ($n = 12$). Indeed, out of the 274 broods that were experimentally manipulated between 2007 and 2010, we were able to capture both breeding adults for 252 broods. In five cases, male captures potentially led to nestling desertion, constraining us to exclude these broods from statistical analyses. We still considered predated broods in our analysis since nest defence behaviour is likely to be involved in key life-history trade-offs (Montgomerie & Weatherhead 1988; Wolf *et al.* 2007), since unpublished results showed that the intensity of nest defence behaviour is correlated with coloration in the tawny owl (Da Silva *et al.* in prep., ***Annexe 1***). To test whether plumage coloration of the rearing parents was associated with variation in fledgling production, we performed a mixed-model ANCOVA including the number of fledglings as dependent variable. Hatching date of the first egg (*Hatching date*) and

brood size before manipulation (*Brood size at hatching*) were introduced as covariates in the model, while brood size manipulation treatment (hereafter *Brood size manipulation* or *BSM*) and coloration of the parents that rear the brood (*Father colour, Mother Colour*) were entered as independent variables, plus all possible interactions between the latter three variables. Since raising an enlarged brood over several consecutive years may have devastating long-term effects on reproductive success, we considered males for which we manipulated their brood in two successive years. We performed a mixed-model ANCOVA including the number of fledglings in the second breeding year as dependent variable. Using hatching date of the first egg (*Hatching date in year 2*) and brood size before manipulation in the second breeding year (*Brood size at hatching in year 2*) as covariates, we introduced class of successive brood size manipulation treatments in year X_1 and year X_2 (*Class of successive treatments*, i.e. enlarged-enlarged; reduced-reduced; enlarged-reduced and reduced-enlarged) and rearing male coloration as independent variables, plus the two-way interaction between both variables. To investigate colour-specific variation in offspring quality, we performed a mixed-model ANCOVA including nestling body mass (i.e. just before they fledged) as dependent variable. We introduced time when nestlings were captured (*Hour of the day*), nestling wing length (*Nestling wing length*) and gender (*Nestling sex*) as covariates. As independent variables, we entered nestling cross-fostering status (*Cross-fostered*, i.e. whether nestlings were raised by biological or foster parents), brood size manipulation treatment (*Brood size manipulation*) and rearing male coloration (*Father colour*), plus all possible interactions between these three variables.

Finally, we tested whether fledgling recapture rate (as breeding adult in following years) covaries with plumage coloration of the rearing father. We performed a GLMM model including recapture rate per brood (i.e. number of recaptured individuals reared in the same brood weighted by the number of nestlings that fledged from this brood) as dependent variable. Mean body mass of fledglings (per brood) and hatching date were introduced as covariates, while brood size manipulation (*BSM*) and coloration of the rearing father (*Father colour*) were entered as independent variables, plus the two-way interaction between both variables.

For each mixed-model, we controlled for pseudoreplication by introducing year and male identity as random factors. We ran full factorial models and then dropped non-significant terms

(starting with non-significant interactions) in a stepwise manner in order to produce minimum adequate final models. Statistical tests are two-tailed, and significance level is set to 0.05. In all models, residuals were normally distributed, and variances were homogeneous between treatments. Mixed-models were performed using JMP IN 8.0 and SAS 9.1.3.

RESULTS & DISCUSSION

Colour-specific survival

According to the most parsimonious model structure that kept survival constant across sexes and years and allowed the recapture rates to vary over the years and between the sexes (Model 7 in Table 1.1), survival was 0.69 (se = 0.02). Interestingly, the addition of colour scores to this parsimonious model resulted in a better fit, indicating that dark melanic individuals survived better than light-melanic ones (Models 1 to 3 in Table 1.1; and Figure 1.1). We found no evidence that the brood manipulation experiment affected survival, either directly (Model 4 in Table 1.1) or through interactions with colour or sex (Models 5 and 8 in Table 1.1).

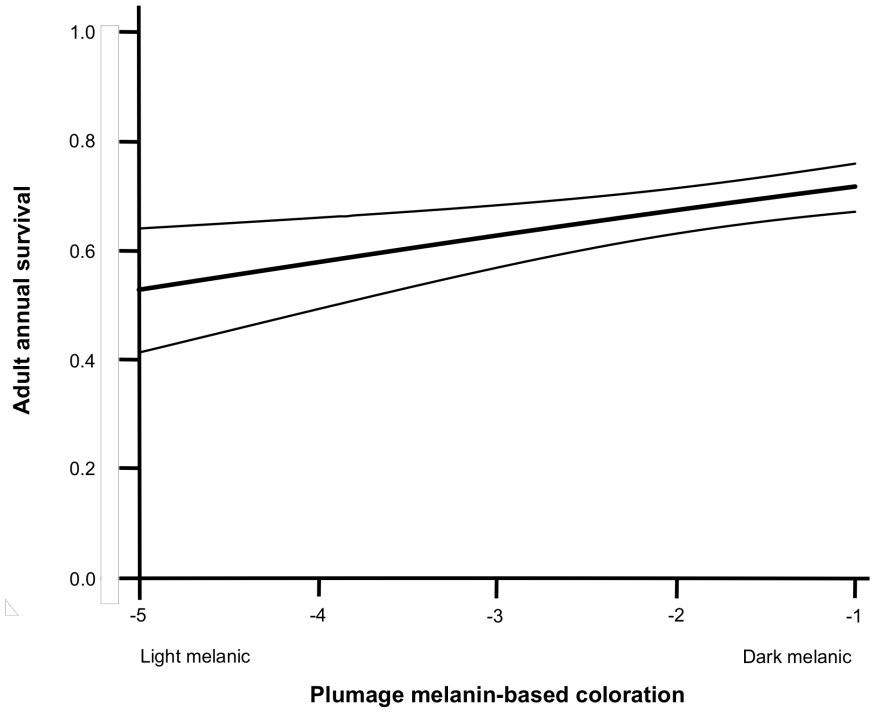

Figure 1.1: Adult annual survival rate in relation to melanin-based plumage coloration | Survival estimates are derived capture-mark-recapture techniques (model 1 in Table 1.1).

Colour-specific reproductive success

Alternative survival rates may thus promote colour-specific life history strategy in our local population, particularly with respect to the resolution of the trade-off between offspring number and quality. Accordingly, we found that the effect of father (but not mother) coloration on reproductive success (i.e. number of fledglings) differed between enlarged and reduced broods (Model A in Table 1.2: $F_{1,238.5} = 4.57$, $P = 0.034$). In enlarged broods, light melanic fathers produced more fledglings than darker melanic ones (Model B in Table 1.2: $F_{1,88.34} = 5.135$, $P = 0.026$), while in the reduced treatment number of fledglings was not significantly associated with male coloration (Model C in Table 1.2: $F_{1,95.77} = 1.07$, $P = 0.30$; Figure 1.2).

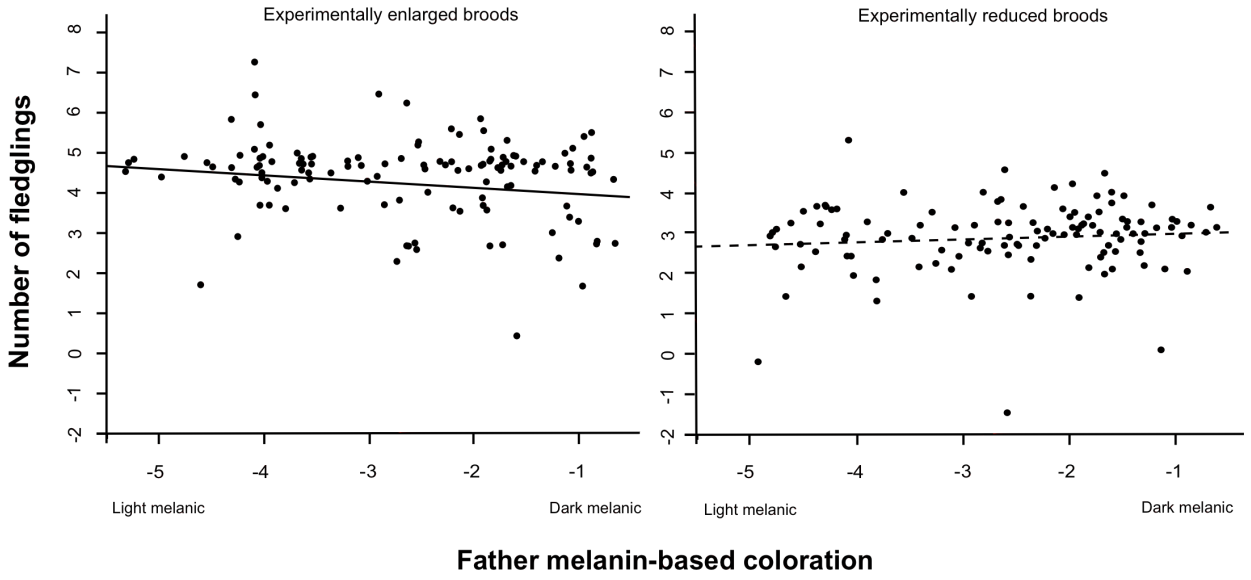

Figure 1.2: Number of offspring in relation to father melanin-based coloration in experimentally enlarged and reduced broods in the tawny owl ⎪Values are derived from models B and C of Table 1.2. Significant linear regressions are illustrated by straight regression line, whereas non-significant linear regressions are illustrated by dashed regression line.

When we considered males for which we manipulated their brood in two successive years, the interaction between the category of successive treatments and father coloration on the number of fledglings produced in the second year (i.e. after males experienced two consecutive experimental treatments) was strong (Model A in Table 1.3; $F_{3,20} = 6.78$, $P = 0.0025$). Light melanic males produced more fledglings than darker melanic ones after having raised an enlarged brood in two consecutive

years (Model B in Table 1.3; $F_{1,6.376} = 20.75$, $P = 0.003$; Figure 1.3), while the opposite was true when raising a reduced brood in two consecutive years (Model C in Table 1.3; $F_{1,5.454} = 11.55$, $P = 0.017$; Figure 1.3). This analysis shows that light melanic males better cope with two consecutive energy-demanding breeding seasons (i.e. two consecutive enlarged treatments), while darker melanic males better manage two consecutive reduced broods (and thereby potentially lower energy-demanding reproductive effort). Of particular interest, the additive effect of the brood size manipulation provides strong evidence of the flexible and constant life-history strategy of light and dark melanic male tawny owls, respectively. Light melanic males seem to increase their parental investment to maximize offspring productivity when necessary (e.g. enlarged-enlarged treatment), while skipping some breeding seasons (Roulin *et al.* 2003) or reducing parental care when broods were smaller (e.g. reduced-reduced treatment; Figure 1.3). In contrast, dark melanic males tend to keep a constant strategy with respect to parental investment in offspring productivity, regardless of the reproductive conditions. Indeed, whatever the brood size treatment, dark melanic males produced about 2-3 fledglings, whereas light melanic males produced on average 0.75 fledglings when brood was experimentally reduced and 5 when enlarged (Figure 1.3).

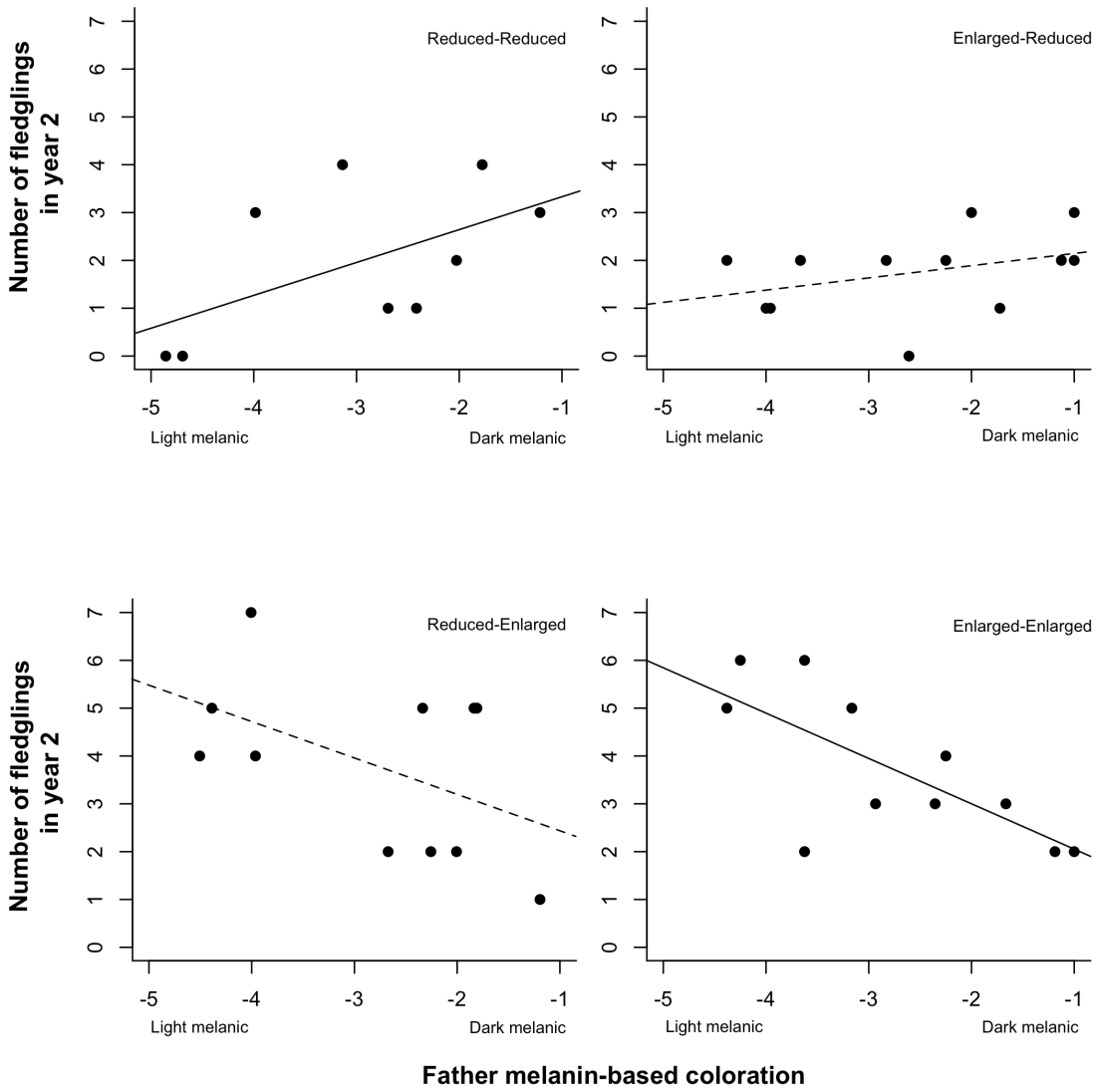

Figure 1.3: Number of fledglings in relation to melanin-based coloration of the father and brood size manipulation experiment in the tawny owl | We considered number of fledglings produced after two years of having manipulated brood size for the same breeding males. Enlarged-Enlarged: number of fledglings in year 2 for males with their brood size enlarged in years 1 and 2 (linear regression, n= 10, *P* = 0.003), Reduced-Reduced: with their brood size reduced in years 1 and 2 (linear regression, n= 9, *P* = 0.017), Reduced-Enlarged: with their brood size reduced in year 1 and enlarged in year 2 (linear regression, n=11, *P* = 0.24), and Enlarged-Reduced: with their brood size enlarged in year 1 and reduced in year 2 (linear regression, n= 12, *P* = 0.89). Significant linear regressions are illustrated by straight regression line, whereas non-significant linear regressions are illustrated by dashed regression line.

Colour-specific offspring quality

The observation that light melanic males produce more offspring than dark melanic ones in experimentally enlarged broods should come at a cost in terms of offspring quality. In agreement with previous study (Roulin *et al.* 2004), we detected a significant interaction between the brood size manipulation experiment and coloration of the rearing male on fledging body mass ($F_{1,341.4}$ = 12.25, P = 0.0005; Table 1.4), independently of nestling origin (i.e. 'Cross-fostered' factor was not significant,

alone or in interaction with coloration of foster father and/or the brood size manipulation, *P*-values > 0.10). In the enlarged treatment, dark melanic males produced fewer offspring that were heavier than light melanic ones, whereas no relationship between offspring body mass and father coloration was detected in the reduced treatment (Figure 1.4 and Table 1.4).

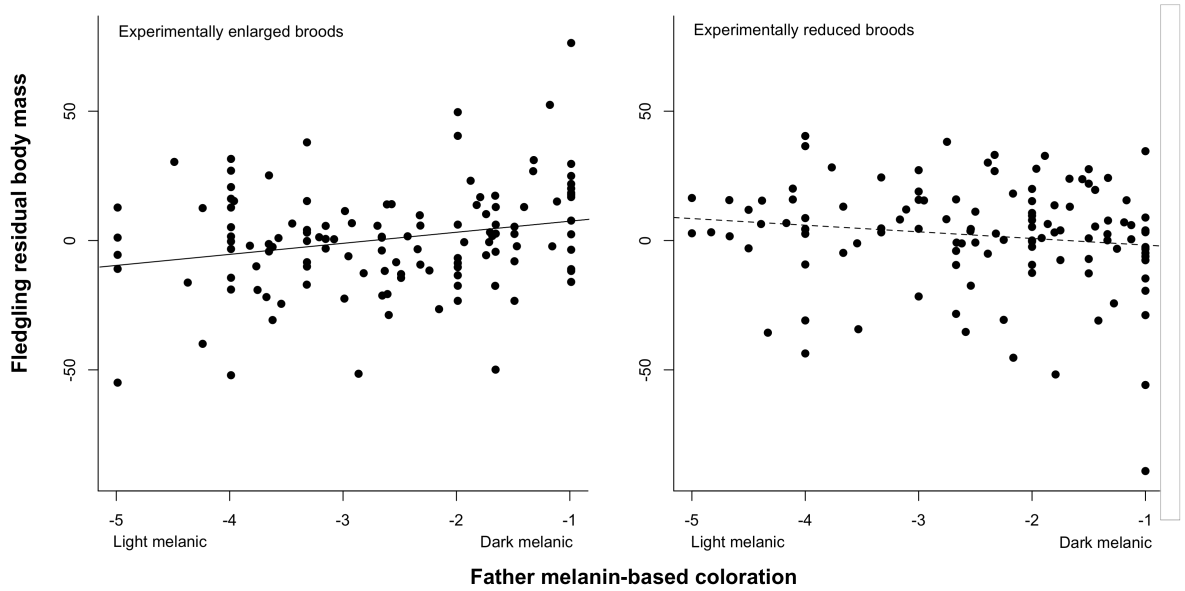

Figure 1.4: Fledging body mass in relation to father melanin-based coloration and brood size manipulation experiment in the tawny owl | For this graph, but not for the analyses, we calculated mean offspring body mass for each male, while controlling for sex, wing length, time of the day and year (i.e. residual body mass). The relationship is significant in experimentally enlarged broods (Pearson's correlation: *r* = -0.23, *n* = 123 different males, *P* = 0.008) but not in experimentally reduced broods (*r* = 0.10, *n* = 115 different males, *P* = 0.28). Significant linear regressions are illustrated by straight regression line, whereas non-significant linear regressions are illustrated by dashed regression line.

Colour-specific recruitment rate

To further investigate whether dark melanic males produce offspring in better condition than lighter melanic conspecifics, we examined whether the likelihood of being recruited in the local breeding population is associated with father coloration. Accordingly, we found a significant interaction between brood size manipulation experiment and coloration of the rearing male on weighted recapture rate per brood (Model A in Table 1.5: $F_{1,37} = 5.39$, $P = 0.031$), independently of the mean offspring body mass ($P = 0.96$, Table 1.5) Nestlings raised by dark reddish males were more

frequently recruited when experiencing an experimentally reduced brood (Model C in Table 1.5, $F_{1,8}$ = 8.38, P = 0.02), whereas in the enlarged treatment there was no relationship between father coloration and the probability of producing at least one recruit (Model B in Table 1.5, $F_{1,12}$ = 0.2, P = 0.66). Note here that this outcome is not a consequence of colour-specific dispersal behaviour, the dispersal distance per recruited fledgling (i.e. distance between nest of fledging and nest of recapture as breeding adult) being independent of recruit coloration ($F_{1,95}$ = 1.16, P = 0.28) or the coloration of the male that reared them ($F_{1,93}$ = 0.38, P = 0.54). Given the finding that nests of dark reddish males were less often depredated (Da Silva *et al.* in prep., ***Annexe 1***), all these outcomes argued for greater offspring quality in broods reared by dark melanic male tawny owls compared to light melanic conspecifics.

Implications on the evolution of colour-specific paces of life

The hypothesis that individuals differ in their pace of life implies that they should also differ in a suite of behavioural, ecological and physiological attributes to sustain either a fast or slow pace of life (Ricklefs & Wikelski 2002; Reale *et al.* 2010). The finding that dark melanic tawny owls have a higher survival prospect than lighter conspecifics suggests that the former individuals have a slower pace of life than the latter. If this hypothesis holds, life-history theory states that individuals have to allocate optimally resources among life-history traits (MacArthur & Wilson 1967; Stearns 1992), a decision rule that can differ between individuals (Pianka 1970; Mcleod *et al.* 1981). In this context, dark melanic owls should adopt rather *K*-life history strategy and lighter melanic owls a rather *r*-life history strategy (Pianka 1970). In line with this prediction, the resolution of the trade-off between offspring number and quality differed between dark and light melanic male tawny owls. Dark melanic males showed a slow-pace of life, characterized by the production of few high quality offspring that were more often recruited in the local breeding population than offspring of light melanic males. These findings are also concordant with previously published results. Indeed, we already showed that light melanic owls are better able to cope with stressful environmental (Piault *et al.* 2009; Karell *et al.* 2011a; Karell *et al.* 2011b) and reproductive conditions (Roulin *et al.* 2003; Roulin *et al.* 2008b; Roulin *et al.* 2011c), an energy-demanding strategy that allows individual to be constantly adapted to

the prevailing environmental conditions. When the environment permits (e.g. high food availability), these light-coloured males are able to increase their parental investment to maximize offspring production (i.e. *r*-selected traits), but at the expense of offspring quality. However, when environmental conditions deteriorate, they are likely to reduce their parental care (e.g. nest defence behaviour (Da Silva *et al.* in prep., ***Annexe 1***)) or occasionally skip reproduction (Roulin *et al.* 2003). In contrast, dark melanic males adopt a slower, but more homogenous strategy, taking good care of a limited number of high-quality offspring (i.e. *K*-selected traits), which facilitates resource investment in maintenance traits (Gasparini *et al.* 2009a) and, in turn, survival.

Although flexibility in behaviour should allow organism to finely track changes in their environment, behavioural plasticity as shown by light coloured males can entail substantial costs (DeWitt *et al.* 1998). Behaving optimally in any situation implies the allocation of resources by trying some suboptimal behaviour, especially if there is little information about the environment. A game theoretic model (McElreath & Strimling 2006) demonstrates that situations where individuals have noisy information about environmental conditions, combined with differences in individual state (e.g. morphological differences such as melanin-based coloration), can lead to evolutionarily stable strategies within a single population. Depending on environmental stochasticity in habitat quality, individuals better perform by displaying alternative behaviours, especially those affecting life history decisions. Adopting a fixed behavioural tactic, as shown by dark melanic males, can be the best solution rather than trying to predict and adapt to all situations as performed by lighter melanic conspecifics (Sih *et al.* 2004; Bell 2007; Wolf *et al.* 2007). This proposition is consistent with our findings that dark melanic males survive longer and produce more recruits than light melanic ones. Here, we suggest that different genetically-inherited colour variants can be maintained within populations because of temporal fluctuations between *r*- and *K*- selective regimes (Hedrick 1986), induced by environmental fluctuations in food availability. When food is abundant, the carrying capacity of the population is likely to be higher than the total number of breeding pairs in our population of tawny owls, a context favouring fast-*r* strategists (i.e. light melanic males). When food is scarce, the carrying capacity of the population is drastically reduced, favouring the competitive slow-*K* strategists (i.e. dark melanic males). Interestingly, the outcomes rising from our intra-

population study seem also applicable at higher taxonomic level. In line with the colour-specific fast-*r* and slow-*K* life histories observed in our population, a recent study suggested that light melanic owl species are adapted to cold climates and dark melanic ones to warmer climates (Roulin *et al.* 2011b). Climatic amplitudes are more pronounced in northern rather than southern climes, resulting in stronger stochastic variation that are favourable for light melanic organisms and their fast and flexible life-history strategy. In a context of global warming, the observed increase in reddish morph frequency (Karell *et al.* 2011b) is not surprising since warmer temperatures are expected to stabilize the environmental conditions. This may lead to directional selection favouring the slow, but stable life-history strategy adopted by dark melanic individuals.

Acknowledgements

We are grateful to Dr. Romain Piault, Pierre-Alain Ravussin, Yves Ménétrey, Dominique Vuille, Sophie Cotting, Sabrina Joye, Esther Gonzalez, Ester Luzio, Arnaud Da Silva, Mélissa Noll, Olympe Arévalo, Charlène Ruppli, Dr. Amélie Dreiss and Dr. Anne-Lyse Ducrest for assistance in the field, and Dr. Pierre Fontanillas and Dr. Thomas Broquet for useful comments on previous versions of the manuscript. This study was financially supported by the National Research Foundation of South Africa and the Swiss National Science Foundation (PPOA-102913 and 3100AO_120517 to Alexandre Roulin).

TABLES

Table 1.1: Selection of capture-mark-recapture models of annual survival in relation to melanin-based coloration and experimental brood size manipulation treatments in adult tawny owls | The models consisted in two features, namely the modelling survival (ϕ) and the modelling recapture rates (P). We examined the effects of year, sex, coloration (col) and brood size treatment (treat), either as main effects only (symbolised by '+') or as main effects and interactions (*). The different treatments were brood size enlargement, reduction, or not manipulated, which were entered as time-varying covariates. Broods of owls that were not captured were not manipulated. Model selection was based on Akaike's Information Criterion. Akaike weights give the relative support a particular model has in relation to the others. K is the number of estimated parameters.

	Model	ΔAIC_c	Akaike Weights	K	Deviance
1	ϕ (col)P(year+sex)	0.000	0.361	9	1471.54
2	ϕ (sex*col)P(year+sex)	0.586	0.269	11	1468.01
3	ϕ (sex+col)P(year+sex)	0.938	0.226	10	1470.42
4	ϕ (col+treat)P(year+sex)	3.215	0.072	11	1470.64
5	ϕ (sex*col*treat)P(year+sex)	5.460	0.024	19	1456.21
6	ϕ ()P(year+sex+col)	6.327	0.015	9	1477.87
7	ϕ ()P(year+sex)	6.924	0.011	8	1480.51
8	ϕ (col*treat)P(year+sex)	7.110	0.010	13	1470.4
9	ϕ (sex)P(year+sex)	7.919	0.007	9	1479.46
10	ϕ (sex)P(year)	9.280	0.003	8	1482.87
11	ϕ (year*sex)P(year)	17.072	0.000	17	1472.02
12	ϕ (year*sex)P(year*sex)	22.538	0.000	22	1466.94

Table 1.2: Number of fledglings in relation to father melanin-based coloration of the father and brood size manipulation experiment in the tawny owl | In Model A the significant interaction between father coloration and brood size manipulation is explained by light melanic males producing more fledglings in experimentally enlarged broods (Model B), while in reduced broods male colour was not associated with number of fledglings (Model C). Brood size was manipulated one or two days after hatching and the number of fledglings was defined as the number of 25 days old nestlings. Mother plumage coloration was initially entered as explanatory variable in the mixed-model, but was not significant.

Number of fledglings in year 1	*F*	*df*	*P*
A. Full model including 247 males (197 different individuals) rearing enlarged or reduced broods in year 1			
Final model			
Hatching date	10.28	1,173.2	0.0016
Brood size at hatching	184.67	1,237.7	<0.0001
Father colour	0.82	1,155.9	0.37
Brood size manipulation in year 1	221.41	1,238	<0.0001
Father colour*Brood size manipulation in year 1	4.57	1,238.5	0.034
Rejected terms			
Mother colour	0.13	1,154.7	0.72
Mother colour*Brood size manipulation in year 1	1.66	1,233.5	0.20
Father colour*Mother colour	0.04	1.182.3	0.84
Father colour*Mother colour*Brood size manipulation in year 1	0.85	1,232.5	0.36
B. Model including 126 males (109 different individuals) rearing an enlarged brood in year 1			
Hatching date	1.53	1,107.7	0.22
Brood size at hatching	127.28	1,117.7	<0.0001
Father colour	5.13	1,88.34	0.026
C. Model including 121 males (105 different individuals) rearing a reduced brood in year 1			
Hatching date	12.79	1,49.96	0.0008
Brood size at hatching	67.36	1,107.8	<0.0001
Father colour	1.07	1,95.77	0.30

Mixed models ANCOVAs with year and male identity as two random variables. Mother identity was initially entered as random factor in model A, but was removed from the model. The final model A shows significant terms and terms involved in significant interactions, whereas rejected terms were not significant. More offspring fledged early than late in the season (term hatching date) and in nests with a larger number of hatchlings before broods were manipulated (term brood size at hatching).

Table 1.3: Number of fledglings in relation to father melanin-based coloration and brood size manipulation experiment carried out in two successive years in the tawny owl |The significant interaction between father coloration and class of successive brood size treatments in Model A is explained by dark melanic males producing more fledglings than light melanic ones after having reared an experimentally reduced brood in two successive years (model B) and by light melanic males producing more fledglings than dark melanic ones after having reared an experimentally enlarged brood in two successive years (model C).

Number of fledglings in year 2	*F*	*df*	*P*
A. Mixed model ANCOVA including 42 males (31 different individuals) rearing experimentally manipulated broods in two consecutive years			
Hatching date in year 2	1.32	1,28.91	0.26
Brood size at hatching in year 2	38.82	1,15.98	<0.0001
Father colour	12.49	1,32.47	0.0013
Class of successive treatments	10.06	3,23.74	0.0002
Father colour*Class of successive treatments	6.78	3,20	0.0025
B. Multiple regression analysis including 10 different males rearing an enlarged brood in year 1 and an enlarged brood in year 2			
Brood size at hatching in year 2	4.54	1,6.871	0.07
Father colour	20.75	1,6.376	0.003
C. Multiple regression analysis including 9 different males rearing a reduced brood in year 1 and a reduced brood in year 2			
Brood size at hatching in year 2	11.71	1,4.701	0.021
Father colour	11.55	1,5.454	0.017
D. Multiple regression analysis including 11 different males rearing a reduced brood in year 1 and an enlarged brood in year 2			
Brood size at hatching in year 2	7.11	1,8	0.029
Father colour	1.74	1.5.519	0.24
E. Multiple regression analysis including 12 different males rearing an enlarged brood in year 1 and a reduced brood in year 2			
Brood size at hatching in year 2	1.56	1,8.888	0.24
Father colour	0.02	1,7.334	0.89

Mixed models ANCOVAs with year and male identity as two random variables in model A and year as single random variable in model B, C, D and E. The final model A shows significant terms and terms involved in significant interactions, whereas rejected terms were not significant. More offspring fledged early than late in the season (term hatching date) and in nests with a larger number of hatchlings before broods were manipulated (term brood size at hatching).

Table 1.4: Fledgling body mass in relation to father melanin-based coloration and brood size manipulation experiment in the tawny owl | The significant interaction between father coloration and treatment in Model A is explained by dark melanic males producing heavier fledglings than light melanic males in experimentally enlarged broods (Model B) while in the reduced treatment male colour was not associated with fledging body mass. Brood size was manipulated one or two days after hatching and fledglings were counted as the number of 25 days old nestlings.

Fledglings body mass	*F*	*df*	*P*
A. Full model including 837 fledglings reared in 128 enlarged and 114 reduced broods in year 1			
Final model			
Hour of the day	22.38	1,537.9	<0.0001
Wing length	68.43	1,828.9	<0.0001
Sex	360.84	1,783.6	<0.0001
Father colour	0.0004	1,171	0.98
Brood size manipulation in year 1	2.18	1,328.8	0.14
Father colour*Brood size manipulation in year 1	12.25	1,341.4	0.0005
Rejected terms			
Cross-fostered	0.12	1,735.3	0.73
*Cross-fostered*Brood size manipulation in year 1*	2.65	1,750	0.10
*Cross-fostered*Father colour*	1.91	1,739.3	0.17
*Cross-fostered*Father colour*Brood size manipulation in year 1*	0.27	1,745.4	0.60
B. Model including 508 fledglings reared in 128 enlarged broods in year 1			
Hour of the day	10.28	1,256.7	0.0015
Nestling wing length	69.31	1,493.2	<0.0001
Nestling sex	276.98	1,435	<0.0001
Father colour	4.11	1,106.1	0.045
C. Model including 329 fledglings reared in 114 reduced broods in year 1			
Hour of the day	5.82	1,176.8	0.0081
Nestling sex	165.4	1,303.7	<0.0001
Father colour	0.47	1,1.885	0.57
Nestling wing length	22.68	1,1.367	0.17

Mixed models ANCOVAs with year and foster brood identity as random variables. The final model A shows significant terms and terms involved in significant interactions, whereas rejected terms were not significant. In an initial model (not presented), the terms 'hatching date' and 'brood size at hatching' were not significant. Offspring were heavier when sampled early than late during the day (term 'Hour of the day'), when their wings were long rather than short (term 'Nestling wing length', i.e. a reliable estimator of nestling age; $F_{1,6464}$ = 68893.0; $P < 0.0001$). Female nestlings were also heavier (mean ± sd = 362.2 ± 31.3) than male nestlings (mean ± sd = 324.6 ± 27.3; term 'Nestling sex').

Table 1.5: Weighted recapture rate per brood in relation to father melanin-based coloration and brood size manipulation experiment in the tawny owl | The recapture rate per brood was estimated as the number of recaptured individuals reared in the same brood weighted by the number of nestlings that fledged from this brood. The significant interaction between father coloration and treatment in Model A is explained by a higher recapture rate of fledglings reared by reddish males when the brood was experimentally reduced (Model C), whereas no difference in recapture rate was found in the enlarged treatment (Model B).

Number of fledgling recaptured, weighted by the number of fledglings	*F*	*df*	*P*
A. Full model including the number of fledglings recaptured in 217 broods			
Final model			
Father colour	6.31	1,37	0.017
Brood size manipulation in year 1	5.92	1,37	0.02
Father colour* Brood size manipulation in year 1	5.39	1,37	0.031
Rejected terms			
Mean body mass of fledglings	0.00	1,37	0.96
Hatching date	2.03	1,37	0.16
B. Model including 116 enlarged broods			
Father colour	0.2	1,12	0.66
C. Model including 101 reduced broods			
Father colour	8.38	1,8	0.02

Mixed models ANCOVAs with year and male identity as random variables. The final model A shows significant terms and terms involved in significant interactions, whereas rejected terms were not significant.

CHAPTER 2

Reproductive effort induces covariation between melanin-based coloration and oxidative balance in the tawny owl (*Strix aluco*)

Guillaume Emaresi[*], Isabelle Henry, Esther Gonzalez, Pierre Bize[‡], and Alexandre Roulin[‡]

Department of Ecology and Evolution, Biophore building, University of Lausanne, 1015 Lausanne, Switzerland

ABSTRACT

Oxidative stress is known to increase with reproductive effort. Yet, it is poorly understood whether individuals from the same population vary in their oxidative status under different reproductive conditions. We investigated this issue in relation to melanin-based plumage coloration in adult tawny owls (*Strix aluco*). We manipulated brood size in 94 nests in order to induce differences in reproductive investment, and in turn variation in oxidative stress assessed with red blood cell (RBC) markers. Our results show that light melanic males (the sex assuming offspring food provisioning) produced more reactive oxygen species (ROS) than darker conspecifics, but only when rearing an enlarged brood. We also found that in both sexes light melanic individuals have a larger pool of intracellular antioxidant glutathione ($_{t}$GSH) than darker owls under relaxed reproductive conditions (i.e. reduced brood), but not when investing substantial effort in current reproduction (enlarged brood). Finally, male and female oxidative status was differently affected by the brood size manipulation experiment (i.e. RBC resistance to free radicals), but independently of plumage coloration. We conclude that under stressful reproductive conditions lighter melanic owls overproduced ROS (especially among males) and were not able to generate large GSH pool as observed when rearing reduced brood. This suggests that differently melanic tawny owls differentially resolve the trade-off between current reproduction and maintenance, at least with respect to antioxidant response. However, according to RBC resistance to free radicals, these strategies developed by dark and light melanic owls appear to be equally successful, since they equally suffered from oxidative damage in the two brood size treatments.

INTRODUCTION

Cell respiration is fundamental to ensure survival, but generates simultaneously reactive oxygen species (ROS), which can induce physiological damages (Frisard & Ravussin 2006; Schallreuter *et al.* 2011). For example, ROS can cause DNA point mutations (Twigg *et al.* 1998), cell membrane rupture (Halliwell 1992) and oxidation of amino acids (Halliwell & Gutteridge 1989). Aerobe organisms have developed various defence systems to counter the deleterious effects of ROS. The ratio between ROS production and these antioxidant defence mechanisms is critical as an over-production of ROS exposes organisms to the so-called oxidative stress (Finkel & Holbrook 2000). Cell oxidative status therefore depends on numerous factors such as ROS production, tissue susceptibility to free radicals and strength of the defence and repair systems (Frisard & Ravussin 2006).

Despite the growing interest in the role of oxidative stress in mediating reproductive investment (Cohen *et al.* 2008; Monaghan *et al.* 2009; Metcalfe & Alonso-Alvarez 2010; Isaksson *et al.* 2011), the oxidative reaction norms of individuals adopting different reproductive strategies remain unclear (see for instance Garratt *et al.* 2011). Inter-individual variation in oxidative balance can be a direct consequence of genetic and environmental factors, but can also arise from alternative life history strategies, highlighting the diversity in trade-off resolutions between reproductive effort and oxidative equilibrium (Monaghan *et al.* 2009). In the zebra finch (*Taeniopygia guttata*) for instance, parents experiencing higher parental investment were more exposed to oxidative stress compared to those experiencing lower investment (Alonso-Alvarez *et al.* 2004; Wiersma *et al.* 2004). The sign and the magnitude of this complex link between the oxidative balance and life history traits can vary according to the species considered (Cohen *et al.* 2008), the sex of individuals (Sullivan *et al.* 2007; Bize *et al.* 2008), their age (Beckman & Ames 1998; Ashok & Ali 1999; Devevey *et al.* 2010) or the environmental conditions (Beaulieu *et al.* 2010). Thus, a central issue is to identify how different genotypes or phenotypes cope with oxidative stress, a key information to apprehend the evolution of individual-specific life history strategies.

Inherited inter-individual variation in the deposition of eumelanin (grey to black) and pheomelanin (yellowish to reddish) pigments in the integuments is common in the animal kingdom (Majerus 1998; Roulin 2004). Variation in coloration is often associated with behavioural and life

history traits (Roulin 2004), but also with physiological processes such as resistance to oxidative stress (Galvan *et al.* 2010; Roulin *et al.* 2011a), the sign and magnitude of these covariations differing between environments (e.g. Roulin *et al.* 2008b; Piault *et al.* 2009; Vergara & Fargallo 2011). This suggests that coloration can advertise either different physiological reaction norms depending on specific environmental conditions (e.g. Roulin *et al.* 2011c), or alternative life history strategies according to the environmental circumstances. This highlights the need of experimental studies where the environmental conditions (e.g. rearing conditions) experienced by individuals are manipulated to clearly identify the adaptive value of a specific colour pattern. For instance, if melanin-based coloration covaries with oxidative balance only when environmental conditions are poor, one can deduce that individuals displaying a given colour trait are particularly adapted to cope with stressful factors. Under this scenario, these individuals would outcompete conspecifics mainly when environmental conditions deteriorate.

In the tawny owl (*Strix aluco*), a species characterized by a continuous variation in melanin-based coloration (from light to dark reddish, 68% of the total variance in plumage coloration is due to pheomelanin and 21% to eumelanin; Gasparini *et al.* 2009a), the least reddish (hereafter referred to as 'light melanic') individuals better cope with stressful reproductive conditions than darker conspecifics, as demonstrated by their nestling growth (Roulin *et al.* 2008b; Piault *et al.* 2009) and parental investment (Roulin *et al.* 2011c), or survival under harsh environmental conditions (Karell *et al.* 2011b). This species is thus a prime model organism to investigate the complex links between heritable melanin-based coloration, oxidative balance and environmental conditions. Given these colour-specific strategies to cope with stressful conditions, we propose herein that differently coloured individuals differentially regulate their oxidative balance according to the environmental conditions. To test this hypothesis, we manipulated brood size in order to induce differences in the level of parental workload and thereby oxidative balance (Alonso-Alvarez *et al.* 2004; Wiersma *et al.* 2004; Christe *et al.* 2011). We evaluated oxidative status of adults rearing an experimentally enlarged or reduced brood through three quantitative measurements performed on red blood cells (RBC), namely ROS production, total intracellular GSH levels ($_{t}$GSH, a major intracellular antioxidant; Reddy *et al.* 1982; Halliwell & Gutteridge 1989) and resistance of cell membrane to free radicals.

METHODS

Model organism

The present study was conducted in 2010 on a population of tawny owls located in western Switzerland. This nocturnal species preys mainly upon small rodents and passerines in woodlands. This monogamous and territorial owl is sexually dimorphic in size (with females 20% bigger than males), with a strong partition of the reproductive roles between sexes. The female incubates the clutch during 28 days, and then remains in the nest to guard her hatchlings and to distribute among them prey items collected by the male. Once owlets are thermo-independent at 15-20 days of age, the female patrols around the nest to protect her offspring from potential predators, while helping her partner with provisioning food to the brood. Nestling growth rate and survival strongly depend on prey availability, especially wood mice (Roulin *et al.* 2009). Offspring leave the nest at 25–30 days of age but are fed by their parents until 90-120 days of age (Sunde 2008).

Experimental design

In 2010, females produced between two and seven eggs (mean ± sd: 5.13 ± 0.94), which hatched between February 21 and May 31 (mean ± sd: March 31 ± 13.3 days). On the basis of similar hatching dates, 94 nests were matched into pairs to decrease (reduced broods) or increase (enlarged broods) parental investment of breeding parents as described in a previous study (Roulin *et al.* 2011c). Among pairs of nests, brood sizes were randomly manipulated, leading to an exchange of 1.74 nestlings on average (sd = 0.6) from nest E (experimentally enlarged, n = 47) and placed in nest R (experimentally reduced, n = 47), while 2.74 hatchlings on average (sd = 0.6) underwent the opposite exchange (i.e. from nest R to nest E). Each family was thus composed of nestlings from two origins, disrupting thereby the potential covariation between brood size and parental genotype and phenotype. This brood size manipulation (BSM) treatment had the intended effect on reproductive effort and subsequently rearing conditions (ANCOVA accounting for initial brood size: $F_{1,141} = 141.9$, $P < 0.0001$); parents assigned to the enlarged brood treatment were rearing a larger number of 10-day-old owlets than those assigned to the reduced brood treatment (mean ± SE number of owlets per enlarged vs. reduced brood: 4.92 ± 1.27 vs. 3.47 ± 1.11). When nestlings were 10 days of age, we captured both parents to investigate the consequences of the brood size manipulation experiment on their body

condition and oxidative balance. Females were captured in the nest box during daylight (8am – 6pm, n = 94), while males were captured at night when provisioning their brood (10pm – 6am, n = 88). Note that 90 females and 24 males were already captured once during egg incubation (i.e. before performing the brood size manipulation). Because of the complexity of capturing male due to their fearful behaviour, we were able to recapture only two out of 24 males (i.e. before and after the brood size manipulation experiment). However, male capture procedure, either before or after experimental manipulation, was not biased with respect to melanin-based coloration (Student's t-test: $t_{83} = 0.83$, $P > 0.4$). Upon capture, each adult was weighed to the nearest g, their left wing length measured to the nearest 1mm and left tarsus to the nearest 0.1mm. We found no differences between the two brood size treatments in adult wing length and tarsus length (Student's t-tests, $P > 0.42$). From the wing vein, we collected 60-200ul of blood in EDTA tubes for ROS production and free radicals resistance analyses and in heparin tubes for $_t$GSH analysis. For each sample, 16µl of whole blood collected in EDTA tube were immediately diluted in 584µl of KRL buffer and stored at 4°C before further analyses, which occurred within 24 hours after blood collection. EDTA tubes were then centrifuged to separate red cells from blood plasma, the latter being collected in microtubes. Heparin, centrifuged EDTA and microtubes were finally frozen in dry ice in the field and transferred at -80°C within 12 hours until later analyses in the laboratory.

Assessment of plumage coloration

Although tawny owls vary continuously in the degree of reddishness, this species is usually considered as colour polymorphic in the literature (Glutz von Blotzheim & Bauer 1980; Galeotti 2001; Brommer *et al.* 2005) and hence we also employ this terminology. Scores of adult plumage coloration were determined either visually in the field or through spectrophotometric analysis in the laboratory. The visual scoring method, based on five distinct colour morphs (Roulin *et al.* 2005) is a reliable approach, evidenced by high inter-annual repeatability of colour scores visually assigned to the same individuals between 2005 and 2010 ($r = 0.89 \pm 0.02$, $F_{174,383} = 13.76$, $P < 0.0001$; Lessells & Boag 1987). In parallel, three feathers collected on the back of adult tawny owls were stuck together on black paper to capture reflectance spectra at four distinct positions using the S2000 spectrophotometer (Ocean Optics, Dunedin, FL) and a dual deuterium and halogen 2000 light source (Mikropackan,

Mikropack, Ostfildern, Germany). From these spectra, we calculated a mean brown chroma score for each individual as described by Montgomerie (2006). In the collected data, both scoring methods were tightly correlated (Pearson's correlation: r = -0.84, n = 270 individuals, $P < 0.0001$), comforting us to consider only visual coloration scores in the present study. This choice is based on the fact that visual colour scores provide a better overall estimation of plumage coloration (as already explained by Brommer *et al.* 2005) than brown chroma, which is assessed with only three back feathers of adult tawny owls, an approach that does not necessarily reflect entire body coloration.

Adult plumage coloration was neither associated with hatching date of the first egg, nor with brood size before and after the manipulation (Student's *t*-tests, *P*–values > 0.38). Although randomly assigned with respect to female coloration (78 out of 80 females being already captured once before their brood size was manipulated, Student's *t*-test, t_{78} = -0.66, P = 0.51), it appeared that males rearing a reduced brood tended to be darker melanic than those rearing an enlarged brood (brood sizes was manipulated without prior knowledge of male plumage coloration, t_{58} = 2.14, P = 0.04). This is however not a major problem for the present study, since our aim is to correlate oxidative balance with coloration within the two brood size treatments. Within pairs of experimental nests, foster and biological parents did not resemble each other with respect to plumage colour scores (Pearson's correlations: -0.16 < r < -0.03, *P*-values > 0.49). Pairing with respect to male and female coloration was not assortative in both treatments (enlarged nests: r = 0.025, n = 32, P = 0.89; reduced nests: r = -0.18, n = 28, P = 0.34). Note also that we failed to detect an effect of the BSM (alone or in interaction with plumage coloration) on adult body mass, the latter depending primarily on the two-way interaction between sex and colour morph ($F_{1,123.9}$ = 4.67, P = 0.03). This interaction was mainly explained by the negative relation between body mass and plumage coloration in females (light melanic females being heavier than darker ones; estimate ± SE = - 9.76 ± 4.48, $F_{1,70}$ = 4.7, P = 0.03), but not in males (estimate ± SE = 3.4 ± 2.9, $F_{1,56}$ = 1.4, P = 0.24; Figure 2.1). Note also that adult wing and tarsus lengths were not associated with coloration in both sexes (Student's *t*-test, *P*-values > 0.12).

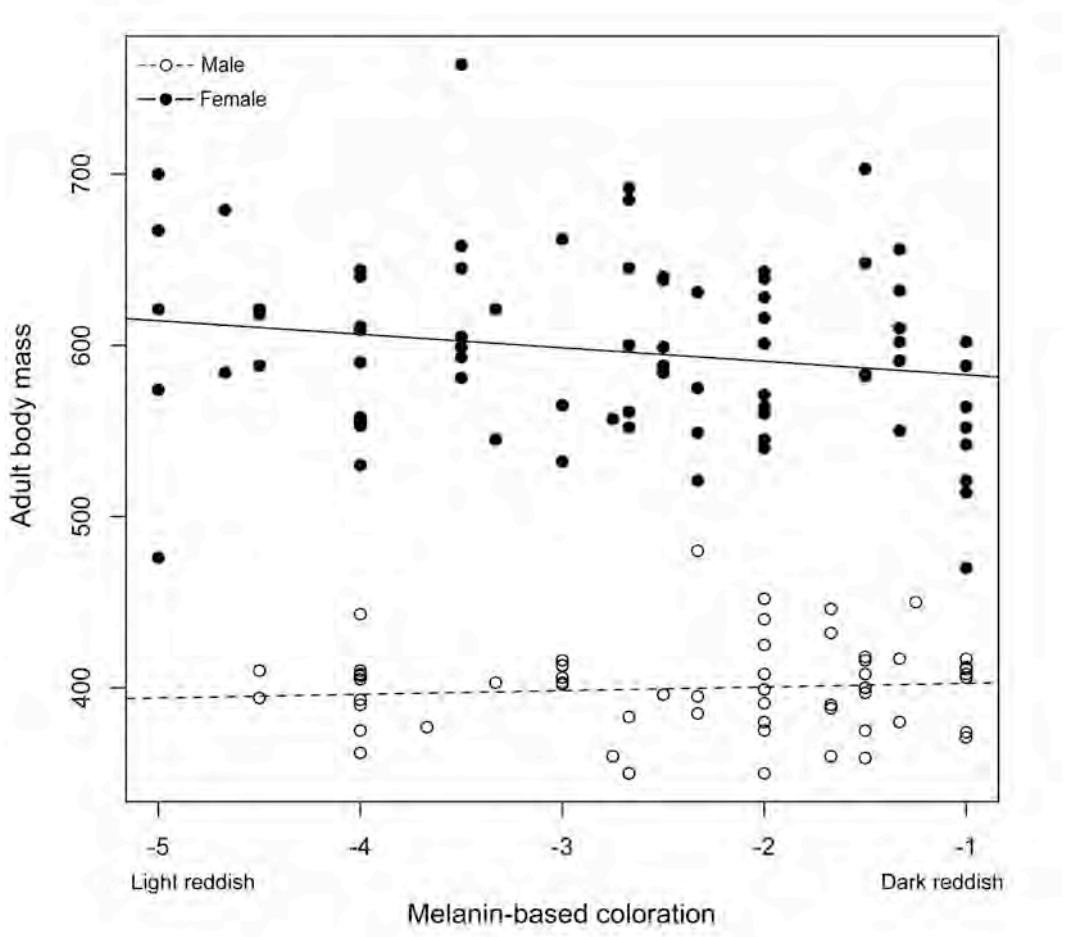

Figure 2.1: Illustration of the mixed-model results testing the relation between male (open circles and dash regression line; estimate ± SE = 3.4 ± 2.9, $F_{1,56}$ = 1.4, P = 0.24) and female body mass (closed circles and straight regression line; estimate ± SE = - 9.76 ± 4.48, $F_{1,70}$ = 4.7, P = 0.03) and melanin-based coloration in breeding tawny owls.

Oxidative stress measurements

Focusing on one tissue sample, namely red blood cells (RBC), our measurements of three distinct markers of oxidative stress provided an overall estimation of RBC oxidative status.

Endogenous production of ROS by RBC mitochondria was analyzed by flow cytometry in combination with the MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen). For each individual (55 males and 69 females), 420µl of KRL-diluted whole blood was centrifuged at 300 rpm and 4°C for 4 minutes. After removing the supernatant, we added 400µl of KRL buffer and 1µl of probe MitoSOX Red. Samples were incubated at 37°C for 30 minutes and finally analyzed by flow cytometry using a BD FACS Calibur, with excitation at 582 nm (FL2). Data were acquired and

analyzed with the software FACSDiva and CellQuest Pro (Olsson *et al.* 2009). For each sample, the mean fluorescence for all 50'000 cells was determined using CellQuest Pro.

Total intracellular GSH concentration ($_t$GSH) was assessed using the DetectX® Glutathione Colorimetric Detection Kit (Arbor Assays) with some modifications. For each sample (60 males and 80 females), 25µl of blood collected in heparin tubes was diluted with 100µl of 5% metaphosphoric acid, stored for 15 minutes on ice and then centrifuged at 14'000 rpm and 4°C for 15 minutes to collect the supernatant. From this extraction, 6µl of the sample was diluted with 114µl of 'Sample Diluent' and loaded in duplicates on a 96 wells microplate, prior to the distribution of 25µl of 'Colourimetric Detection Reagent' and 25µl of 'Reaction Mix' per well. As mentioned in the protocol, optical densities (OD) were measured at a wavelength of 405nm after 20 minutes. Computed OD was finally box-cox transformed before statistical procedures, to enable the use of models with a Gaussian-distributed error.

RBC resistance to free radicals provides a general assessment of the full range of antioxidants present in the blood to counter this free radical attack (Blache & Prost 1992; Pieri *et al.* 1996; Stocker *et al.* 2003), and thus not only GSH. Accordingly, inter-individual variation in such resistance is likely to reflect aspects of individual genetic quality and/or phenotypic quality (Bize *et al.* 2008; Kim *et al.* 2011). We assessed this measurement as the time required to haemolyse 50% of RBC exposed to a controlled free-radical attack (Alonso-Alvarez *et al.* 2006), using the KRL bioassay (Brevet Spiral V02023, Courernon, France) adapted to avian physiological parameters (see Bize *et al.* 2008). Loaded in duplicates on a 96 wells microplate (intra-plate repeatability: $r = 0.99$, $P < 0.0001$), 90µl of KRL-diluted whole blood (58 males and 74 females) was submitted to a ROS attack at 40°C by adding a solution of 150mM of 2,2'-azobis-(aminodinopropane) hydrochloride diluted in 153µl of KRL buffer (Bize *et al.* 2008). The lyses of red blood cells were monitored with a microplate reader device as the decrease of optical density at the wavelength of 540nm. Note that the time between blood collection and measurements of ROS production (mean ± sd = 10h37 ± 6.2, range = [2h – 22h15]) or RBC resistance to free radicals (mean ± sd = 10h04 ± 6.2, range = [1h30 – 22h])) was neither associated with the brood size manipulation (*P*-values > 0.78), nor with male or female plumage coloration (*P*-values > 0.2).

Statistical procedure

We performed two-tailed statistical analyses using the statistical software JMP IN 8.0. Preliminary analyses on potential inter-connection between the three oxidative stress markers revealed no significant pairwise correlations (after Bonferroni adjustment for multiple testing, Table 2.1). This lack of significance validates the use of separate statistical models to investigate the influence of BSM and plumage coloration on these measurements of oxidative stress. In three separate linear mixed-models, mitochondrial ROS production, intracellular $_{t}$GSH levels and RBC membrane resistance to free radicals were entered as response variables. BSM, sex of the corresponding parent and its plumage coloration were entered as explanatory variables, plus all possible interactions between these three variables. Apart from one marginal relationship (heavier individuals showing lower $_{t}$GSH levels, $P = 0.07$), time of blood sampling (in hours), body mass (corrected for wing length) and age or age^2 of each individual (in years) did not significantly covary with our oxidative measurements (P-values > 0.2) and were finally removed from our mixed-models. Moreover, hatching date of the first egg and brood size before BSM were introduced as covariates in preliminary analyses, but did not modify our conclusions. For this reason, we removed them from the analyses presented in the result section for the sake of clarity.

In each mixed model, we controlled for the effect of the nesting site on the response variable by including nest identity as random factor. Starting with full models, a backward stepwise procedure was used to sequentially remove non-significant terms ($P > 0.05$), starting with the least significant higher order interactions, until we obtained the best-fitting models. For each model, we verified that the distributions of errors were homogenous and normally distributed.

RESULTS

Oxidative balance prior to manipulation of brood size

During the incubation period, 24 male and 90 female tawny owls were already captured and blood-sampled once, most of them experiencing the brood size manipulation afterwards (2 males and 78 females). Initial measurements of mitochondrial ROS production, intracellular $_{t}$GSH levels and RBC membrane resistance to free radicals performed on these blood samples did not covary with adult plumage coloration (linear mixed-models, *P*-values > 0.31). This lack of significant covariation indicates that the following relationships found between melanin-based coloration and measurements of adult oxidative stress when owlets were 10 days old resulted from colour-specific reaction norms to the BSM experiment.

Colour-specific ROS production

As expected, ROS production was significantly affected by the manipulation of parental workload. When comparing initial and post-BSM measurements, we found that breeding pairs rearing an enlarged brood increased significantly more their ROS production (mean difference of both parents ± se = 0.6 ± 0.1) than the breeding pairs rearing a reduced brood (mean difference of both parents ± se = 0.27 ± 0.1; $F_{1,76}$ = 5.04, P = 0.028). More importantly, ROS production, measured in adults when their offspring were 10-day-old, was significantly explained by the interaction between BSM, sex and colour (linear mixed model: $F_{1,115.7}$ = 8.03, P = 0.005; Table 2.2A). Follow-up analyses performed separately for each sex and each brood size treatment showed that this interaction resulted mainly from differences in males rather than females. Light melanic males produced significantly more ROS than dark melanic ones when rearing an enlarged brood (linear mixed model: colour effect, estimate ± SE = -0.38 ± 0.11, $F_{1,21}$ = 11.8, P = 0.003), but not when rearing a reduced brood (another linear mixed model: estimate ± SE = 0.03 ± 0.1, $F_{1,30}$ = 0.07, P = 0.8; Figure 2.2).

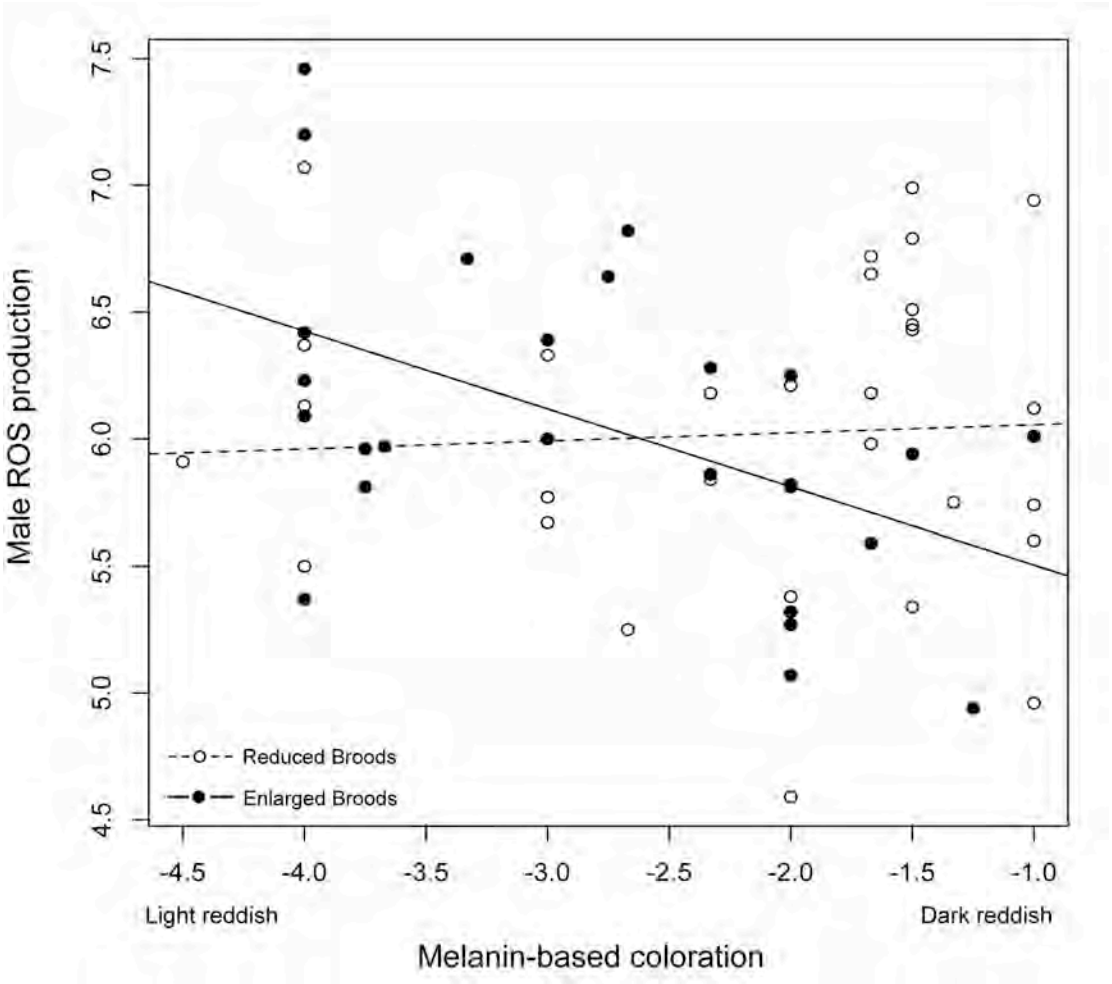

Figure 2.2: Endogenous production of reactive oxygen species (ROS) by red blood cell (RBC) mitochondria, in relation to melanin-based coloration in male tawny owls raising an experimentally reduced (open circles and dash regression line; linear mixed-model, estimate ± SE = 0.06 ± 0.1, $F_{1,28}$ = 0.41, P = 0.53) or enlarged brood (closed circles and straight regression line; another linear mixed-model, estimate ± SE = -0.39 ± 0.11, $F_{1,19}$ = 12.1, P = 0.003).

Colour-specific $_t$GSH production

Box-cox transformed $_t$GSH levels were significantly explained by the two-way interaction between BSM and colour (linear mixed model: $F_{1,133.8}$ = 5.13, P = 0.025; Table 2.2B); no effect of factor sex was observed, neither alone, nor in interaction with BSM. Models performed separately for each treatment group showed that adult $_t$GSH levels significantly covaried with the degree of plumage melanism when tawny owls were rearing an experimentally reduced brood (another linear mixed model: estimate ± SE = -49.5 ± 23.7, $F_{1,65.97}$ = 4.4, P = 0.04), but not an experimentally enlarged brood (another linear mixed model: estimate ± SE = 41.6 ± 29.3, $F_{1,63.89}$ = 2.0, P = 0.16; Figure 2.3).

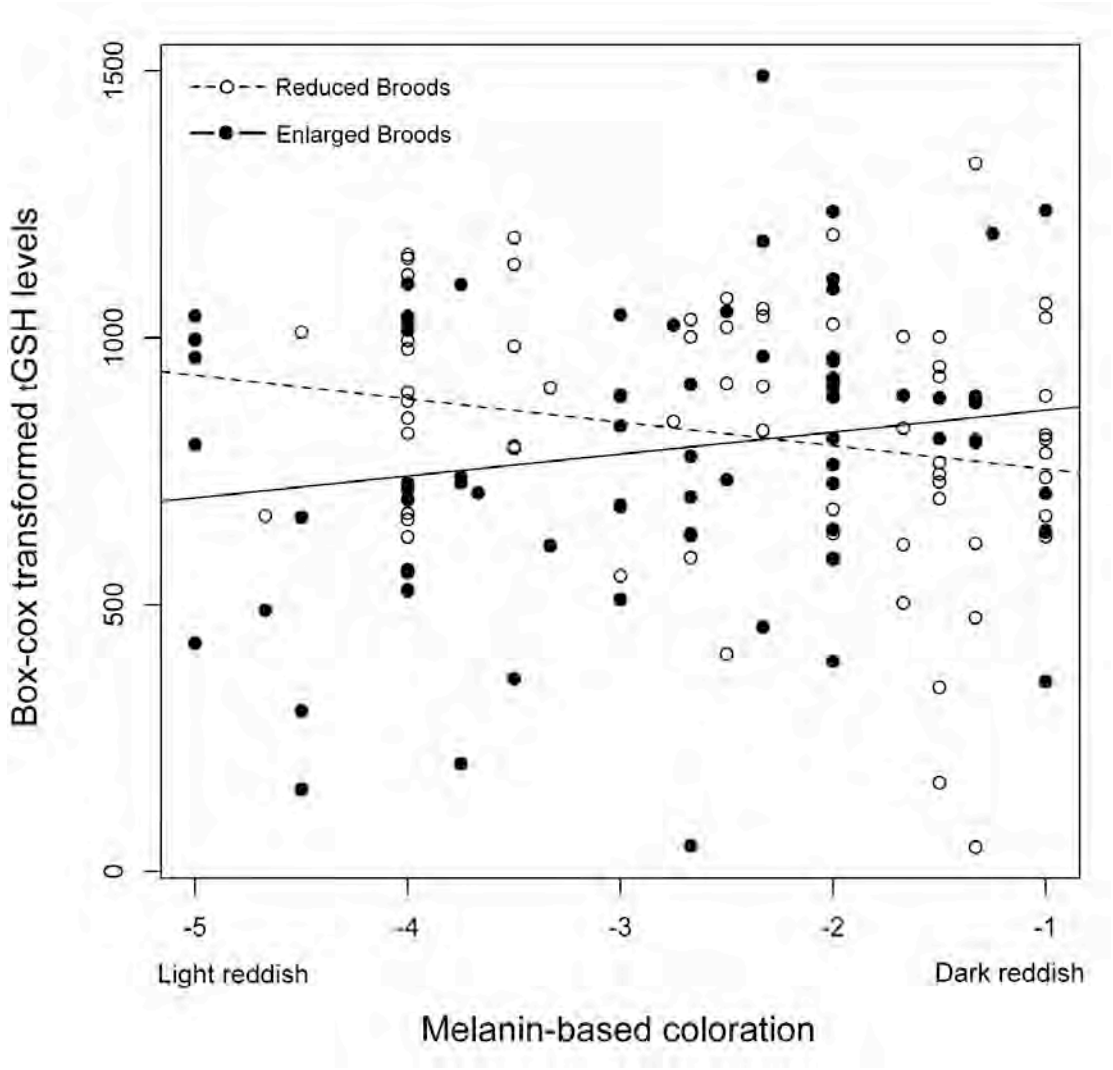

Figure 2.3: Box-cox transformed total intracellular glutathione ($_t$GSH) levels (μM), in relation to melanin-based coloration in breeding tawny owls raising an experimentally reduced (open circles and dash regression line; linear mixed-model, estimate ± SE = -49.5 ± 23.7, $F_{1,65.97}$ = 4.4, P = 0.04) or enlarged brood (closed circles and straight regression line; another linear mixed-model, estimate ± SE = 41.6 ± 29.3, $F_{1,63.89}$ = 2.0, P = 0.16).

Sex-specific resistance to free radicals

RBC membrane resistance to free radicals was significantly explained by the two-way interaction between BSM and sex (linear mixed model: $F_{1,75.58}$ = 8.85, P = 0.004, Table 2.2C); no effect of factor 'Colour' was observed, neither alone, nor in interaction with BSM. Models performed separately for each sex showed that the significant BSM by sex interaction was due to RBC membranes being significantly more vulnerable to oxidative stress in females rearing an enlarged rather than reduced brood (another linear mixed model: $F_{1,72}$ = 11.76, P = 0.001), whereas no

significant difference was found in males rearing an enlarged or reduced brood (another linear mixed model: $F_{1,56} = 1.36$, $P = 0.25$; Figure 2.4).

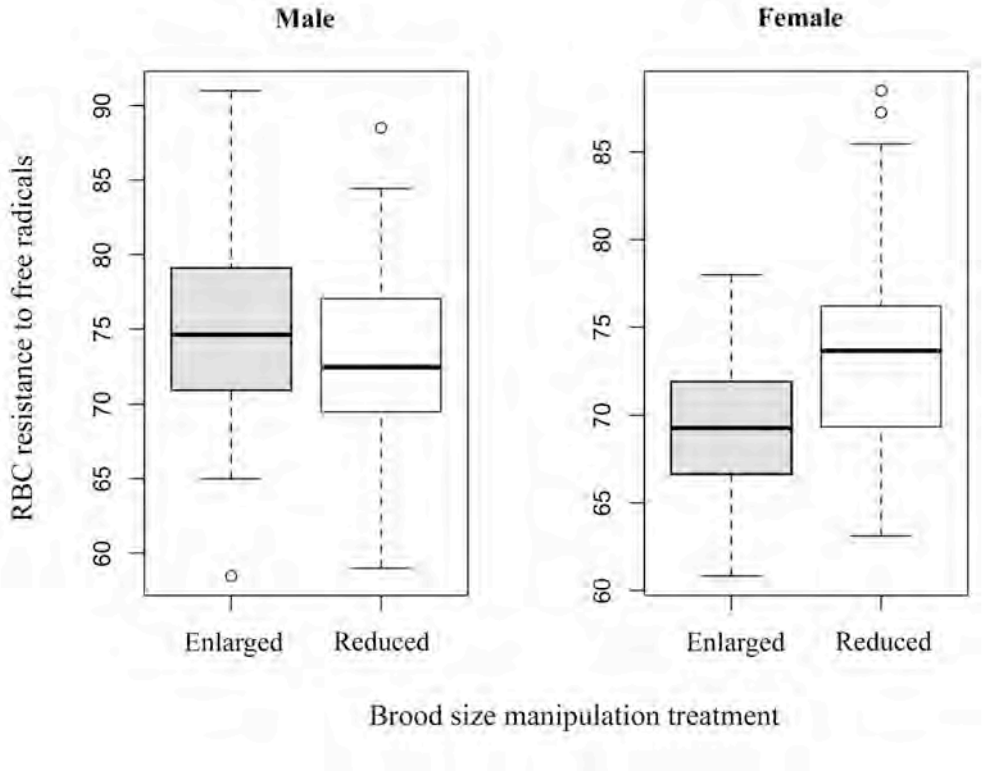

Figure 2.4: Red blood cell (RBC) resistance to an exogenous free radical attack, in relation to the brood size manipulation in adult tawny owls. Males rearing an enlarged brood (grey box-plot) showed no difference in their RBC resistance than those rearing a reduced brood (empty box-plot, *n* = 58, *P* = 0.25). By contrast, RBC resistance to free radicals was lower in females rearing an enlarged (grey box-plot), rather than a reduced brood (empty box-plot, *n* = 74, *P* = 0.001).

DISCUSSION

We investigated the relationship between melanin-based coloration and oxidative stress in adult tawny owls rearing experimentally reduced or enlarged broods. As expected, we found that our experimental manipulation of parental investment affected tawny owl oxidative balance, the expression of RBC oxidative biomarkers also depending on the sex of individuals and/or their plumage coloration. In males, ROS production was significantly higher in light compared to dark melanic owls, but only when rearing an enlarged brood. In both sexes, intracellular $_t$GSH levels were significantly higher in light compared to dark melanic adults, but this time when rearing a reduced

brood. Although we found that light and dark melanic birds underwent different levels of oxidative stress, as measured by ROS production, and differentially regulated one antioxidant component (i.e. $_{t}$GSH), we nevertheless found that resistance to a standardized oxidative attack was not associated with plumage coloration. Hereafter, we discuss how colour-specific adaptation to heterogeneous environments and sex-specific reproductive tasks may shape the oxidative status of adult tawny owls.

Colour-specific oxidative balance

Melanin-based coloration displays tight and complex links with the oxidative balance (i.e. antioxidant properties of melanin pigments, pleiotropic effects of the melanocortin system or the biochemical role of $_{t}$GSH in melanogenesis). These effects raised the hypothesis of colour-specific strategy in the regulation of tawny owl oxidative balance according to environmental conditions.

To our knowledge, the present study is the first to reveal a covariation between ROS production and adult melanin-based coloration. Light melanic males produced more ROS than dark melanic ones when rearing enlarged broods, while no significant difference was found between differently coloured males rearing a reduced brood. Previous works on this species provided empirical evidences that light melanic individuals better cope with stressful environmental conditions caused either by an experimental brood enlargement or by food deprivation (Roulin *et al.* 2005; 2008b; Piault *et al.* 2009; Roulin *et al.* 2011c). As a consequence of this adaptive strategy, light melanic individuals are likely to suffer from physiological costs. In adults for instance, food provisioning to larger broods can increase male metabolic rate (Nilsson 2002), such constraint leading to an increase of ROS production (Robinson *et al.* 1997; Domenicali *et al.* 2001). Although, we did not specifically measure paternal investment in the two brood size treatments, seven years of population monitoring revealed that light melanic males invest more effort than darker conspecifics when their brood was experimentally enlarged (Emaresi *et al.* in prep, ***Chapter 1***). Higher ROS production observed in light melanic males rearing enlarged broods may be therefore a by-product of their specific life history strategy.

Intracellular antioxidants such as GSH have a key role in scavenging reactive oxygen species, but can also interact biochemically within important molecular pathways such as pheomelanogenesis (Ozeki *et al.* 1997). This dual role has led to the hypothesis that melanocytes, in which eumelanogenesis prevails at the expanse of pheomelanogenesis, suffer higher oxidative stress since

they require low thiol group conditions, and thereby low $_t$GSH concentrations (Ito 2003; Galvan & Solano 2009). Observations from experimental studies on great tits (*Parus major*) and red-legged partridges (*Alectoris rufa*) were consistent with this hypothesis (Galvan & Alonso-Alvarez 2008; 2009); nestlings with experimentally reduced $_t$GSH levels expressed eumelanic traits to a larger degree, sometimes at the expense of pheomelanic traits (Galvan & Alonso-Alvarez 2009). These outcomes suggested close associations between the production of melanin pigments and $_t$GSH (Galvan & Solano 2009). However, it remained unclear whether these inter-individual variations in coloration were the consequence of drastic experimental inhibition of intracellular $_t$GSH levels or whether similar relationship between melanin-based coloration and $_t$GSH could also occur under natural situations (across a natural range of tGSH variation; see for instance Galvan *et al.* 2010). The present study was designed in this sense, investigating if tawny owl melanic plumage (mainly due to deposition of pheomelanin) revealed resistance abilities against oxidative stress in different circumstances, as a potential consequence of colour-specific $_t$GSH expression. Given their adaptive abilities to perform in stressful reproductive or environmental conditions (Roulin *et al.* 2008b; Piault *et al.* 2009), we expected light melanic individuals to express more $_t$GSH to counter the detrimental effects induced by their ROS production (as observed when rearing enlarged broods). Our results confirmed this assumption, $_t$GSH levels significantly covarying with melanin-based coloration. A closer look at the models performed separately for each treatment revealed that light melanic owls exhibited higher levels of $_t$GSH than darker conspecifics when rearing an experimentally reduced brood, whereas such covariation was not significant for tawny owls rearing an experimentally enlarged brood. Due to the lack of strong trends in both treatment groups, these results need to be interpreted with caution. However, due to the adaptive strategy of light melanic individuals to cope with stressful reproductive conditions as it can be the case when rearing an enlarged brood (see also Roulin *et al.* 2003; 2008b; Piault *et al.* 2009; Emaresi *et al.* 2011; Karell *et al.* 2011b), one can raise the hypothesis that these individuals are likely to endure physiological constraints, such as ROS overproduction (especially in males, the sex assuming offspring food provisioning in this species). And because of the trade-off resolution between current reproduction and antioxidant response (Monaghan *et al.* 2009), their energy-demanding strategy is also likely to decrease their larger GSH pool under stressful conditions,

but not under prime rearing conditions such as when we reduced brood size. This may explain why light melanic individuals produced more $_{t}$GSH than dark melanic conspecifics only under relaxed rearing conditions. Nevertheless, our measurements on RBC resistance to free radicals RBC resistance, a marker assessing the full range of antioxidants present in the blood to counter a free radical attack (Blache & Prost 1992; Pieri *et al.* 1996; Stocker *et al.* 2003), suggested that differently coloured tawny owls were likely to suffer from similar oxidative damage. This outcome advocates for a larger consumption of GSH by light melanic tawny owls to counter the detrimental effects of their ROS overproduction. This hypothesis points out the central issue of GSH expression and expenditure. The global pool of GSH ($_{t}$GSH) being composed in two forms, namely $_{red}$GSH (amount of available antioxidants) and $_{ox}$GSH (amount of $_{t}$GSH recently used), the percentage of each fraction may indeed covary with melanin-based coloration, an issue that needs to be addressed in future studies.

Sex-specific links between oxidative balance and life histories

Tawny owl parents display clear division of duties during reproduction. Males deliver food to their offspring and partner (Sasvari *et al.* 2009), while females guard the brood and distribute the prey items among the progeny. Our brood size manipulation experiment is primarily expected to increase male parental effort and reduce female food resources. Thus, this treatment is likely to differentially affect male and female oxidative status in this species. An important consequence of an increase in parental investment in males (i.e. increased food supply) is an increase in metabolic rate (Nilsson 2002) and potentially in ROS production (Halliwell & Gutteridge 1989; Finkel & Holbrook 2000). In the present study, we partly verified this scenario, ROS production mainly differing among males of different plumage coloration. Yet, we did not measure daily variation in metabolic rate of males rearing reduced and enlarged broods and studies are still needed to demonstrate a clear association between increased metabolic rate and ROS production in the tawny owl.

Concordant with experimental studies on zebra finch (Wiersma *et al.* 2004; Alonso-Alvarez *et al.* 2006), our experiment also showed a significant decrease in RBC membrane resistance in females (but not in males) rearing an enlarged, rather than reduced brood, independently of coloration. Because of their brooding behaviour, females could suffer from reduced food intake until owlets are thermo-independent at 15-20 days of age, especially when rearing an enlarged brood. Therefore, they

could have experienced a shift in their diet that lowered their acquisition of antioxidants (i.e. uric acid, carotenoids, vitamin E; Cohen *et al.* 2009). Both processes are likely to induce higher oxidative stress to females rearing enlarged broods, as suggested in this study by the observed decline in RBC resistance to free radicals after their brood had been enlarged. The exact proximate mechanisms driving the evolution of these sex-specific differences remain poorly understood. One mechanism could be the opposite action of sex steroids on oxidative balance, namely the positive and negative effect of testosterone and oestrogen, respectively (Gupta & Thapliyal 1985; Vina *et al.* 2005; Tobler & Sandell 2009). Another hypothesis is the effect of body mass on oxidative status, individuals generating more free radicals as a consequence of higher basal metabolic rate imposed by larger body mass. This hypothesis is however not supported by our data, since we failed to detect a relationship between body mass (corrected for wing length) and ROS production or RBC resistance to free radicals in adult tawny owls (*pers. comm.*, but see Marko *et al.* 2011 for similar results in the collared flycatcher *Ficedula albicollis*).

Conclusion

The present study demonstrated that oxidative balances of males and females were differently affected by experimental manipulation of their parental effort. In agreement with previous studies, our results revealed that the adaptation of light melanic tawny owls to stressful reproductive conditions (i.e. brood enlargement) leads them to overproduce ROS, but are likely to afford it by consuming larger amount of $_{red}$GSH (and potentially other antioxidants that we did not measure). Unlike barn owls (Roulin *et al.* 2011a), our results suggested that light melanic tawny owls were likely to suffer from similar oxidative damages than dark melanic conspecifics, but potentially at the expense of survival (e.g. Karell *et al.* 2011b). Finally, this experiment pointed out the complexity of formulating general predictions on the link between the production of melanin pigments, and hence melanin-based coloration, $_{t}$GSH levels and oxidative stress. As suggested herein, the oxidative balance and melanin-based coloration were not necessarily directly linked, but could be indirectly related via alternative colour-specific life history strategies.

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TABLES

Table 2.1: Pearson's correlation coefficients between the three measures of oxidative stress, with respect to the sex of an individual and the brood size manipulation treatment in adult tawny owls (to account for the fact that covariations between measurements of oxidative stress can differ between sexes and treatment groups). Scores of the *Reduced* treatment are in italics above the diagonal, while scores of the *Enlarged* treatment are below the diagonal. The significant pairwise *P*-value is given in bold, but is no more significant after Bonferroni adjustment for multiple testing.

Female

Enlarged vs. *Reduced* treatment

	Resistance	ROS production	GSH levels
Resistance		***0.36***	*0.15*
ROS production	-0.12		*0.17*
GSH levels	-0.09	0.23	

Male

Enlarged vs. *Reduced* treatment

	Resistance	ROS production	GSH levels
Resistance		*-0.04*	*-0.04*
ROS production	0.23		*-0.1*
GSH levels	-0.007	-0.1	

Table 2.2: Results of linear mixed models for adult tawny owls, investigating the effects of the brood size manipulation, sex of individuals, plumage coloration and their interactions on (*a*) endogenous production of ROS by RBC mitochondria, (*b*) box-cox transformed total intracellular GSH levels, and (*c*) RBC resistance to free radicals. We controlled for the effect of the nesting site on each response variable by including its identity as a random factor in each mixed model. Starting with full models, a backward stepwise procedure was used to sequentially remove non-significant terms (*P* > 0.05), starting with the least significant higher order interactions, until we obtained the best-fitting models (considered variables are given in bold).

Source of variation	n	estimate	se	*df*	*F*	*P*
A. ***Ros production***						
BSM[E]	**124**	**0.02**	**0.06**	**1,56.64**	**0.10**	**0.75**
Sex[F]		**-0.15**	**0.06**	**1,58.12**	**5.74**	**0.02**
BSM[E] : Sex[F]		**-0.01**	**0.06**	**1,58.12**	**0.01**	**0.93**
Colour		**-0.09**	**0.06**	**1,115.8**	**2.78**	**0.10**
BSM[E : Colour		**-0.05**	**0.06**	**1,115.8**	**0.67**	**0.42**
Sex[F] : Colour		**0.09**	**0.06**	**1,115.7**	**2.64**	**0.11**
BSM[E] : Sex[F]: Colour		**0.16**	**0.06**	**1,115.7**	**8.03**	**0.005**
B. ***Box-cox transformed total GSH levels***						
BSM[E]	**140**	**-16.63**	**20.41**	**1,69.71**	**0.66**	**0.42**
Colour		**-3.60**	**18.71**	**1,133.8**	**0.04**	**0.85**
BSM[E] : Colour		**42.36**	**18.71**	**1,133.8**	**5.13**	**0.025**
Sex[F]		-27.29	23.11	74.5	1.39	0.24
BSM[E] : Sex[F]		-9.63	23.20	73.26	0.17	0.68
Sex[F] : Colour		10.67	19.88	129.7	0.29	0.59
BSM[E] : Sex[F]: Colour		19.69	19.94	129.9	0.97	0.33
C. ***RBC membrane resistance to free radicals***						
BSM[E]	**132**	**-0.51**	**0.53**	**1,75.88**	**0.93**	**0.34**
Sex[F]		**-1.50**	**0.53**	**1,75.58**	**8.04**	**0.006**
BSM[E] : Sex[F]		**-1.57**	**0.53**	**1,75.58**	**8.85**	**0.004**
Colour		-0.60	0.46	1,127	1.71	0.19
BSM[E] : Colour		0.53	0.46	1,126	1.30	0.26
Sex[F] : Colour		0.20	0.48	1,124.8	0.17	0.68
BSM[E] : Sex[F]: Colour		0.27	0.49	1,123.8	0.30	0.59

CHAPTER 3

Genetic *versus* environmental determinism of intracellular glutathione levels in pheomelanin-based colour polymorphic tawny owls (*Strix aluco*)

Guillaume Emaresi*, Pierre Bize, and Alexandre Roulin

Department of Ecology and Evolution, Biophore building, University of Lausanne, 1015 Lausanne, Switzerland

ABSTRACT

A fundamental trade-off resolution in living organism is the resource allocation between reproduction and somatic self-maintenance traits, such as antioxidant response. Glutathione (GSH) is a major component of the intracellular antioxidant defence, scavenging for instance deleterious by-products of oxygen consumption, i.e. reactive oxygen species (ROS). Interestingly, recent studies pointed out the plausible role of intracellular GSH levels in melanin synthesis, suggesting therefore a trade-off resolution in GSH allocation between oxidative stress and melanin production. To better understand and capture how intracellular GSH is expressed and used between differently coloured individuals and at different life stages, we report here an experimental study where we measured the global pool of GSH produced ($_{t}$GSH), but also the accurate amount of GSH already consumed ($_{ox}$GSH) and remaining ($_{red}$GSH) in adult and nestling tawny owls, a species displaying continuous variation in pheomelanin-based coloration and colour-specific life history strategies. Based on the idea of GSH dependence of pheomelanin-based colour traits, we expected a greater consumption of GSH (i.e. higher $_{ox}$GSH levels) in dark melanic nestlings. However, this was not the case, suggesting that the expression of melanin-based coloration is independent of the pool of GSH available in this species. However, we found that $_{t}$GSH levels and marginally $_{red}$GSH levels quantified in adult tawny owls were associated with melanin-based coloration, light melanic individuals showing higher levels than dark melanic ones. This latter covariation is likely to arise due to adaptive response of light melanic tawny owls to an oxidative challenge induced by their energy-demanding life history strategy and their adaptation to stressful environmental conditions.

INTRODUCTION

Living organisms are constantly confronted to environmental sources of stress, such as parasites and predators, harsh climate conditions or food depletion. These organisms can adapt their strategy to these stressful conditions through sophisticated metabolic or physiological changes (e.g. enzymatic or hormonal activities), but which entails a redistribution of resources allocation in certain trade-off resolutions. Under certain circumstances, these adaptations can trigger negative consequences for other traits. For instance, individuals investing heavily in reproduction have fewer resources for maintenance mechanisms, highlighting the trade-off between survival and reproduction (Partridge *et al.* 2005; Harshman & Zera 2007). Accordingly, insights on the origin of variation in animal physiology are therefore particularly interesting to better understand the capacity of given genotypes/phenotypes to cope with heterogeneous environments, and potentially the evolution of life history strategies (Monaghan *et al.* 2009).

In this context, a prime candidate mechanism that gains increasing attention in animal physiology is the oxidative balance, i.e. the homeostasis between the productions of deleterious reactive oxygen species (ROS) and antioxidant defence systems. This ratio is critical as an excessive ROS production over antioxidants expose organism to oxidative stress and important intracellular damages, such as DNA point mutations (Twigg *et al.* 1998), cell membrane rupture (Halliwell 1992) and oxidation of amino acids in proteins (Halliwell & Gutteridge 1989). In this context, reproductive strategies in current reproduction are traded-off against somatic self-maintenance and repair mechanism of oxidative balance (Cohen *et al.* 2010; Isaksson *et al.* 2011; Kim *et al.* 2011). In the same example, individuals investing heavily in reproduction are likely to produce more ROS, constraining them either to suffer from oxidative stress, or to adopt a strong antioxidant response, both strategy leading to negative impact on survival. The major component of the intracellular antioxidant defences is glutathione (i.e. $_{red}$GSH, Reddy *et al.* 1982; Halliwell & Gutteridge 1989). Found in relatively high cellular concentration in most organisms, this tripeptide of glutamic acid, cysteine, and glycine (Hopkins 1929) is known to scavenge ROS in a reaction catalyzed by the glutathione peroxidase (GPx, Meister 1994). This redox process leads to the formation of glutathione disulfide (GSSG), derived from two $_{ox}$GSH molecules. Hence, the global pool of intracellular glutathione

($_{t}$GSH) is composed by reduced and oxidized GSH fractions (i.e. $_{t}$GSH = $_{red}$GSH + $_{ox}$GSH), both providing an accurate estimation of cellular redox state. $_{red}$GSH gives information on the amount of available antioxidants, while $_{ox}$GSH sheds in light how much $_{t}$GSH was recently used either to scavenge ROS or in alternative metabolic or biochemical processes (e.g. protein turnover and pheomelanogenesis; see below). In healthy mammalian tissues, the $_{t}$GSH pool is composed by more than 90% of $_{red}$GSH and less than 10% of $_{ox}$GSH (Pastore *et al.* 2003). Although GSH synthesis requires the action of two distinct enzymes, i.e. glutamate cysteine ligase and GSH synthase (Meister & Anderson 1983; Anderson 1998; Lu 2000), its production can be physiologically limited by the conditionally essential amino acid cysteine (Meister & Anderson 1983). Accordingly, one can raise the hypothesis that the production of intracellular GSH is genetically controlled (Board *et al.* 1974; Rizzi *et al.* 1988; Krogmeier *et al.* 1993), but also condition-dependent due to the need of acquiring cysteine from the diet (Lu 2000). Surprisingly, very few studies investigated the genetic *versus* environmental determinism of intracellular GSH regulation. Specifically, our knowledge of how different genotypes or phenotypes adjust their overall GSH pool (i.e. $_{t}$GSH), especially in terms of recent (i.e. $_{ox}$GSH) and future (i.e. $_{red}$GSH) expenditures, remains surprisingly scarce in wild populations.

In this context, melanin-based coloration is a promising system to consider the relationship between genotype and phenotype for important ecological or physiological traits, such as variation in GSH antioxidant response. This widespread pigmentation system (Majerus 1998) is composed by two types of pigment, namely eu- (grey to black) and pheomelanin (yellow to reddish-brown) and their synthesis is under strong genetic control (Roulin 2004; Hoekstra 2006; Ducrest *et al.* 2008). Interestingly, recent studies pointed out the plausible role of intracellular GSH levels in the synthesis of melanin pigments (Benedetto *et al.* 1981; Ozeki *et al.* 1997). Within melanocytes, the concentration of sulfhydryl compounds, in particular GSH, can modulate tyrosinase activity (Benedetto *et al.* 1981; 1982; Land & Riley 2000), which is the key enzyme controlling the switch between eu- and pheomelanogenesis (Barsh 1996; Ito *et al.* 2000). This biochemical link led researchers to formulate extensive predictions on the expression of GSH in relation to melanin-based coloration. For instance, they expect dark pheomelanic individuals to present higher GSH levels than lighter pheomelanic (but

darker eumelanic) morphs due to the fact that GSH can be used as substrate during pheomelanogenesis (Galvan & Solano 2009). In line with this hypothesis, melanocytes have lower intracellular levels of GSH when producing eumelanin, rather than pheomelanin pigments (Benathan *et al.* 1999). Moreover, experimental inhibitions of GSH levels in nestling great tits (*Parus major*), red-legged partridges (*Alectoris rufa*) or greenfinches (*Carduelis chloris*) induced the production of smaller eumelanin-based plumage traits and conversely larger pheomelanic ones (Galvan & Alonso-Alvarez 2008; 2009; Horak *et al.* 2010). The observation that natural variations in the degree of eu- and pheomelanin-based coloration can be associated with resistance to oxidative stress (Roulin *et al.* 2011a) is suggestive that GSH may be implicated in both the oxidative balance and melanin synthesis. This stimulated researchers to propose that the use of GSH in the production of melanin pigments is traded-off against resistance to oxidative stress (e.g. Galvan & Alonso-Alvarez 2008). However, even if GSH is implicated in melanogenesis, associations between GSH, melanin-based coloration and oxidative stress are not necessarily the outcome of trade-off resolution in GSH allocation. Indeed, differently coloured individuals may adopt alternative life history strategies, each requiring specific levels of antioxidant defences. Accordingly, melanin-based polymorphism is frequently reported to covary with life history strategies (Roulin 2004), and more recently with variation in resistance to oxidative stress (Roulin *et al.* 2011a). Under this scenario, one can predict that, if melanin-based coloration signals alternative life history strategies, variation in GSH levels between eu- and pheomelanic morphs could be the outcome of alternative need for antioxidant defences rather than *vice versa* (i.e. variation in GSH availability between eu- and pheomelanic morphs engendering alternative life history strategies). Both scenarios illustrate the complexity of predicting whether the relationship between eu- and pheomelanin-based coloration and GSH levels should be positive or negative. Indeed, the sign of this relationship will depend on five factors, i.e. (1) the nature of the colour polymorphism (eu- vs. pheomelanin-based coloration), (2) if there is a genetically-based polymorphism in the production of GSH, some individuals being programmed to produce more GSH than others, (3) if the production of GSH is condition-dependent (i.e. more GSH produced when needed to resist oxidative stress), (4) if there is a trade-off in GSH allocation between melanin colour traits and resistance to oxidative stress, and finally (5) if differently coloured individuals adopt alternative GSH-independent life history

strategies that generate various levels of oxidative stress. These factors being non-mutually exclusive and their relative importance unknown, it is extremely difficult to propose *a priori* predictions regarding how melanin-based coloration should covary with GSH. Descriptive studies where coloration and GSH is measured under natural conditions (i.e. without drastic manipulation of GSH levels) are therefore needed (see Galvan *et al.* 2010 for an example, although the colour polymorphism in this study remains unclear). Particular attention on the global pool of GSH produced ($_{t}$GSH), and specifically on the amount of GSH that has already been consumed ($_{ox}$GSH) *versus* the amount of GSH still available ($_{red}$GSH), should help improving our understanding of the role and the use of GSH in differently coloured individuals (with respect to their life histories), an approach that has not yet been considered.

Herein, we examined this issue in the colour polymorphic tawny owl (*Strix aluco*). This owl shows continuous variation in the degree of plumage reddishness (from light to dark), which is positively correlated to the amount of pheomelanin pigments and to a lesser extent to eumelanin pigments (Gasparini *et al.* 2009a). This colour polymorphism is segregated in accordance with Mendelian's law of inheritance (Karell *et al.* 2011b) and is therefore highly heritable (Gasparini *et al.* 2009a). There is accumulating evidences that, in the tawny owl, inter-individual variation in melanin-based coloration is associated with indices of individual performance, such as immunocompetence (Galeotti & Sacchi 2003; Gasparini *et al.* 2009a) or offspring growth (Roulin *et al.* 2004; 2008b; Piault *et al.* 2009), potentially signalling alternative physiological strategies or metabolic needs to cope with heterogeneous environments (Galeotti & Cesaris 1996; Brommer *et al.* 2005; Emaresi *et al.* 2011; Karell *et al.* 2011b). Of particular interest, recent findings indicated that variation in plumage coloration in breeding male tawny owls was associated with differences in ROS production and in red blood cell $_{t}$GSH levels (Emaresi *et al.* submitted, ***Chapter 2***). Since 2011 was characterized by harsher rearing environmental conditions (i.e. decreased food availability, *unpublished data*), we decided to replicate the same design as in Emaresi et al. (submitted, ***Chapter 2***; i.e. brood size manipulation experiment), but addressing here complementary issues. We specifically focused on GSH levels (i.e. $_{t}$GSH, $_{red}$GSH and $_{ox}$GSH) of non-moulting adults and their cross-fostered nestlings (rather than considering exclusively $_{t}$GSH in breeding adults), two life stages characterized by low and high

melanogenic activities, respectively, in this species. The main objectives of this study were (i) to establish whether inter-individual intracellular GSH levels are genetically inherited or environmentally mediated, and (ii) to determine the sign of the potential relationship between GSH levels and coloration in both breeding adults and growing nestlings. To test for genetic GSH inheritance, we performed a cross-fostering experiment where eggs or hatchlings were swapped between randomly chosen nests in order to allocate genotypes randomly among different rearing environments. In addition, we manipulated brood size to induce various levels of oxidative stress (e.g. Wiersma *et al.* 2004; Christe *et al.* 2011), individuals raised (i.e. nestlings) or rearing (i.e. parents) an experimentally enlarged broods experiencing higher levels of stress than individuals allocated to experimentally reduced broods. Because environmentally-induced covariation between coloration and GSH levels could be due to condition-dependent expression of coloration and/or GSH levels, we first tested whether the expression of melanin-based coloration is indeed not condition-dependent in our local population of tawny owls. To this end, we investigated whether coloration of breeding adults rearing an experimentally enlarged brood changes differently compared to adults rearing an experimentally reduced brood (Griffith 2000). This test was possible because we performed brood size manipulation experiments since 2005, providing therefore a large dataset to investigate change in coloration between two successive breeding seasons. We also investigated whether nestling colour traits were affected by the brood size manipulation experienced during their early growth (see Piault *et al.* in press for similar test in the kestrel).

METHODS

The tawny owl is a medium-sized bird, commonly found in woodlands across Eurasia (Galeotti 2001). It is a monogamous and philopatric nocturnal species, living up to 20 years. Females are 16-20% larger than males (Baudvin & Dessolin 1992), both sexes showing a strong partition of the reproductive roles (Sunde *et al.* 2003b). Females incubate their clutch during 28 days and remain inside the cavity with their owlets until they are thermo-independent at 15-20 days of age. Then, the mother stays around the nest, protecting the offspring against potential predators. Males are primarily engaged in supplying food to their brood. Nestling growth rate and survival strongly depend on prey

availability, especially wood mice (*Apodemus* sp.; Roulin *et al.* 2009). In our 911 km^2 Swiss study area, 2011 was characterized by a small number of breeding pairs (n = 57) compared to 2007 (n = 125) or 2010 (n = 139). Clutches were composed of 1-5 eggs (2.7 eggs ± 0.77), deposited between March 3 and April 22 (mean ± sd = March 19 ± 12 days), while the mean number of nestlings that fledged per breeding pair was small (mean ± sd = 1.36 fledglings ± 1.34). Offspring leave the nest at 25–30 days of age, but are fed by their parents until 90-120 days of age (Sunde 2008).

Experimental design

To test experimentally whether the expression of melanin-based coloration is sensitive to investment in reproductive activities (Griffith 2000) or to rearing conditions experienced at the nestling stage (see Piault *et al. in press* for example), we manipulated brood size between 2005 and 2010. As previously shown, this experimental approach has significant effect on adult (e.g. Roulin *et al.* 2011c) and nestling body condition (e.g. Roulin *et al.* 2008b).

In 2011, we repeated the same brood size manipulation experiment to test whether the covariation between GSH and coloration differs between rearing environments. We matched 42 nests in pairs based on the criteria that clutches were initiated on a similar date (Pearson's correlation: r = 0.92, $P < 0.0001$). Among pairs of nests, brood sizes were randomly manipulated, leading to an exchange of 2.11 hatchlings or eggs on average (sd = 0.6) from nest E (experimentally enlarged) and placed in nest R (experimentally reduced), while 3.11 hatchlings or eggs on average (sd = 0.6) underwent the opposite exchange (i.e. from nest R to nest E). Out of the 42 manipulated broods, eight broods were predated leading to a small imbalance between the two treatments (16 experimentally reduced broods vs. 18 experimentally enlarged broods). As expected, this brood size manipulation (BSM) had the intended effect on brood size soon after hatching and thereby on rearing conditions; parents assigned to the enlarged brood treatment were rearing a larger number of nestlings than those assigned to the reduced brood treatment (mean ± SE number of nestlings per enlarged vs. reduced brood: 3.17 ± 0.13 vs. 2.37 ± 0.14; Student's t-test: t_{32} = -4.07, P = 0.0003). As previously described (Emaresi *et al.* submitted, ***Chapter 2***), adults and their offspring were captured post-manipulation, when nestlings were 10 days of age (mean ± sd = 9.73 ± 2.7). Females and their offspring were captured in the nest box during daylight (8am – 6pm), while males were captured at night (when

provisioning their brood; 10pm – 6am). Individual body mass, tarsus length, and wing length were measured to the nearest 0.1 g, 0.1 mm, and 1 mm, respectively. Adult wing length and tarsus length were not significantly associated with the brood size manipulation experiment (Student's t-tests, $P > 0.3$). For each individual, we collected 60-200ul of whole blood from the brachial vein, using heparin microtubes, which were immersed in dry ice in the field and transferred at -80°C within 8 hours until later analyses in the laboratory.

Assessment of plumage coloration

Although tawny owls vary continuously in the degree of reddishness, this species is usually considered as colour polymorphic in the literature (Glutz von Blotzheim & Bauer 1980; Galeotti 2001; Brommer *et al.* 2005), and hence we also employ this terminology. Adults were classified into one of five colour morphs (from light to dark reddish; Roulin *et al.* 2005), based on plumage coloration from different body areas (i.e. breast, flanks, back, head, wings). This visual determination of adult plumage coloration is a highly reliable scoring method ($r = 0.89 \pm 0.02$, $F_{174,383} = 13.76$, $P < 0.0001$; Emaresi *et al.* submitted, ***Chapter 2***), providing a good estimation of overall coloration (Brommer *et al.* 2005). Moreover, colour scores assigned visually were found to be strongly correlated with brown chroma scores derived from spectrometric measurements (Pearson's correlation: $r = -0.84$, n = 270, $P < 0.0001$; Emaresi *et al.* submitted, ***Chapter 2***). At the nestling stage, plumage coloration is much less variable, which makes difficult the classification of nestling plumage coloration into one of five colour morphs. For this reason, nestling plumage coloration was assessed by spectrometric measurements. To this end, three feathers collected on the back of each nestling were overlaid on black paper to capture reflectance spectra at four distinct positions using the S2000 spectrophotometer (Ocean Optics, Dunedin, FL) and a dual deuterium and halogen 2000 light source (Mikropackan, Mikropack, Ostfildern, Germany). Based on these spectra, a mean brown chroma score was calculated for each nestling as described by Montgomerie (2006).

Adult plumage coloration was neither associated with clutch size, brood size before or after the manipulation (Student's *t*-tests, *P*-values > 0.39), nor with the experimental treatment (Student's *t*-test by gender, $P > 0.59$). Moreover, adult wing and tarsus lengths were not associated with coloration in both sexes (Student's *t*-test, *P*-values > 0.3). Pairing with respect to plumage coloration was

random, since there was no correlation between male and female coloration within breeding pairs (r = 0.01, n = 28, P = 0.96). Within pairs of experimental nests, foster and biological parents did not resemble each other with respect to plumage colour scores (females: r = -0.02, n = 20, P = 0.94; males: r = -0.58, n = 9, P = 0.1). Nestling body mass was unaffected by the experimental treatment ($F_{1,8.7}$ = 0.56, P = 0.47) and did not covary with plumage coloration ($F_{1,45.3}$ = 1.28, P = 0.26; Appendix 3.1A). By cons, our brood size manipulation experiment influenced adult body mass, owls rearing a reduced brood being significantly heavier than those rearing an enlarged brood ($F_{1,31.03}$= 9.01, P = 0.005), but independently of plumage coloration ($F_{1,45.63}$ = 0.2, P = 0.66; treatment by coloration interaction: $F_{1,39.77}$ = 1.29, P = 0.26; Appendix 3.1B).

GSH measurements

We measured intracellular total and oxidized intracellular glutathione levels ($_t$GSH and $_{ox}$GSH, respectively) in red blood cells using the Glutathione Colorimetric Detection Kit (Arbor Assays), following instructions provided by the kit manufacturer. For each blood sample (i.e. 58 adults and 75 cross-fostered nestlings), a minimum of 10µl of blood collected in heparin tube was diluted with an equal volume of 5-sulfosalicylic acid dihydrate (SSA) solution at 5% weight/volume (1g of SSA per 20 mL of water) to remove protein. After 15 minutes of incubation on ice, the diluted samples were centrifuged at 14'000 rpm and 4°C for 15 minutes to collect the supernatant. This latter solution was then diluted 1:2.5 with Assay Buffer and stored in two aliquots for final analyses. After a final dilution of 1:20 with Sample Diluent, 50 µL of extracted samples from the first aliquot were loaded in duplicates on a 96 wells microplate to assess total glutathione concentrations (i.e. $_t$GSH). The second aliquot of SSA-diluted samples was treated with 2-Vinylpyridine to block any free GSH (5 µL of 2VP solution for every 250 µL of sample) and incubated at room temperature for one hour. This solution was then diluted 1:20 with Sample Diluent, loaded in duplicates on 96 wells microplates to assess oxidized glutathione concentrations (i.e. $_{ox}$GSH). Based on best standard curves, optical densities (OD) were measured at a wavelength of 405nm after 10 minutes. $_t$GSH and $_{ox}$GSH concentrations were finally calculated from OD data using the standard dilution curve, while $_{red}$GSH levels were simply obtained by subtracting $_t$GSH and $_{ox}$GSH values. Inter-plate repeatability of $_t$GSH and $_{ox}$GSH scores

demonstrated the reliability of this colorimetric assay ($_{t}$GSH : r = 0.97 ± 0.005, $F_{12,29}$ = 96.95, P < 0.0001; $_{ox}$GSH : r = 0.99 ± 0.0005, $F_{12,29}$ = 955.6, P < 0.0001; Lessells & Boag 1987).

Statistical analyses

In adults, based on data collected between 2005 and 2011, we tested whether plumage colour traits changed between consecutive breeding seasons, as a consequence of rearing a reduced or enlarged brood. Using an ANCOVA model with visual coloration scores as response variable ('ColourMorph'), we entered adult gender, coloration scores determined the year before ('ColourMorph$_{X-1}$') and the brood size manipulation experienced the year before ('BSM$_{X-1}$') as explanatory variables, plus the interaction between both factors. In this model, we controlled for year, individual identity and nest site by including them as random variables. In nestlings, we tested whether rearing conditions affected their plumage colour traits on the basis of data collected between 2008 and 2011. In this ANCOVA model, we entered nestling brown chroma ('Brownchroma') as response variable, while brood size manipulation treatment (hereafter BSM), nestling gender, wing length (i.e. a reliable estimator of nestling age; $F_{1,73}$ = 470.65; P < 0.0001) and residual body mass before fledging (i.e. corrected for wing length; $F_{1,73}$ = 261.8; P < 0.0001) were added as explanatory variables. We controlled for an effect of the foster nest site and year by including both variables as random factors.

After preliminary statistical exploration in which we performed full mixed-models, we decided to simplify our statistical procedure for the sake of clarity. Melanin-based coloration being neither associated with date and time of the day when individuals were sampled (Pearson's correlations: -0.14 < r < 0.08, P-values > 0.29), nor with brood size before treatment, individual age (in years for adults and in days for nestlings) or wing length (Student's t-tests, P-values > 0.39), these factors could not confound or blur our results and were removed from the mixed-models. Consequently, our linear mixed models contained only explanatory variables of interest, i.e. coloration, brood size manipulation (plus their interaction). Although its effect was negligible in 2010 (Emaresi *et al.* submitted, ***Chapter 2***), tawny owl body mass is likely to be related to GSH expression in 2011, as a consequence of stronger selective pressure due to environmental conditions (i.e. decreased food availability, *unpublished data*). Accordingly, we also entered residual body mass (corrected for gender in adults and wing length in nestlings) as covariate in specific mixed-models.

Note here that the parsimonious mixed-models gave similar results as with the full mixed-models (i.e. involving all covariates).

To test for genetic and environmental components of GSH expression, we compared GSH concentrations measured in cross-fostered nestlings with the levels of their biological (i.e. effect of origin probably explained by genetic inheritance) and foster parents (i.e. environmental effect). Preliminary statistical examination revealed that $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels of biological parents were not correlated with those of foster parents (Pearson's correlations: $-0.42 < r < 0.57$, *P*-values > 0.14). In separate linear mixed models, we introduced thus nestling $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels as response variables, while BSM and GSH values of either biological (i.e. $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels of Genetic Father and Genetic Mother) or foster parents (i.e. $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels of Foster Father and Foster Mother) were entered as explanatory variables, plus the two-way interactions between these variables. In these models, we controlled for the effect of the genetic (models with biological parents) or foster nest site (models with foster parents) by including either genetic or foster brood identity as random factor. Note that we could not implement values of biological and foster parents in the same analyses because of the limited number of available broods. However, given that their values were not related (see above), a relationship between GSH values found in the offspring and their biological parents is unlikely to be inflated by the values measured in their foster parents and *vice versa*.

To investigate the link between melanin-based coloration and the concentrations of $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH, we first tested whether GSH levels measured in a given individual are correlated with its own coloration (within-individual comparisons). Then, we performed parent-offspring comparisons, investigating whether GSH levels measured in nestlings are associated with coloration scores of their biological (i.e. colour-genetic determinism) or foster parents (environmental determinism). For the within-individual comparisons, we performed separate ANCOVA models with either adult or cross-fostered nestling $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH levels as response variables. In addition of residual body mass (corrected for gender in adults and wing length in nestlings) as covariate, we entered BSM and adult or nestling plumage coloration (i.e. 'ColourMorph' or 'Brownchroma', respectively) as explanatory variables, plus the interaction between both variables. In these six mixed-

models, we controlled for the effect of the breeding site by including nest identity as random factor. For the parent-offspring comparisons where nestling GSH levels were the response variables, plumage coloration of their genetic parents ('GFatherMorph' and 'GMotherMorph') or foster parents ('FFatherMorph' and 'FMotherMorph') were entered as explanatory variables in separate linear mixed-models, plus the three-way interactions between these variables. In these models, we included nestling residual body mass as covariate and we controlled for the effect of the nest of origin or of rearing by including genetic or foster brood identity as random factor.

Starting with full models, a backward stepwise procedure was used to sequentially remove non-significant terms ($P > 0.05$), starting with the least significant higher order interactions, until obtaining best-fitted models. For each model, we visually inspected that the distribution of errors was homogenous and normally distributed. Statistical analyses were performed using JMP IN 8.0.

RESULTS

Adult and nestling tawny owls presented slight differences in the intensity of their GSH response, breeding parents showing larger amounts of GSH than their offspring (Figure 3.1). Adults had on average 1578.5 µM of $_{t}$GSH (sd: 488.5 µM, range: [555.5 - 2702.0]), divided in 683.9 µM of $_{ox}$GSH (sd: 189.8 µM, range: [228.8 - 1120.1]) and 910.0 µM of $_{red}$GSH (sd: 366.9 µM, range: [326.7 - 2050.6]). Nestlings had on average 1290.6 µM of $_{t}$GSH (sd: 376.0 µM, range: [197.0 - 2127.4]), divided in 466.2 µM of $_{ox}$GSH (sd: 162.2 µM, range: [97.0 - 1051.7]) and 825.5 µM of $_{red}$GSH (sd: 251.9 µM, range: [100.1 - 1339.1]). The proportions of $_{t}$GSH levels in the oxidized form (i.e. $_{ox}$GSH/$_{t}$GSH) were higher than expected (Pastore *et al.* 2003) and differed between adults and nestlings (Student's *t*-test, $t_{126} = -5.2$, $P < 0.0001$), with $_{ox}$GSH constituting 43.3% of $_{t}$GSH levels in adults ($_{red}$GSH: 56.7%) and 36.1% of $_{t}$GSH levels in nestlings ($_{red}$GSH: 63.9%).

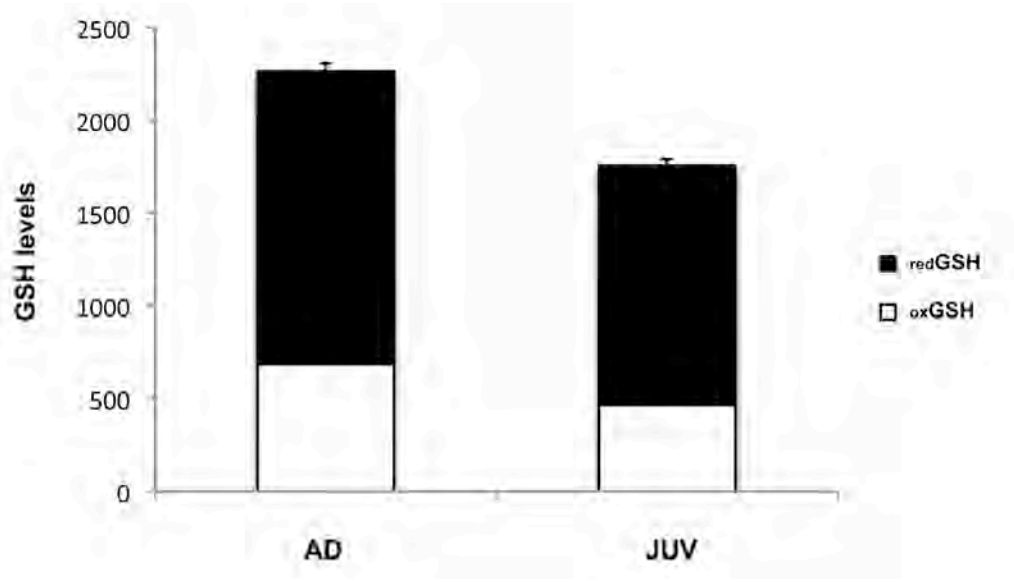

Figure 3.1: Corresponding concentrations (µM) of $_{ox}$GSH (white bar) and $_{red}$GSH (black bar) levels in adult and nestling tawny owls. Accordingly, $_{t}$GSH pool is composed on average by 43.3% and 36.1% of $_{ox}$GSH in adult and nestling tawny owls, respectively.

Expression of melanin-based coloration is not condition-dependent

Based on data collected between 2004 and 2011, adult plumage coloration scores were closely associated with those determined the year before (i.e. $ColourMorph_{X-1}$; n = 228, $F_{1,226}$ = 825.27, P < 0.0001), independently of gender ($F_{1,225}$ = 1.28, P = 0.26) and the brood size manipulation treatment experienced the year before (BSM_{X-1}; $F_{1,199}$ = 0.4, P = 0.53). The two-way interaction between $ColourMorph_{X-1}$ and BSM_{X-1} was not significant ($F_{1,198}$ = 2.2, P = 0.14). In the same vein, nestling coloration scores were independent of gender (n = 329, $F_{1,306.9}$ = 0.94, P = 0.33), wing length ($F_{1,320.4}$ = 0.36, P = 0.55) and brood size manipulation treatment experienced during growth ($F_{1,190.1}$ = 1.12, P = 0.29). There was a slight tendency for heavier nestlings to be more darkly melanic ($F_{1,325.5}$ = 3.0, P = 0.08). Altogether, these results suggest that in the tawny owl the expression of melanin-based coloration is not or only weakly sensitive to environmental effects.

Comparison between parental and offspring GSH levels

$_{t}$GSH levels measured in cross-fostered nestlings were significantly and positively related to $_{t}$GSH levels of their genetic father (estimate ± se = 0.29 ± 0.12, $F_{1,15.43}$ = 6.07, P = 0.03; Table 3.1A; Figure 3.2A) and both foster parents (father: estimate ± se = 0.38 ± 0.11, $F_{1,20.65}$ = 6.83, P = 0.02 ; mother: estimate ± se = 0.16 ± 0.11, $F_{1,18.69}$ = 5.37, P = 0.03 ; Table 3.1B, Figure 3.2B). Note here that we found also a significant relationship between nestling $_{t}$GSH levels and the average $_{t}$GSH levels of

foster parents (n = 54, $F_{1,19}$ = 13.64, P = 0.002), but not with the average levels of genetic parents ($F_{1,8.353}$ = 0.76, P = 0.41).

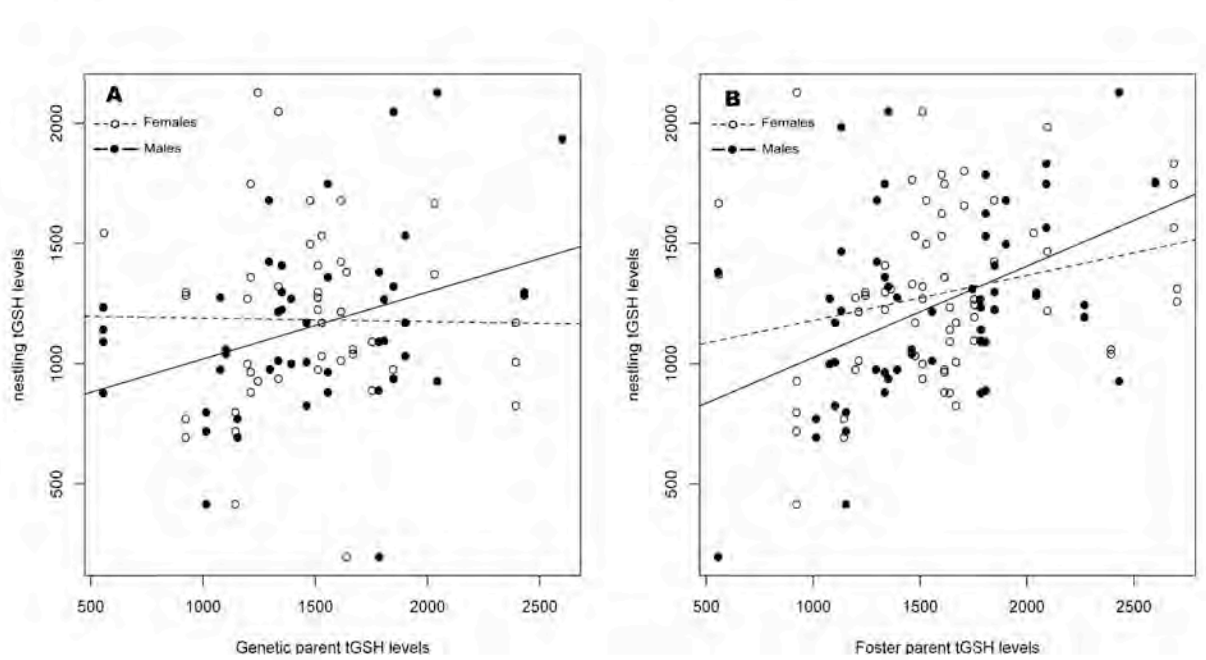

Figure 3.2: Relationship between nestling $_t$GSH levels and those of their (A) genetic father (closed circles and straight regression line; n = 48, estimate ± se = 0.29 ± 0.12, $F_{1,15.43}$ = 6.07, P = 0.03) and mother (open circles and dash regression line; n = 46, estimate ± se = -0.02 ± 0.17, $F_{1,11.48}$ = 0.01, P = 0.91) or (B) foster father (closed circles and straight regression line; n = 60, estimate ± se = 0.38 ± 0.11, $F_{1,20.65}$ = 6.83, P = 0.02) and mother (open circles and dash regression line; n = 66, estimate ± se = 0.16 ± 0.11, $F_{1,18.69}$ = 5.37, P = 0.03).

Similarly, $_{ox}$GSH levels measured in these nestlings were significantly associated with $_{ox}$GSH levels of their genetic father (estimate ± se = 0.32 ± 0.13, $F_{1,13.54}$ = 5.92, P = 0.03; Table 3.1C, Figure 3.3A) and foster father (estimate ± se = 0.35 ± 0.13, $F_{1,25.85}$ = 7.15, P = 0.01; Table 3.1D, Figure 3.3B).

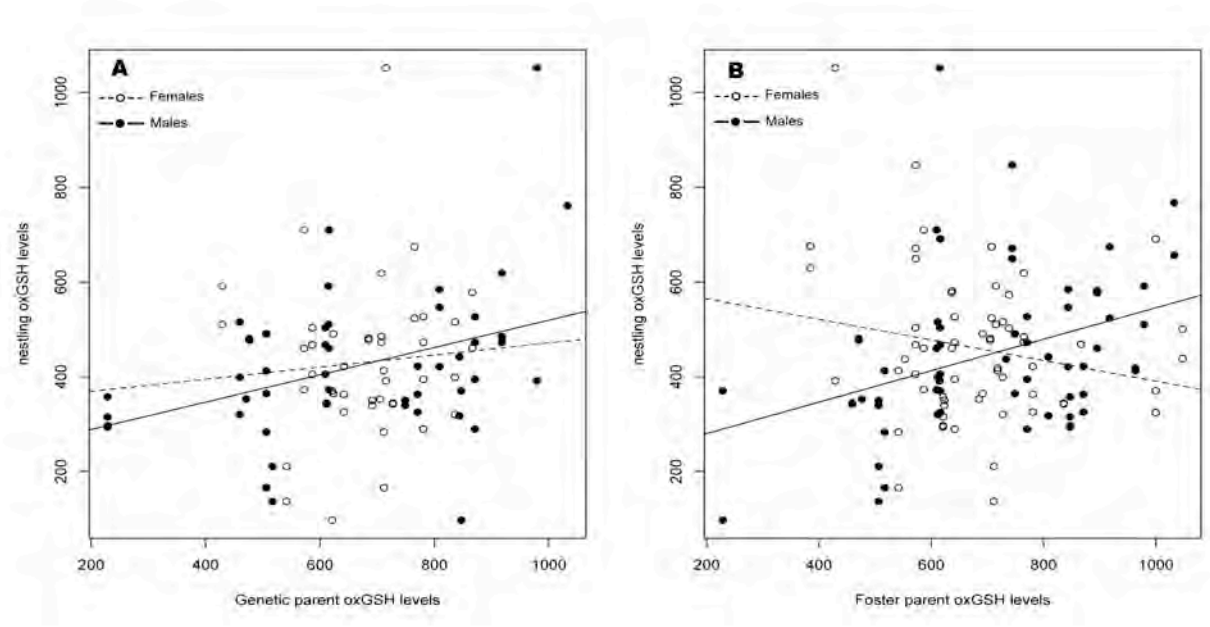

Figure 3.3: Relationship between nestling $_{ox}$GSH levels and those of their (A) genetic father (closed circles and straight regression line; *n* = 49, estimate ± se = 0.32 ± 0.13, $F_{1,13.54}$ = 5.92, *P* = 0.03) and mother (open circles and dash regression line; *n* = 43, estimate ± se = 0.16 ± 0.3, $F_{1,10.66}$ = 1.23, *P* = 0.29) or (B) foster father (closed circles and straight regression line; *n* = 61, estimate ± se = 0.35 ± 0.13, $F_{1,25.85}$ = 7.15, *P* = 0.01) and mother (open circles and dash regression line; *n* = 62, estimate ± se = -0.24 ± 0.17, $F_{1,18.66}$ = 0.45, *P* = 0.51).

However, no significant relation was found when considering the average $_{ox}$GSH levels of genetic and foster parents (*P*-values > 0.28). Finally, $_{red}$GSH levels measured in these nestlings were significantly associated with $_{red}$GSH levels of their foster mother (estimate ± se = 0.27 ± 0.08, $F_{1,22.5}$ = 11.41, *P* = 0.003; Table 3.1F, Figure 3.4B), but not with $_{red}$GSH levels of their foster father ($F_{1,19.18}$ = 0.94, *P* = 0.34), nor those of their genetic parents (Table 3.1E, Figure 3.4A).

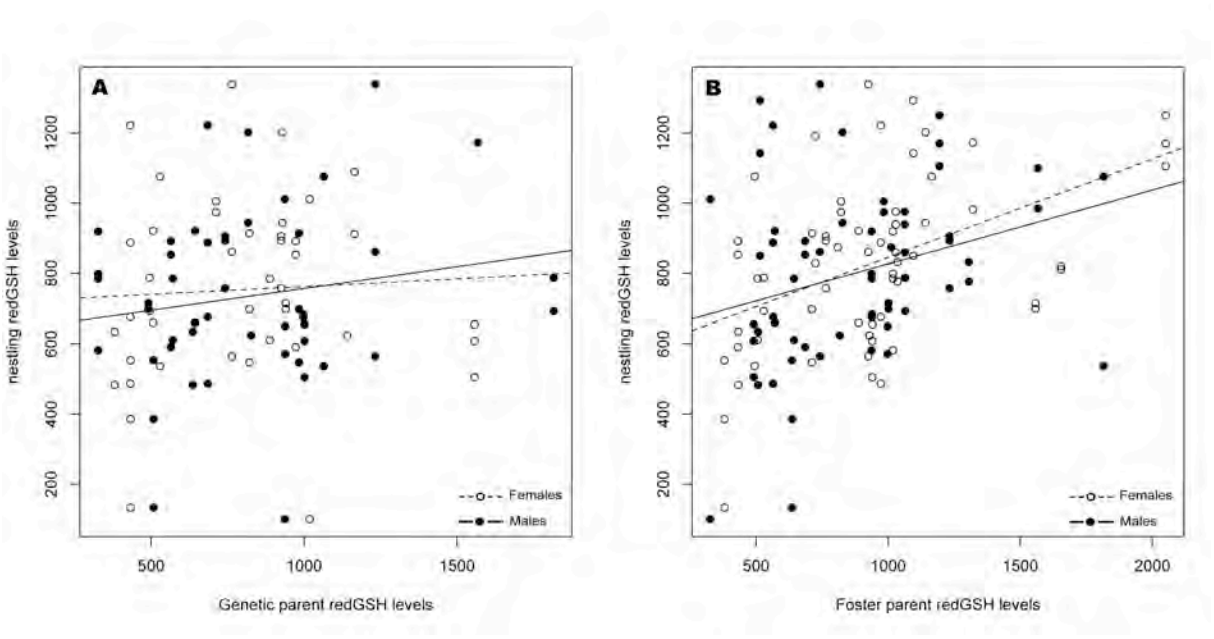

Figure 3.4: Relationship between nestling $_{red}$GSH levels and those of their (A) genetic father (closed circles and straight regression line; *n* = 48, estimate ± se = 0.13 ± 0.11, $F_{1,19.18}$ = 1.34, *P* = 0.26) and mother (open circles and dash regression line; *n* = 43, estimate ± se = 0.05 ± 0.14, $F_{1,11.57}$ = 0.01, *P* = 0.91) or (B) foster father (closed circles and straight regression line; *n* = 60, estimate ± se = 0.21 ± 0.11, $F_{1,19.18}$ = 0.94, *P* = 0.34) and mother (open circles and dash regression line; *n* = 62, estimate ± se = 0.27 ± 0.08, $F_{1,22.5}$ = 11.41, *P* = 0.003).

When considering the average $_{red}$GSH values of genetic and foster parents, our model revealed a significant relation between nestling $_{red}$GSH levels and the average $_{red}$GSH levels of foster parents (*n* = 52, $F_{1,18.23}$ = 10.59, *P* = 0.004), but not with the average levels of genetic parents ($F_{1,10.23}$ = 0.16, *P* = 0.70). Apart from one marginal relationship (nestlings showed higher levels of $_{ox}$GSH when experiencing enlarged rather than reduced broods, *P* = 0.06; Table 3.1D), we found no evidence that brood size manipulation experiment influenced nestling $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH levels (*P*-values > 0.26; Table 3.1).

Covariation between melanism and GSH levels: within-individual comparison

Nestling $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH levels were not significantly related to plumage coloration ($_{t}$GSH*:* $F_{1,44.01}$ = 1.93, *P* = 0.17; $_{ox}$GSH: $F_{1,42.67}$ = 0.98, *P* = 0.33; $_{red}$GSH: $F_{1,47.93}$ = 1.59, *P* = 0.21; Table 3.2 A-C). There was however tight association between nestling GSH concentrations and body mass ($_{t}$GSH: $F_{1,56.87}$ = 9.84, *P* = 0.003; $_{ox}$GSH: $F_{1,55.24}$ = 8.70, *P* = 0.005; $_{red}$GSH: $F_{1,62.95}$ = 6.33, *P* = 0.015), with heavier nestlings having higher levels of $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH (Figure 3.5).

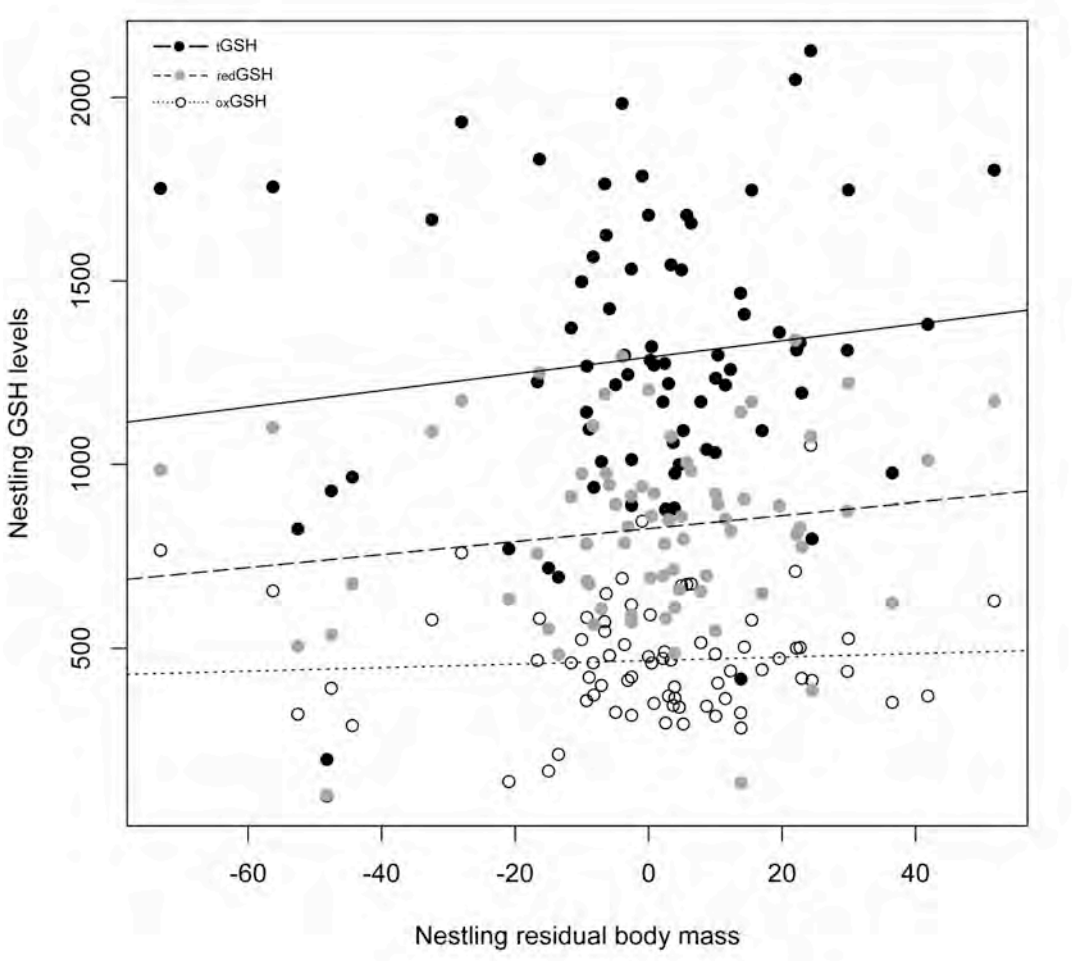

Figure 3.5: Nestling residual body mass (corrected for wing length) in relation to their $_{t}$GSH (closed circles and straight regression line; r^2 = 0.02, n = 73, $F_{1,56.87}$ = 9.84, P = 0.003), $_{ox}$GSH (open circles and small dash regression line; r^2 = 0.004, n = 74, $F_{1,55.24}$ = 8.70, P = 0.005) and $_{red}$GSH (gray circles and long dash regression line; r^2 = 0.02, n = 73, $F_{1,62.95}$ = 6.33, P = 0.015).

In adults, $_{t}$GSH levels were significantly explained by their plumage coloration, light melanic breeding adults showing higher $_{t}$GSH levels than darker melanic ones ($F_{1,52.69}$ = 4.35, P = 0.04; Table 3.2D, Figure 3.6). Interestingly, we found that adult melanin-based coloration was not significantly associated with $_{ox}$GSH levels ($F_{1,48.97}$ = 0.49, P = 0.49; Table 3.2E, Figure 3.6), but marginally with $_{red}$GSH levels ($F_{1,51.99}$ = 3.80, P = 0.057; Table 3.2F, Figure 3.6). Note also that we did not find any effect of the BSM experiment (alone or in interaction with coloration, P-values > 0.09; Table 3.2), even when removing residual body mass from these models.

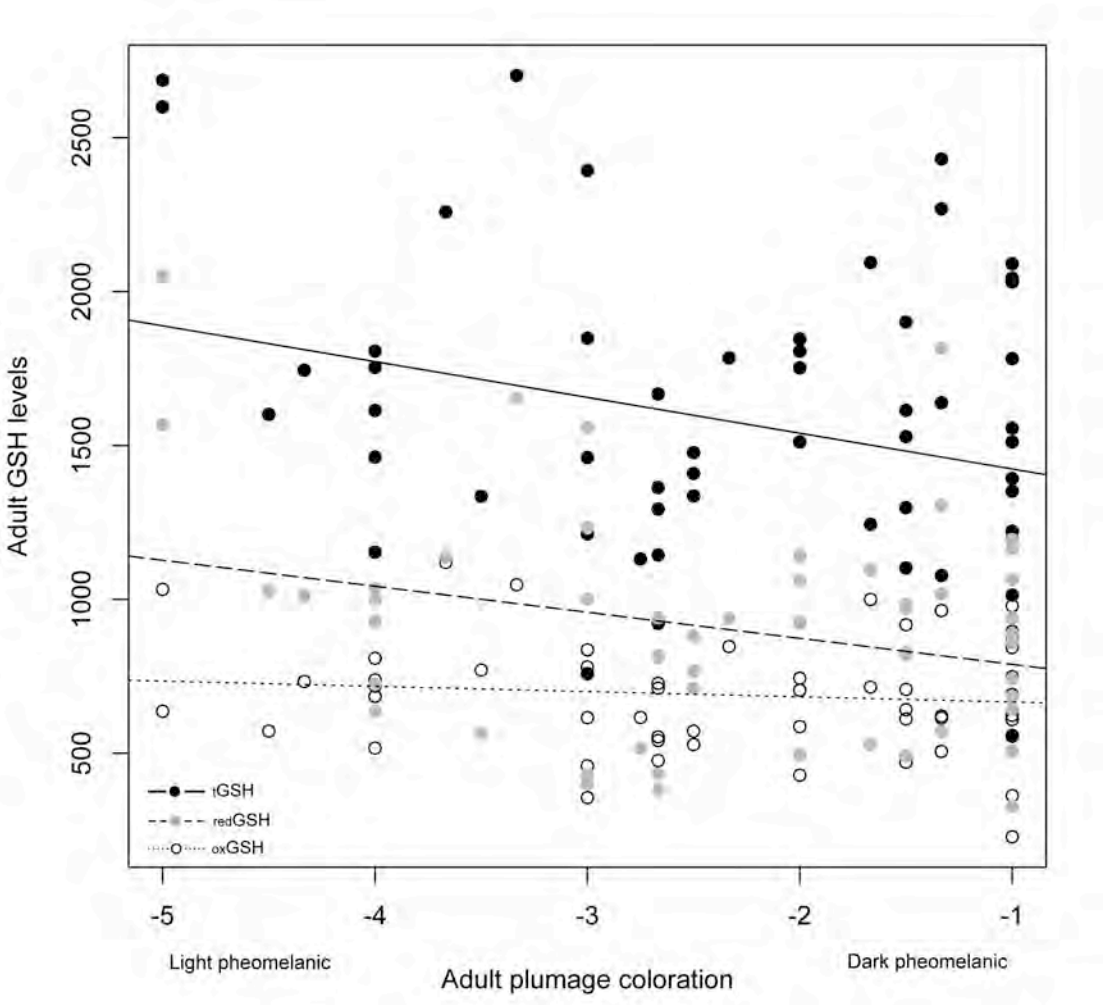

Figure 3.6: Plumage reddishness in relation to $_{t}$GSH (closed circles and straight regression line; r^2 = 0.077, n = 56, $F_{1,52.69}$ = 4.35, P = 0.04), $_{ox}$GSH (open circles and small dash regression line; r^2 = 0.01, n = 54, $F_{1,48.97}$ = 0.49, P = 0.49) and $_{red}$GSH levels (gray circles and long dash regression line; r^2 = 0.075, n = 54, $F_{1,51.99}$ = 3.80, P = 0.057) in adult tawny owls.

Covariation between melanism and GSH levels: parent-offspring comparison

Plumage coloration of the biological mother was not associated with $_{t}$GSH levels measured in their cross-fostered offspring (linear mixed-model, $F_{1,15.39}$ = 0.16, P = 0.70), but we found a marginal trend for higher $_{t}$GSH levels in nestlings born from light melanic males ($F_{1,18.25}$ = 3.10, P = 0.10, Appendix 3.2A). However, we found no covariation between coloration of both biological parents and nestling $_{ox}$GSH (another linear mixed-model, father: $F_{1,18.58}$ = 1.99, P = 0.17; mother: $F_{1,16.5}$ = 0.02, P = 0.88) and $_{red}$GSH levels (another linear mixed-model, father: $F_{1,19.5}$ = 2.85, P = 0.11; mother: $F_{1,15.92}$ = 0.31, P = 0.59; Appendix 3.2A-C). Note also that no relationships were found when considering the mean coloration of both genetic parents in these models (P-values > 0.38). Similarly, plumage

coloration of the foster parents was neither related to nestling $_{t}$GSH (linear mixed-model, father: $F_{1,22.88}$ = 0.005, P = 0.95; mother: $F_{1,25.93}$ = 1.97, P = 0.17), nor to $_{ox}$GSH (another linear mixed-model, father: $F_{1,22.72}$ = 0.08, P = 0.78; mother: $F_{1,25.93}$ = 2.37, P = 0.14) and $_{red}$GSH levels (another linear mixed-model, father: $F_{1,23.77}$ = 0.01, P = 0.91; mother: $F_{1,28.17}$ = 1.29, P = 0.26; Appendix 3.2D-F). Again, no relationships were found between GSH concentrations and the mean coloration of both foster parents (P-values > 0.56). As mentioned above, nestling GSH levels were primarily dependent on their body mass, but were unaffected by the BSM (alone or in interaction with parental colour scores, P-values > 0.1; Appendix 3.2).

DISCUSSION

Although not questioning its genetic basis, our study demonstrated that the expression of GSH response was condition-dependent in our local population of tawny owls, conversely to melanin-based coloration. Indeed, we showed that nestling $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH levels were strongly related to body mass, while nestling $_{t}$GSH and $_{red}$GSH levels were mainly shaped by foster rearing conditions. Based on the idea of GSH dependence of pheomelanin-based colour traits, we expected a greater consumption of GSH (i.e. higher $_{ox}$GSH levels) in dark melanic nestlings, which were producing melanic feathers at the time when we blood-sampled them. This was however not the case. In contrast, we found that in adults, which were not moulting and hence not in a state of intense melanisation, melanin-based coloration was negatively correlated with $_{t}$GSH and $_{red}$GSH levels, dark melanic individuals showing lower $_{t}$GSH and $_{red}$GSH concentrations. In the following, we discuss potential mechanisms leading to covariations between adult $_{t}$GSH and $_{red}$GSH levels and their plumage coloration.

Genetic vs. environmental determinism of GSH expression

Covariations between melanin-based coloration and GSH may arise because of condition-dependent expression of either coloration and/or GSH. Our results in adult and nestling tawny owls revealed that the expression of melanin-based coloration was not or only weakly sensitive to environmental effects. This points out that the sign or the magnitude of a covariation between

coloration and GSH levels is more likely to be shaped by the condition-dependence of the GSH response in this species.

To sustain adequate levels, GSH is synthesized by a two-step biosynthetic pathway (Meister & Anderson 1983; Anderson 1998; Lu 2000), raising the hypothesis of strong genetic inheritance of GSH expression (Board *et al.* 1974). In Holstein cows (*Bos taurus*) for instance, heritability of GSH concentration in erythrocytes was estimated at 0.61 ± 0.16 in red blood cell samples and at 0.67 ± 0.17 in whole blood samples (Krogmeier *et al.* 1993). But this antioxidant response is also physiologically dependent on cysteine availability, which is partly regulated by food intake (Lu 2000). Yet, there are (to our knowledge) very few evidences of genetic or environmental determinism of GSH expression. The present study aimed to estimate the proportion of variation explained by these two types of determinism in the tawny owl. Despite the lack of influence of the brood size manipulation experiment on adult or nestling GSH levels, our results revealed that nestling $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH levels were strongly associated with body mass. Heavier nestlings were in better condition and probably more active, leading them to increase their metabolic rate and, in turn, their GSH concentrations. Despite the suggestive existence of a genetic basis (e.g. significant relation between nestling and genetic father $_{t}$GSH levels), average GSH concentrations measured in parents nevertheless emphasized that nestling $_{t}$GSH and $_{red}$GSH levels were primarily associated with those of their foster parents, highlighting the strong influence of foster rearing conditions on nestling GSH expression. In this context, food supply by the foster male is likely to play an essential role in the regulation of GSH expression, especially during poor breeding season like in 2011. First, because it provides resources (or energy) that can be allocated to different maintenance traits, favouring thereby their overall condition. Second, because it enables the replenishment of nestling GSH pool by increasing cysteine availability, a limiting factor in the GSH biosynthesis. In line with this hypothesis, a recent laboratory study showed that rat supplied with fish oil complements (i.e. a source of cysteine) presented higher levels of GSH in their brain (Denny Joseph & Muralidhara 2012), attenuating thereby the oxidative stress and mitochondrial dysfunctions induced by a neurotoxicant. Moreover, in humans, a restricted dietary supply of methionine and cysteine mixture slowed the rate of whole blood GSH synthesis (Lyons *et al.* 2000). Nevertheless, the role and expenditure of dietary cysteine in GSH expression still need to be tackled

further in natural systems, with experimental manipulations of either food supply or dietary cysteine levels (see for instance Badaloo *et al.* 2002).

Covariation between melanin-based coloration and GSH expression

Eu- and pheomelanogenesis are principally mediated by the binding of key peptides of the melanocortin system (i.e. α-MSH) and their antagonists (i.e. *Agouti*) to the Melanocortin-1-Receptor (MC1R), a transmembrane G-protein-coupled receptor expressed at high levels in melanocytes. These bindings up- or down-regulate the production of intracellular $_{c}$AMP and thereby the activity of tyrosinase, leading to the production of eumelanin or pheomelanin, respectively (Barsh 1996; Ito *et al.* 2000). Because of its sulfhydryl compounds, key cysteine-containing GSH plays an important regulatory role in tyrosinase activity (Benedetto *et al.* 1981; 1982; Land & Riley 2000), and, in turn, in the switch between eu- and pheomelanogenesis (Ozeki *et al.* 1997). Given the covariations observed between melanin-based coloration and resistance to oxidative stress (Roulin *et al.* 2011a), one can raise the assumption that GSH allocation in melanin-based coloration is traded-off against resistance to oxidative stress (e.g. Galvan & Alonso-Alvarez 2008; Galvan *et al.* 2010). Although not contradicting this trade-off resolution (especially in nestlings that are producing large amounts of pigments), we believe that an association between GSH, melanin-based coloration and oxidative balance can also occur as a consequence of colour-specific life histories. In the tawny owl, colour morphs differ in their life-history strategies (Roulin *et al.* 2003; Roulin *et al.* 2004; Roulin *et al.* 2005; Roulin *et al.* 2008b; Gasparini *et al.* 2009a; Piault *et al.* 2009; Emaresi *et al.* 2011), potentially leading them to differentially resolve the trade-off between current reproduction vs. antioxidant response (Emaresi *et al.* submitted, ***Chapter 2***). In this context, it is particularly difficult to predict whether eu- and pheomelanin-based coloration should be positively or negatively related to GSH levels, the sign and the magnitude of this relationship depending on too many factors (e.g. species and life stage considered, trade-off in GSH allocation, alternative GSH-independent life history strategies or polymorphism in GSH production). To better understand and capture how intracellular GSH is expressed and used between differently coloured individuals and at different life stages (i.e. when producing little or large amounts of pigments), we report here an experimental study where we

measured the global pool of GSH produced ($_{t}$GSH), but also the accurate amount of GSH already consumed ($_{ox}$GSH) and remaining ($_{red}$GSH) in adult and nestling tawny owls.

In agreement with previous study (Emaresi *et al.* submitted, ***Chapter 2***), we found that $_{t}$GSH levels and marginally $_{red}$GSH levels quantified in adult tawny owls were associated with melanin-based coloration, light melanic individuals showing higher levels than dark melanic ones. This indicated that light melanic breeding tawny owls generated greater GSH pool and had more GSH reserved for future use (i.e. $_{red}$GSH levels) compared to dark melanic ones. However, it is of particular interest to note that the different colour morphs were using similar amounts of GSH (i.e. $_{ox}$GSH levels) during this breeding season. Whereas we expected to find a greater GSH consumption (i.e. higher $_{ox}$GSH levels) in dark reddish nestlings because of the GSH dependence of pheomelanogenesis, we found no significant covariations between nestling $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH levels and melanin-based coloration (with respect to their own plumage coloration or those of their genetic parents), contradicting the strong claim that such an association should be universal (Galvan & Alonso-Alvarez 2008; Galvan & Alonso-Alvarez 2009; Galvan & Solano 2009). This latter outcome suggests that the expression of melanin-based coloration is not dependent on the pool of GSH available ($_{red}$GSH) and does not require specific amount of GSH ($_{ox}$GSH) in this species. Altogether, our results pointed out the GSH-independence of pheomelanogenesis in nestling tawny owls, but colour-specific differences in $_{t}$GSH and $_{red}$GSH concentrations in adults. Although we did not measure adult ROS production in 2011, greater $_{t}$GSH levels in light melanic adults are likely to be an adaptive response to an oxidative challenge. Because of their energy-demanding life history strategies (Emaresi *et al.* in prep, ***Chapter 1***) and their adaptation to stressful environmental or reproductive conditions (Roulin *et al.* 2003; 2004; 2008b; Piault *et al.* 2009; Emaresi *et al.* 2011), these individuals are more likely to face stronger metabolic constraints, as suggested by adult ROS production (Emaresi *et al.* submitted, ***Chapter 2***), forcing them to develop a stronger antioxidant response. Another hypothesis is that differently coloured individuals own different physiological strategies or metabolic rates, which require specific protein turnover. GSH being involved in protein metabolism as a source of cysteine, colour-specific difference in $_{t}$GSH and/or $_{red}$GSH concentrations may thus arise because of different needs in cysteine availability rather than antioxidant activity. Finally, another interesting hypothesis is the role of GSH

reductase (i.e. GR) in regenerating $_{red}$GSH from $_{ox}$GSH molecules. Under specific conditions, differently coloured tawny owls may have different patterns of GR expression, leading to colour-specific rates of GSH regeneration. If true, one can raise the idea that the colour morph regenerating faster its $_{red}$GSH stock may require consequently a smaller GSH pool ($_{t}$GSH levels). Additional studies are called to further investigate these alternative, but non-mutually exclusive hypotheses on the proximate reasons leading to colour-specific variations in GSH expression.

Conclusion

In this study, we highlighted the complexity of predicting the link between eu- and pheomelanin-based coloration and intracellular GSH response. The sign and the magnitude of this relation may differ according to the species considered, and hence the nature of the colour polymorphism (eu- vs. pheomelanin-based coloration), the life stage considered (individuals producing little or large amount of pigments), genetically-based polymorphism in the production of GSH or conversely the condition-dependence of the GSH response, and finally colour-specific differences in physiological constraints (e.g. various levels of oxidative stress or protein turnover). These factors being non mutually exclusive, it is extremely difficult to propose *a priori* predictions without a complete knowledge of the biology of the model species.

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TABLES

Table 3.1: Results of linear mixed models testing the genetic and environmental components of GSH expression in nestling tawny owl. In separate models, we investigated the relationship between $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels of cross-fostered nestlings and the levels of their biological (i.e. $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels of Genetic Father and Genetic Mother, models *A,C,E*) and foster parents (i.e. $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels of Foster Father and Foster Mother, models *B,D,F*). In all models, we also tested for an effect of the brood size manipulation treatment (BSM) on nestling GSH concentrations, plus the two-way interactions between BSM and corresponding parental GSH levels. We controlled for an effect of the genetic (models with biological parents) or foster nest site (models with foster parents) by including either genetic or foster brood identity as random factor. Backward stepwise procedure was used to remove non-significant terms (*P* > 0.05), until obtaining best-fitted models (bold values).

Source of variation	n	*df*	*F*	*P*		n	*df*	*F*	*P*
A. Nestling $_{t}$GSH levels					**D. Nestling $_{ox}$GSH levels**				
$_{t}$GSH GeneticMale	48	**1,15.43**	**6.07**	**0.03**	**$_{ox}$GSH FosterMale**	61	**1,25.85**	**7.15**	**0.01**
BSM		1,16.15	0.84	0.37	BSM		1,24.49	3.94	0.06
$_{t}$GSH GeneticFemale		1,11.48	0.01	0.91	$_{ox}$GSH FosterFemale		1,18.66	0.45	0.51
BSM x $_{t}$GSH GeneticMale		1,10.86	2.96	0.11	BSM x $_{ox}$GSH FosterFemale		1,18.09	1.38	0.25
BSM x $_{t}$GSH GeneticFemale		1,11.14	0.07	0.8	BSM x $_{ox}$GSH FosterMale		1,17.21	0.001	0.98
B. Nestling $_{t}$GSH levels					**E. Nestling $_{red}$GSH levels**				
$_{t}$GSH FosterMale	54	**1,20.65**	**6.83**	**0.02**	$_{red}$GSHGeneticMale	48	1,19.18	1.34	0.26
$_{t}$GSH FosterFemale		**1,18.69**	**5.37**	**0.03**	BSM		1,17.3	0.16	0.69
BSM		1,19.68	0.79	0.38	$_{red}$GSH GeneticFemale		1,11.57	0.01	0.91
BSM x $_{t}$GSH FosterMale		1,20.32	0.69	0.42	BSM x $_{red}$GSH GeneticFemale		1,9.54	1.73	0.22
BSM x $_{t}$GSH FosterFemale		1,17.54	0.18	0.68	BSM x $_{red}$GSH GeneticMale		1,14.18	0.48	0.5
C. Nestling $_{ox}$GSH levels					**F. Nestling $_{red}$GSH levels**				
$_{ox}$GSH GeneticMale	49	**1,13.54**	**5.92**	**0.03**	**$_{red}$GSH FosterFemale**	62	**1,22.5**	**11.41**	**0.003**
BSM		1,14.05	1.38	0.26	$_{red}$GSH FosterMale		1,19.18	0.94	0.34
$_{ox}$GSH GeneticFemale		1,10.66	1.23	0.29	BSM		1,17.35	0.01	0.92
BSM x $_{ox}$GSH GeneticMale		1,10.21	1.12	0.31	BSM x $_{red}$GSH FosterFemale		1,15.51	2	0.18
BSM x $_{ox}$GSH GeneticFemale		1,9.047	0.01	0.91	BSM x $_{red}$GSH FosterMale		1,15.06	1.24	0.28

Table 3.2: Within-individual linear mixed models investigating the link between melanin-based coloration and the levels of $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH in nestling (*A-C*) and adult (*D-F*) tawny owls. In addition of residual body mass (corrected for wing length in nestlings and gender in adults) as covariate, we entered BSM and nestling or adult plumage coloration (i.e. 'Brownchroma' in models *A-C* and 'ColourMorph' in model *D-F* or, respectively) as explanatory variables, plus the two-way interaction between both variables. We controlled for the effect of the breeding site by including nest identity as random factor. Backward stepwise procedure was used to remove non-significant terms (*P* > 0.05), until obtaining best-fitted models (bold values).

Source of variation	n	*df*	*F*	*P*
A. $_{t}$GSH levels in nestlings				
Residual Mass	**73**	**1,56.87**	**9.84**	**0.003**
Brownchroma		1,44.01	1.93	0.17
BSM		1,23.92	0.34	0.56
BSM x Brownchroma		1,52.37	0.02	0.89
B. $_{ox}$GSH levels in nestlings				
Residual Mass	**74**	**1,55.24**	**8.7**	**0.005**
Brownchroma		1,42.67	0.98	0.33
BSM		1,22.96	0.57	0.46
BSM x Brownchroma		1,51.65	0.31	0.58
C. $_{red}$GSH levels in nestlings				
Residual Mass	**73**	**1,62.95**	**6.33**	**0.015**
Brownchroma		1,47.93	1.59	0.21
BSM		1,24.84	0.13	0.72
BSM x Brownchroma		1,53.72	0.02	0.88

	n	*df*	*F*	*P*
D. $_{t}$GSH levels in adults				
ColourMorph	**56**	**1,52.69**	**4.35**	**0.04**
BSM		1,23.75	3.15	0.09
Residual Mass		1,48.44	0.01	0.91
ColourMorph x BSMA		1,47.95	0.02	0.9
E. $_{ox}$GSH levels in adults				
ColourMorph	54	1,48.97	0.49	0.49
Residual Mass		1,40.43	0.13	0.72
BSM		1,21.45	0.02	0.9
ColourMorph x BSMA		1,44.36	0.34	0.56
F. $_{red}$GSH levels in adults				
ColourMorph	54	1,51.99	3.8	0.057
BSM		1,26.45	2.93	0.1
Residual Mass		1,46.99	0.13	0.72
ColourMorph x BSMA		1,45.72	0.03	0.85

APPENDIX

Appendix 3.1: Results of linear mixed models investigating the effects of the brood size manipulation and plumage coloration and their interactions on (*A*) nestling and (*B*) adult body mass. In all models, we entered individual gender (Sex) and wing length (Wing), date and time of the blood sampling (i.e. (Date and Hour), and brood size before treatment (Brood size) as covariates to control for mass variability. Backward stepwise procedure was used to remove non-significant terms (*P* > 0.05), until obtaining best-fitted models (bold values).

Source of variation	n	*df*	*F*	*P*
A. Nestling body mass				
Wing	**75**	**1,28.48**	**262.05**	**<.0001**
Brood size		**1,10.3**	**7.97**	**0.02**
Sex		1,58.9	2.95	0.09
Hour		1,10.38	1.09	0.32
BSM		1,8.7	0.56	0.47
Brownchroma		1,45.3	1.28	0.26
BSM x Brownchroma		1,43.25	2.88	0.10
Date		1,15.86	0.83	0.38
Sex x BSM		1,38.71	0.93	0.34
Sex x Brownchroma		1,34.72	0.0006	0.98
Sex x BSM x Brownchroma		1,35.1	0.52	0.48
B. Adult body mass				
Hour	**55**	**1,47.51**	**13.33**	**0.0007**
Sex		**1,43.19**	**70.40**	**<.0001**
BSM		**1,31.03**	**9.01**	**0.005**
Date		1,50	3.63	0.06
Sex x BSM		1,32.47	3.11	0.09
Brood size		1,30.28	1.45	0.24
ColourMorph		1,45.63	0.20	0.66
Sex x ColourMorph		1,44.55	0.18	0.68
Wing		1,40.95	1.11	0.30
ColourMorph x BSM		1,39.77	1.29	0.26
Sex x ColourMorph x BSM		1,38.92	1.80	0.19

Appendix 3.2: Results of linear mixed models investigating the link between adult plumage melanin-based coloration and the levels of $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH in nestling tawny owls. In addition of nestling residual body mass (corrected for wing length) as covariate, we entered BSM and coloration scores of their genetic ('GFatherMorph' and 'GMotherMorph', models *A*-*C*) or foster parents ('FFatherMorph' and 'FMotherMorph', models *D*-*F*) as explanatory variables, plus the three-way interactions between these variables. We controlled for an effect of the nest of origin or of rearing by including genetic or foster brood identity as random factor. Backward stepwise procedure was used to remove non-significant terms (*P* > 0.05), until obtaining best-fitted models (bold values).

Source of variation	n	*df*	*F*	*P*
A. $_{t}$GSH levels in nestlings				
Residual Mass	**73**	**1,56.87**	**9.84**	**0.003**
GFatherMorph		1,18.25	3.1	0.1
GMotherMorph		1,15.39	0.16	0.7
GFatherMorph x GMotherMorph		1,16.37	3.88	0.07
BSM		1,15.35	1.56	0.23
GMotherMorph x BSM		1,14.93	0.17	0.68
GFatherMorph x BSM		1,12.53	0.07	0.8
GFatherMorph x GMotherMorph x BSM		1,12.41	0.23	0.64
B. $_{ox}$GSH levels in nestlings				
Residual Mass	**74**	**1,55.24**	**8.7**	**0.005**
GFatherMorph		1,18.58	1.99	0.17
GMotherMorph		1,16.5	0.02	0.88
GFatherMorph x GMotherMorph		1,16.4	2.25	0.15
BSM		1,15.34	1.5	0.24
GMotherMorph x BSM		1,14.65	0.24	0.63
GFatherMorph x BSM		1,12.71	0.23	0.64
GFatherMorph x GMotherMorph x BSM		1,12.38	1.05	0.32
C. $_{red}$GSH levels in nestlings				
Residual Mass	**73**	**1,62.95**	**6.33**	**0.015**
GFatherMorph		1,19.5	2.85	0.11
GMotherMorph		1,15.92	0.31	0.59
GFatherMorph x GMotherMorph		1,18.02	3.54	0.08
BSM		1,16.68	0.88	0.36
GMotherMorph x BSM		1,16.73	0.06	0.8
GFatherMorph x BSM		1,13.01	0	0.98
GFatherMorph x GMotherMorph x BSM		1,13.08	0	0.99

	n	*df*	*F*	*P*
D. $_{t}$GSH levels in nestlings				
Residual Mass	**73**	**1,56.87**	**9.84**	**0.003**
FMotherMorph		1,25.93	1.97	0.17
BSM		1,24.78	1.39	0.25
FFatherMorph		1,22.88	0.005	0.95
BSM x FMotherMorph		1,22.48	1.85	0.19
FFatherMorph x FMotherMorph		1,20.69	0.14	0.71
BSM x FFatherMorph		1,21.8	0.01	0.94
BSM x FFatherMorph x FMotherMorph		1,19.66	1	0.33
E. $_{ox}$GSH levels in nestlings				
Residual Mass	**74**	**1,55.24**	**8.7**	**0.005**
FMotherMorph		1,25.93	2.37	0.14
BSM		1,24.55	2.86	0.1
FFatherMorph		1,22.72	0.08	0.78
BSM x FMotherMorph		1,22.45	2.29	0.14
FFatherMorph x FMotherMorph		1,20.36	1.73	0.2
BSM x FFatherMorph		1,21.49	0	1
BSM x FFatherMorph x FMotherMorph		1,19.62	2.16	0.16
F. $_{red}$GSH levels in nestlings				
Residual Mass	**73**	**1,62.95**	**6.33**	**0.015**
FMotherMorph		1,28.17	1.29	0.26
BSM		1,26.37	0.42	0.52
FFatherMorph		1,23.77	0.01	0.91
BSM x FMotherMorph		1,23.13	1.1	0.31
BSM x FFatherMorph		1,23.67	0.07	0.8
FFatherMorph x FMotherMorph		1,19.52	0.06	0.81
BSM x FFatherMorph x FMotherMorph		1,19.62	0.32	0.58

CHAPTER 4

Pale and dark reddish melanic tawny owls differentially regulate the level of blood circulating POMC prohormone in relation to environmental conditions

Alexandre Roulin[1], Guillaume Emaresi[1], Pierre Bize[1], Julien Gasparini[1,2], Romain Piault[1,3] and Anne-Lyse Ducrest[1]

[1]*Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland*

[2]*Laboratoire Ecologie et Evolution, Université Pierre et Marie Curie, CNRS UMR 7625, F-75005 Paris, France*

[3]*Animal Ecology Group, University of Groningen, Kerklaan 30, NL-9751 NN Haren, The Netherlands*

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ABSTRACT

Knowledge of the hormonal pathway controlling genotype-specific norms of reaction would shed light on the ecological factors to which each genotype is adapted. Environmentally-mediated changes in the sign and magnitude of covariations between heritable melanin-based coloration and fitness components are frequent, revealing that extreme melanin-based phenotypes can display different physiological states depending on the environment. Yet, the hormonal mechanism underlying this phenomenon is poorly understood. One novel hypothesis proposes that these covariations stem from pleiotropic effects of the melanocortin system. Melanocortins are post-translationally modified bioactive peptides derived from the POMC prohormone that are involved in melanogenesis, anti-inflammation, energy homeostasis and stress responses. Thus, differential regulation of fitness components in relation to environmental factors by pale and dark melanic individuals may be due to colour-specific regulation of the POMC prohormone. Accordingly, we found that the degree of reddish melanic coloration was negatively correlated with blood circulating levels of the POMC prohormone in female tawny owls (*Strix aluco*) rearing a brood for which the size was experimentally reduced but not when enlarged, and in females located in rich but not in poor territories. Our findings support the hypothesis that the widespread links between melanin-based coloration and fitness components may be mediated, at least in part, by the melanocortin system.

INTRODUCTION

Organisms usually experience temporal and spatial variation in environmental conditions. This phenomenon generates fluctuating selective pressures, potentially favouring different genotypes that vary in their phenotypic response to environmental changes, denoted norms of reaction or genotype by environment interaction (Hedrick 2006). Norms of reactions are shaped by key regulators such as hormones (e.g. sex steroids: Shahjahan *et al.* 2010) that are adjusted throughout the life cycle according to environmental conditions. Thus, our understanding of the proximate basis of reaction norms requires the measurement, along an environmental gradient, of candidate hormones. In the present study, we adopt such an approach to investigate the proximate basis of the adaptive function of melanin-based coloration. We consider this phenotypic trait because the biochemistry of melanogenesis is well known (Sturm 2006). As for most multigenic phenotypic traits, several genes and hormones known to alter melanogenesis can explain inter-individual variation in coloration (e.g. 127 loci in mice; Silvers 1979; Bennett & Lamoreux 2003). Here, we focus only on one set of genes belonging to the melanocortin system.

From a proximate perspective, the melanocortin system is involved in the regulation of multiple physiological functions, and may thus underlie covariations between melanin-based coloration and other phenotypic traits such as energy homeostasis, immunity, aggressiveness, sexual behaviour, resistance to oxidative stress and activation of the stress response and its further modulation (reviewed in Ducrest *et al.* 2008). Melanocortins are peptides derived from the proopiomelanocortin gene (*POMC*), which is translated to a POMC prohormone. This molecule undergoes a series of cell-specific proteolytic cleavages and modifications that result in the generation of several peptides, namely the melanocortins (α-, β- and γ-melanin-stimulating-hormones MSH, the adrenocorticotropic hormones ACTH), and the endorphins (Pritchard *et al.* 2002). In vertebrates, the *POMC* gene is mainly expressed in the pituitary gland, but also in the central nervous system; in most peripheral tissues, neural and pituitaric melanocortins act as neurocrine and endocrine factors (Cone 2006), whereas peripherally produced melanocortins have paracrine and autocrine functions (Slominski *et al.* 2000).

Feather melanisation occurs during growth in chicks and moulting in adults. Melanin coloration is generated by the deposition of mixed eu- (brown/black to grey) and pheomelanin (yellow to reddish brown) pigments. In the first of three steps of melanogenesis, the rate-limiting enzyme tyrosinase oxidases L-tyrosine to produce dopaquinone that is further transformed to cysteinyldopa in the presence of cysteine derivatives. In a second step, pheomelanin is produced and in the last stage when cysteine donors are depleted eumelanin polymers are synthesized (Ito & Wakamatsu 2008). Therefore, the quantity and the ratio of eu- to pheomelanin depend primarily on the activity of tyrosinase and the presence of substrates cysteine and tyrosine in the melanosomes. Two major regulators of melanogenesis, namely the melanocortins and the Agouti protein (ASIP) control the level and activity of the tyrosinase via binding to the melanocortin 1 receptor (MC1R Ito & Wakamatsu 2010; Walker & Gunn 2010). Binding of melanocortins, particularly α-MSH, to MC1R induces transcription and activity of eumelanic genes such as MITF, tyrosinase, TRP1 and DCT, and therefore increases the production of black/brown eumelanin. In contrast, the inverse-agonist/antagonist ASIP blocks the transcription of eumelanic genes (Le Pape *et al.* 2009) and hence switches the balance between eumelanogenesis towards pheomelanogenesis (Lin & Fisher 2007).

The different melanocortin peptides not only bind the MC1R but four other melanocortin receptors that regulate morphological, physiological and behavioural traits. Because of the numerous pleiotropic effects of the *POMC* gene, we predict an association between the different traits regulated by the melanocortins binding to the different MCRs (Ducrest *et al.* 2008). Hence, in species in which melanocortins account for part of the inter-individual variation in coloration, eumelanin- and pheomelanin-based coloration may be correlated positively and negatively, respectively, with the levels of the POMC prohormone and the levels of melanocortins that bind to MC2-5Rs.

Interestingly, recent studies showed that covariations between the degree of melanic coloration and other phenotypic traits are more easily detected under specific environmental conditions (Roulin *et al.* 2008b; Piault *et al.* 2009; Roulin 2009). In the tawny owl (*Strix aluco*), for instance, we found that nestlings produced by pale and dark reddish melanic females have different reaction norms. Among nestlings raised by foster parents, nestlings born from dark reddish mothers grew faster than

those born from paler reddish mothers when brood size was experimentally reduced but not when it was experimentally enlarged with dark and pale individuals growing at similar rates (Roulin *et al.* 2008b). Thus, in the tawny owl the genetic correlation between melanic coloration and offspring growth was stronger in some environments than in others. Because in the tawny owl the expression of reddish melanism is strongly heritable and not or weakly condition-dependent (Gasparini *et al.* 2009a), this observation is likely to be the result of the action of some genes involved in growth trajectories and for which offspring born from differently coloured mothers should display either distinct alleles or different expression levels in relation to rearing conditions.

To test whether regulation of the POMC prohormone is colour-specific, we performed a brood size manipulation experiment in breeding female tawny owls (i.e. these females raised a brood for which we either added or removed one hatchling) in order to induce changes in the level of parental workload and thus modify the level of stress experienced by parents. Assuming that melanocortin hormones mediate colour-specific growth patterns found in previous experiments (Roulin *et al.* 2008b; Piault *et al.* 2009), we measured the levels of blood circulating POMC prohormone (Barna *et al.* 1998; Bell *et al.* 2005; Myers *et al.* 2005) in these breeding females. We thus investigated whether the levels of the POMC prohormone covary with the degree of reddish melanic coloration differentially in females rearing an experimentally reduced or enlarged brood. Interestingly, beechnut (*Fagus sylvatica*) production was particularly pronounced (pers. obs.), a food source for wood mice (*Apodemus* sp.) and bank voles *Clethrionomys glareolus* (Abt & Bock 1998; Margaletic *et al.* 2005), the staple prey species in the study area (Roulin *et al.* 2008a). Thus, beechnut densities should be proportional to prey densities. As a consequence, we examined whether the levels of the POMC prohormone covaries with the degree of reddish coloration differently in females rearing a progeny located in rich and poor territories with respect to beech density.

The present study is a first step towards the understanding of the potential role played by melanocortins in generating reaction norms. Significant results should stimulate more detailed studies on the pleiotropic effects of the melanocortin system, its importance in generating covariations between melanin-based coloration and other phenotypic traits, and more generally why environmental

heterogeneity can promote the evolution of local adaptation (Kawecki & Ebert 2004). Our aim is therefore not to test whether inter-individual variation in reddish coloration is the result of the melanocortin system, but whether differently coloured owls differentially regulate the POMC prohormone in relation to environmental factors.

METHODS

The study was carried out in 2007 in western Switzerland, where we installed 366 nest boxes in forests located within a 911 km^2 area, at a mean altitude of 672 m (range: 458-947 m). Nest boxes were hung up on trees in forest patches of at least 4'000 m^2; the mean distance between two nest boxes was 627 m with a minimal distance of 500 m. The landscape consists of managed forests (26.6%) and farmland (55.5%), with 116 villages of 100 to 1'000 inhabitants dispersed on the whole area. Forest patches ranged from 0.0038 km^2 to 32 km^2 and were composed mainly of beeches followed by oaks (*Quercus spp.*), pure spruce (*Picea abies*), European silver fir (*Abies alba*) and common ash (*Fraxinus excelsior*). Farmlands consist mainly in cereal fields, pastures, truck farming, fruit orchards and fallows. In 2007, the 54 breeding females for which we measured levels of the POMC prohormone laid between 2 and 7 eggs (mean ± SD: 4.8 ± 1.0) from 1 February to 8 March (mean ± SD: 24 February ± 7 days). Ninety-one percent of the eggs hatched and 1 to 7 nestlings per nest took their first flight (mean ± SD: 4.2 ± 1.4). Nestlings grow rapidly and leave their nest at 25-30 days of age (Galeotti 2001).

Measurement of reddish coloration

For each breeding female, we collected three feathers located on their back 5 cm below the neck. Feathers were then stuck with adhesive tape onto a black paper, placed in a black box equipped with a fluorescent tube (8w/20-640 bl-super), and individually photographed with a digital camera (Dimage A200, Konika Minolta) fixed at a distance of 27 cm to the feather. Pictures were imported in the software Adobe Photoshop to measure individual spectral hues, saturation and brightness. For each individual we calculated a mean value over the three feathers and then extracted the first component (PCA1) of a principal components analysis, which explained 72% of the total variance. We multiplied PCA1 values with -1 to obtain a scale from pale to dark reddish, a methodology that we did not use in

previous papers. Measurements of coloration (i.e. PCA1) were shown to be repeatable, and strongly correlated with colour scores obtained with a spectrophotometer, and colour morph estimated in the field (Pearson's correlation: $r = 0.80$, $n = 54$, $P < 0.0001$). The concentration of pheomelanin pigments stored in feathers accounts for 68% of the total variance in reddish coloration and eumelanin pigment concentrations for only 21% (Gasparini *et al.* 2009a). Additional analyses based on the data collected by these authors showed that the ratio of pheomelanin/eumelanin feather content and the total amount of melanins contained in feathers are associated with PCA1 ($r = 0.57$, $n = 15$, $P = 0.027$ and $r = 0.87$, $n = 15$, $P < 0.0001$, respectively).

Experimental procedure

In 2007, we matched 90 nests in pairs with similar hatching date (Pearson's correlation, $P < 0.0001$) and manipulated brood sizes by exchanging hatchlings between nests of the same pair; we took on average 2.4 nestlings from a nest E (enlarged) and brought them in another nest R (reduced) where we took on average 3.4 hatchlings to be brought in nest E. Each family was thus composed of nestlings from two origins with half of the nests being experimentally enlarged by one nestling and the other half of the nests experimentally reduced by one nestling. Out of the 90 initial nests, we were able to collect a blood sample to measure POMC prohormone in adult females in 27 of the reduced nests and 27 of the enlarged nests. Age of the offspring when their mother was blood sampled did not differ between the two treatments (14 ± 3.7 days, range: 9-21; Student's *t*-test: $t_{52} = 1.19$, $P = 0.24$). Clutch size of enlarged and reduced nests was similar ($t_{52} = 0.70$, $P = 0.49$). Breeding females from the two brood size treatments did not differ in wing, tail and tarsus lengths, body mass at the time of blood sampling and plumage coloration (Student's *t*-tests, *P*-values > 0.30). Within pairs of experimental nests foster and biological mothers did not significantly resemble each other with respect to reddish coloration ($r = -0.13$, $n = 35$, $P = 0.46$) and pairing with respect to coloration was not significantly disassortative in both the reduced and enlarged brood size treatment (Pearson's correlation comparing coloration of female and male partners: $r = -0.13$, $n = 27$, $P = 0.53$ vs. $r = -0.09$, $n = 27$, $P = 0.67$). We successfully created broods with a different number of nestlings, as at the time when we blood sampled breeding females their nest contained significantly more nestlings in the enlarged than reduced treatment (mean ± SE, 4.9 ± 0.2 vs. 3.6 ± 0.2; Student's *t*-test: $t_{52} = 3.88$, $P = 0.0002$). To

investigate the long-term effect of the brood size manipulation experiment, we captured breeding individuals in 2008 and examined whether females rearing an enlarged brood in 2007 were less likely to breed in 2008 than females rearing a reduced brood in 2007.

Because beechnut production was very high in the autumn 2006, we recorded the proportion of beech trees during the winter 2006-2007, walking 75 m in the four cardinal points around each nest box. This proportion (mean is 32%, range: 0-92%) was $\log_{10}+1$ transformed to obtain a normal distribution. In 2007, owls bred earlier in forests where beeches were more abundant (r = -0.25, n = 95, P = 0.015) indicating that abundance in beeches is an appropriate surrogate of some aspects of territory quality in the study year. The proportion of beech trees in territories of experimentally enlarged and reduced broods was similar (P = 0.17). Within each brood size treatment female plumage coloration was not significantly correlated with female body size (i.e. wing, tail, tarsus and mass) and nestling age when females were blood sampled (Pearson's correlations, P-values > 0.18) nor with the proportion of beeches (ANCOVA: colour: $F_{1,50}$ = 0.95, P = 0.33; treatment: $F_{1,50}$ = 2.21, P = 0.14; interaction: $F_{1,50}$ = 2.90, P = 0.10).

Assessment of blood circulating levels of the POMC prohormone

Blood sample of each of the 54 breeding females was collected in tubes containing EDTA, immediately centrifuged to separate the plasma from the blood cells, and frozen in liquid nitrogen in the field until placed on the same day in the laboratory at –80°C. We quantified the amount of blood circulating POMC prohormone using the human OCTEIA POMC ELISA kit (IDS Ltd, Boldon, UK). We carried out the analyses using 50 µl of plasma. This kit consists of a sandwich assay using two antibodies, which bind to POMC prohormone accordingly to its relative concentration. The sensitivity of the assay is 8 pmol/l, the inter-assay precision is 10% and the cross-reactivity for ACTH is 3.6% and for α-MSH 2.2%. We box-cox transformed POMC prohormone values to normalize the dataset. POMC prohormone levels were not correlated with time of the day when blood samples were collected (mean is 1245 hours; range: 0745 and 1745 hours) (r = 0.04, n = 54, P = 0.79), the time taken between capture and blood sampling (mean is 3.2 min; range: 0.1 and 4.3 min; r = 0.10, n = 54, P = 0.49) and nestling age (r = -0.12, n = 54, P = 0.37). These variables were therefore not considered in further analyses. In six females and one male we collected two blood samples on two occasions.

POMC prohoromone levels measured on these two occasions were strongly correlated (r = 0.82, n = 7, P = 0.025). To avoid pseudo-replication we calculated mean values over the two measurements. Finally, it is worth noting that the level of POMC prohormone we measured is not the amount of melanocortins directly involved in melanogenesis, since the feathers collected for colour assessment were not growing anymore.

Statistics

We performed statistical analyses using JMP IN 7.0.0. We used a stepwise ANCOVA including the box-cox transformed levels of POMC prohormone as the dependent variable, and brood size treatment ('treatment'), proportion of trees that were beeches ('beeches') and reddish coloration measured as PCA1 ('colour') as three independent variables, plus all possible interactions. Non-significant interactions were subsequently removed from the final model. The analyses are two-tailed and significance level is set to 0.05. In all models, residuals were normally distributed, and variances were homogeneous between treatments.

RESULTS

The brood size manipulation experiment had the intended effect on breeding females. Out of the 27 females rearing an experimentally enlarged brood in 2007 only eight (29.6%) of them were breeding in 2008, whereas 16 out of 27 (59.3%) females rearing an experimentally reduced brood in 2007 were breeding in 2008 (chi-square test: χ^2 = 4.80, P = 0.028). Mean concentration in the POMC prohormone was 68.5 pmol/l (SD = 85.4; range = 6 and 346.7). After stepwise backward simplification of the model, the interactions 'treatment' by 'colour' and 'beeches' by 'colour' were both significant (Table 4.1).

Table 4.1. Analysis of covariance testing POMC prohormone levels (box-cox transformed) in relation to brood size manipulation experiment and the proportion of trees that were beeches (an index of food abundance) in breeding female tawny owls. The term 'colour' refers to the degree of reddish coloration, 'treatment' to the brood size manipulation experiment (enlarged vs. reduced broods), and 'beeches' to the proportion of trees that were beeches in forests where nest boxes were erected. We removed from the final model the triple interactions and the two-way interaction 'Treat x beeches' which were not significant (*P*-values > 0.40).

	Test statistics		
Source of variation	*F*	*df*	*P*
Colour	2	1, 48	0.16
Treatment	0.09	1, 48	0.84
Beeches	0.11	1, 48	0.74
Colour x Beeches	**4.48**	**1, 48**	**0.039**
Colour x Treatment	**4.8**	**1, 48**	**0.033**

When brood size was experimentally reduced, darker reddish females showed lower levels in the POMC prohormone than pale reddish females (multiple regression analysis, colour: $F_{1,24} = 4.90$, $P = 0.037$; beech: $F_{1,24} = 0.51$, $P = 0.48$), whereas there was no relationship between coloration and levels of the POMC prohormone in the enlarged treatment (multiple regression analysis, colour: $F_{1,24} = 0.02$, $P = 0.88$; beech: $F_{1,24} = 0.99$, $P = 0.33$; Figure 4.1).The interaction 'beeches' by 'colour' was significant because darker reddish females had lower levels of the POMC prohormone when their territory was located in a forest where beeches were abundant (territories with proportion of beeches above the median, ANCOVA, colour as covariate: $F_{1,25} = 5.57$, $P = 0.026$; treatment as factor: $F_{1,25} = 0.12$, $P = 0.73$) but not when relatively rare (territories with proportion of beeches below the median, ANCOVA, colour as covariate: $F_{1,23} = 0.03$, $P = 0.87$; treatment as factor: $F_{1,23} = 0.37$, $P = 0.55$).

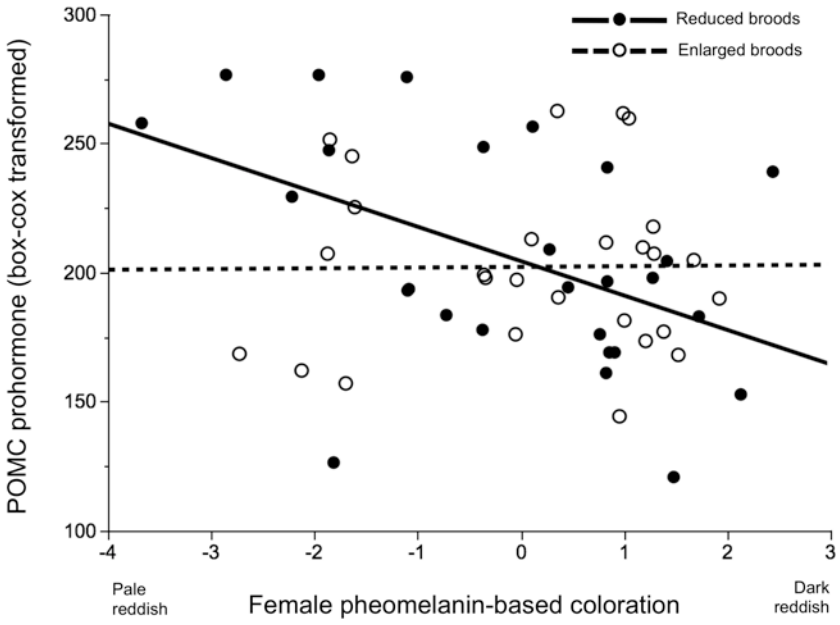

Figure 4.1: Relationship between POMC prohormone levels (box-cox transformed) in relation to reddish coloration in breeding female tawny owls raising an experimentally reduced (closed circles and straight regression line; r = -0.48, n = 27, P = 0.012) or enlarged brood (open circles and broken regression line; r = 0.01, n = 27, P = 0.95).

DISCUSSION

The *POMC* gene is mainly expressed in the pituitary gland and also in the brain and in peripheral tissues. The *POMC* mRNA is translated to the POMC prohormone, which through tissue-specific processing results in the different melanocortins (α-, β-, and γ-MSH, ACTH as well as α-, β-, and γ-endorphins) that exert neurocrine, endocrine and paracrine actions (Pritchard *et al.* 2002; Boswell & Takeuchi 2005). Because these different melanocortins are able to bind to five distinct melanocortin receptors (MC1-5Rs), controlling melanogenesis but also behavioural and physiological traits, a recent genetic model (Ducrest *et al.* 2008) proposed the hypothesis that the melanocortin system generates phenotypic correlations between melanin-based coloration and other attributes. This hypothesis assumes that the level of melanocortin activity in one tissue is proportional to activity in other tissues. Connections between the different tissues, particularly between the pituitary and the skin may exist through endocrine and neurocrine circuits. Homozygous knockout mice for Tpit (a Tbox transcription factor) that is restrictively expressed and regulates the pituitary development, used as a

model for isolated ACTH defect, exhibit a yellowish belly (Pulichino *et al.* 2003) as POMC KO mice (Yaswen *et al.* 1999) and POMC deficient human (Krude *et al.* 1998). This suggests that in mice pituitaric α-MSH circulates in the blood stream and regulates melanogenesis in skin. Based on this kind of observation, we predict that the baseline level of circulating POMC prohormone may be correlated with the baseline activity of the melanocortin bioactive peptides that regulate the degree of melanin-based coloration.

We found that darker reddish female tawny owls had lower levels of the POMC prohormone than paler reddish females when rearing an experimentally reduced (but not enlarged) brood and when they were located in forests where the proportion of beech trees was high (but not when low), an indirect measure of the abundance of their staple prey. In the following, we discuss the potential physiological effects of circulating POMC prohormone, why the levels of circulating POMC prohormone can be associated with coloration in prime environments, whether covariation between coloration and levels of POMC prohormone is adaptive, and how future studies should proceed.

Potential physiological effects of the circulating POMC prohormone

The level of the circulating POMC prohormone in the tawny owl was 68.5 pmol/l (range: 6 and 346.7), a value of similar order of magnitude to the values reported in dogs (range: 15-108 pmol/l) and sheeps (40-75 pmol/l) using the same assay (Granger 2004; Bell *et al.* 2005). To investigate the physiological effects of circulating melanocortins, researchers administrated these hormones or their analogues orally, subcutaneously, intraperitoneally, intravenously or intracerebrally. Systemic injection of melanocortins resulted in the darkening of skin (Lerner & McGuire 1979; Ugwu *et al.* 1997), in a reduction of inflammatory reactions, septic shock and fever (Chiao *et al.* 1996; Gonindard *et al.* 1996; Grabbe *et al.* 1996; Huang *et al.* 1998; Getting 2006) as well as stress-induced corticosterone levels (Daynes *et al.* 1987), and an increase in sexual behaviour (Van der Ploeg *et al.* 2002; Wessells *et al.* 2003) and aggressiveness (Morgan & Cone 2006). As indicated above melanocortin peptides control many important physiological pathways, suggesting that these active peptides should be tightly regulated at the levels of their activity (acetylation, amidation, phosphorylation), their processing by convertases (PC1, PC2, carboxypeptidase E, PAM; Wilkinson 2006), and expression of the POMC prohormone.

Proximate mechanisms underlying covariation between the levels of blood circulating POMC prohormone, coloration and stress

Melanin pigments account for a large part of the variation in animal coloration. The synthesis of melanin is controlled, in part, by melanocortins produced and processed in the skin (paracrine and autocrine; Slominski *et al.* 2000; Rousseau *et al.* 2007; Schallreuter *et al.* 2008). Endocrine melanocortin control of coloration cannot be excluded, since stress stimulates a release of plasma POMC-derived polypeptides in man (Meyerhoff *et al.* 1988), or increases plasma levels of α-MSH, ACTH and further induces skin darkening in the arctic charr (*Salvelinus alpinus*; Hoglund *et al.* 2000). Moreover, mice deficient for Tpit, a transcription factor that regulates pituitary development, exhibit a yellowish belly fur (Pulichino *et al.* 2003).

Since melanocortins are involved in the control of important physiological pathways such as stress control via the hypothalamic-pituitary-adrenocortical axis (HPA) through the action of ACTH, which is derived from the POMC prohormone, it is expected that *POMC* expression and in turn the POMC prohormone are environmentally-regulated. Accordingly, stress induced through a week of immobilization induced an increase in pituitaric POMC mRNA, plasma ACTH and corticosterone in rats (Noguchi *et al.* 2006). In near-term ovine fetus long-term hypoxia increased plasma POMC prohormone and ACTH but reduced the ratio POMC prohormone to ACTH compared to control sheep (Myers *et al.* 2005).

In tawny owls, pale reddish females decreased their level of plasma POMC prohormone when experiencing a higher level of stress (i.e. experimentally enlarged broods), whereas dark reddish females produced POMC prohormone independently of the brood size manipulation treatment. Different scenarios can account for this observation. Firstly it is possible that, under stressful conditions, more ACTH is necessary and more plasma POMC prohormone are processed to ACTH (Myers *et al.* 2005) resulting in a reduction of plasma POMC prohormone in pale reddish owls. Secondly, certain stress such as fasting induces a reduction in pituitaric POMC mRNA. Experiments carried out in laboratory animals showed that fasting usually induces a decrease in the expression of the *POMC* gene (Dallman *et al.* 1999; Bertile *et al.* 2003; Sanchez *et al.* 2004; Myers *et al.* 2005; Schwartz & Porte 2005). Accordingly, pale melanic females may decrease the level of *POMC* gene

expression because melanocortins may induce too many costly activities, which would be detrimental when resources are scarce.

Another scenario is based on the assumption that the POMC prohormone and ACTH levels needed under stressful conditions are colour-specific, with differently coloured individuals requiring different amount of ACTH to withstand a stressful situation, as shown for feeding behaviour in different rat strains, where fasting reduces hypothalamic POMC mRNA in Brown Norway rats and induces POMC mRNA in Fisher rats (Kappeler *et al.* 2004). As different sources of stress induce an increased or reduced expression level of the *POMC* gene (Noguchi *et al.* 2006; Chen *et al.* 2008), pale reddish individuals had lower levels of POMC prohormone under stressful conditions, i.e. when rearing an enlarged brood compared to when rearing a reduced brood (Figure 4.1) and dark reddish females may increase POMC prohormone levels under stressful conditions.

Studies on the proximate mechanisms occurring under stressful conditions are rapidly expanding but little is known about spatial and temporal variation in *POMC* gene expression, PC1/2 activation and ACTH and α-MSH levels between different colour morphs, along an environmental gradient (e.g. different intensity and type of stress). However, these different scenarios stem for stronger covariations between coloration and the levels of the POMC prohormone under prime environmental conditions. A number of biochemical factors regulate *POMC* gene expression, translation and further processing and modification. Such factors include steroids, glucocorticoids, cytokines, prostaglandines, cathecholamine and other neurotransmitters (Slominski *et al.* 2004; Schallreuter *et al.* 2008) potentially allowing individuals to adjust melanocortin levels in relation to environmental or social factors but also to life stages (Ellis *et al.* 2008; Palermo *et al.* 2008). Knowledge of the identity of these factors, their effect on melanocortin levels and physiological traits, and of how they are themselves regulated is key to determine when individuals switch on/off the expression of the *POMC* gene.

Are life history strategies of dark and pale reddish tawny owls mediated by melanocortins?

From an ultimate point of view, why did the POMC prohormone circulate at higher levels in pale reddish owls raising a reduced compared to an enlarged brood? Furthermore, why did melanocortin precursors circulate in the blood stream at relatively constant levels in dark reddish owls, while in paler owls levels were more variable? A likely explanation is that the net benefit of circulating POMC prohormone is higher in situations of low than high stress, particularly in pale individuals. Given the numerous physiological effects of melanocortins, their overproduction may lead individuals to invest resources into a wide range of activities, which in turn could be detrimental under stressful conditions. Interestingly, even under weakly stressful situations dark reddish birds also showed low levels of POMC prohormone suggesting that they avoid investing into too many activities regulated by melanocortins. Dark reddish individuals may thus invest resources in traits regulated by melanocortins relatively independently of changes in the environment, while pale conspecifics regulate these traits more finely in relation to variation in environmental factors. Accordingly, various studies showed that pale and dark reddish tawny owls invest resources differentially into various traits including body growth and maintenance, immunity and reproduction. Pale birds seem to adopt less risky strategies than dark conspecifics: when conditions are poor they skip reproduction more often (Roulin *et al.* 2003), invest less effort in mounting a strong immune response following an immune challenge to limit body mass loss (Gasparini *et al.* 2009a; Gasparini *et al.* 2009b), and produce nestlings that are better able to maintain body mass under low food supply (Piault *et al.* 2009). If pale individuals are better able to buffer variation in environmental factors by finely regulating melanocortin levels, dark reddish birds may have some other advantages such as offspring growing faster in body mass when preys are provided *ad libitum* (Roulin *et al.* 2008b; Piault *et al.* 2009), and when challenged with antigens they maintain a stronger level of antibody for a longer period of time compared to pale individuals (Gasparini *et al.* 2009a).

Conclusion and perspectives

Melanin-based coloration is a multigenic trait and inter-individual variation in the degree of melanism may be caused in part by melanocortins. Regardless of the exact role played by melanocortins in generating variation in coloration, our study demonstrates that differently melanic female tawny owls differentially regulate the POMC prohormone in relation to environmental factors. Because in the tawny owl the expression of reddish coloration is strongly heritable and not or weakly condition-dependent (Gasparini *et al.* 2009a), an environmentally-induced change in the magnitude of the covariation between coloration and the levels of the POMC prohormone must be due to a change in the level of the POMC prohormone but not to a change in coloration.

The present study is a first step into an understanding of the potential role of hormones in generating norms of reaction (Boswell & Takeuchi 2005; Ducrest *et al.* 2008), and hence our reasoning goes beyond the melanocortin system. Knowledge of the regulators of phenotypic correlations is helpful to predict how their sign and magnitude can change along an environmental gradient. In this context, melanin-based coloration is a promising model system, and the present study raises a number of issues to tackle the ecological role of the melanocortin system. First, we intend to measure expression levels of the *POMC* gene in relation to coloration in several tissues. The idea is to investigate whether expression levels of this gene are coordinated across organs, which is plausible given that melanocytes have a neuroendocrine regulatory function (Slominski 2009). This is an important issue because melanin production is determined, in part only, by the expression of the *POMC* gene found in feather buds, whereas many phenotypic traits that covary with melanin-based coloration are influenced by *POMC* gene copies found in the pituitary gland that control for instance energy homeostasis (Coll *et al.* 2004). Second, our aim is to assess the levels of prohormone convertases (PC1/2) processing the POMC prohormone and the levels of the resulting melanocortin bioactive peptides, especially under the stress experienced with the brood size manipulation. The final step will be an experimental injection of melanocortins to confirm their role in generating inter-individual colour variation and to investigate whether they generate covariations between melanin-based coloration and other phenotypic traits. Furthermore, the inverse-agonist/antagonist agouti

protein (ASIP) should also be considered in future studies as it may play another key role in the balance between pheo- and eumelanogenesis, and thus may determine coloration and regulation of many other traits (Ducrest *et al.* 2008). A thorough study of the melanocortin system should provide a proximate explanation as to how norms of reaction are regulated by differently melanic individuals, and help understand the adaptive function of variation in melanin-based coloration. It may also provide an appropriate system to tackle issues about genotype by environment interactions.

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CHAPTER 5

Postranslational modification of the propopiomelanocortin prohormone is associated with melanin-based coloration in the tawny owl (*Strix aluco*)

Guillaume Emaresi[*1], Anne-Lyse Ducrest[1], Pierre Bize[1], Hannes Richter[2], Céline Simon[1] & Alexandre Roulin[1]

[1]*Department of Ecology and Evolution, Biophore building, University of Lausanne, 1015 Lausanne, Switzerland*

[2]*Genomic Technologies Facility, Center for Integrative Genomics, University of Lausanne, Genopode Building, Lausanne, Switzerland*

ABSTRACT

The adaptive function of melanin-based coloration is a long-standing debate, principally because of limited knowledge of the underlying proximate mechanism. A recent genetic model suggested that the melanocortin system could account for covariations observed between melanin-based coloration and behavioural, morphological, physiological or life history traits. In the present study, we adopted a candidate gene approach to explore the genetic architecture of the melanocortin system in the tawny owl (*Strix aluco*), a species characterized by continuous variation in the degree of plumage reddishness (from light to dark). To this end, we collected developing feather in nestling tawny owls, a life stage characterized by the production of melanin pigments. After screening for SNP sites in the coding sequence of nine melanogenic genes and examined how feather bud expression of eu- and pheomelanogenesis-related genes covaries with plumage coloration in interaction with a brood size manipulation experiment. While we failed to detect non-synonymous mutations in the coding sequence of these melanogenic genes, we found interesting age-, sex- and colour-specific patterns of gene expression in the growing feather buds. Even though we could not show colour-specific pattern of the proopiomelanocortin (*POMC*) gene expression, we demonstrated that prohormone convertase 1 (*PC1*) gene was more expressed in offspring born from light rather than dark melanic tawny owls. Accordingly, offspring born from light melanic parents are likely to have a greater capacity of *POMC* processing to melanocortin peptides (i.e. ACTH and α-MSH). This raises the hypothesis that *PC1* gene expression may be a potential proximate mechanism regulating the pleiotropic effects of melanocortins in the tawny owl.

INTRODUCTION

Variation in coloration is very common throughout the animal and plant kingdoms (Jones 1977; Majerus 1998; Shine *et al.* 1998; Hoffman & Blouin 2000; Warren & Mackenzie 2001). The adaptive function of colour patterns can be diverse. Empirical studies highlighted a role of coloration in camouflage (Endler 1988), thermoregulation (Margalida *et al.* 2008) or social interaction (Endler 1988; Andersson 1994; Hill 2006; Bond 2007; Roulin & Bize 2007). In many species, coloration signals phenotypic or genetic attributes to potential mates in order to enhance mating success (Møller 1990; Shutler & Weatherhead 1990; Olson & Owens 1998; Smiseth & Amundsen 2000; Roulin 2004; Caro 2005; Weiss 2006; Kemp & Rutowski 2007). The exact individual qualities that a colour trait conveys are still debated as well as the underlying mechanisms linking quality to coloration. Two broad categories of mechanisms can be advanced. First, the expression of coloration is condition-dependent so that only individuals in prime condition can afford to pay the costs of producing the brightest colour trait. Condition being considered as a multifactorial trait, covariations between coloration and its associated qualities are likely to be regulated by a multitude of genes (Rowe & Houle 1996). Second, the expression of coloration is not or only weakly sensitive to condition, but strongly heritable, as it is the case in the so-called colour polymorphic species. In this case, covariations between coloration and physiological processes or behaviours could be due to a limited number of genes that may pleiotropically regulate other phenotypic traits (Strand 1999; Millington 2006; Ducrest *et al.* 2008). Identifying the proximate genetic mechanism underlying this type of covariation is not trivial and requires appropriate model systems.

Melanin-based coloration is a promising framework to investigate the proximate basis of the adaptive function of coloration. Although the adaptive values of melanic attributes remains a long-standing debate (Gray 1996; Hill & McGraw 2006; Gray & McKinnon 2007), the finding that melanin-based coloration frequently covaries with morphological, behavioural, physiological or life-history traits raised the hypothesis that variation in these colour traits can signal to conspecifics alternative strategies adapted to specific environmental or reproductive conditions (Roulin 2004; Roulin & Altwegg 2007; Roulin *et al.* 2007; Forsman *et al.* 2008; Galvan & Alonso-Alvarez 2009; Galvan *et al.* 2010; Jacquin *et al.* 2011). Since these covariations are often more easily detected under

specific environmental conditions (Roulin *et al.* 2008b; Piault *et al.* 2009; Roulin 2009; Roulin *et al.* 2011c), natural selection can locally and temporally favour alternative genotypes that differ in their phenotypic response to environmental conditions, also known as norms of reaction (Hedrick 1986; 2006). Recently, a genetic model suggested that the melanocortin system can account for these covariations (Ducrest *et al.* 2008). This system includes α-, β-, γ-melanocyte-stimulating-hormone (MSH) and adrenocorticotropic hormone (ACTH), posttranslational bioactive peptides derived from the cleavage of the proopiomelanocortin gene (*POMC*; Gantz & Fong 2003; Slominski *et al.* 2004; Millington 2006) by two prohormone convertases (PC1 and PC2; Bell *et al.* 1998; Laurent *et al.* 2004; Helwig *et al.* 2006; Pritchard & White 2007). In human anterior lobe of the pituitary, PC1 cleaves POMC prohormone to ACTH and β-lipotropin (β-LPH), whereas, in the intermediate pituitary, PC2 cleaves ACTH and β-LPH to produce α-, γ–MSH and β-endorphin, respectively (Pritchard & White 2007; Wardlaw 2011). Here, it is noteworthy that *POMC* processing by PC1 and PC2 also occurs in rodent and human epidermal tissues (Mazurkiewicz *et al.* 2000; Rousseau *et al.* 2007). Inverse agonist and antagonist of melanocortins are Agouti-signalling- and related-proteins (ASIP and AGRP), two proteins encoded by pheomelanogenesis-related *Agouti* genes in epidermal and brain tissues, respectively (Ito 1993; Barsh *et al.* 2000; Abdel-Malek *et al.* 2001; Bonilla *et al.* 2005; Mundy & Kelly 2006; Lin & Fisher 2007; Lightner 2009). Of particular interest, bindings of the melanocortins and the antagonists to the five melanocortin-receptors (MC1-5R; Schioth 2001; Butler & Cone 2002), a family of transmembrane G-protein-coupled receptors well conserved among vertebrates (Schioth *et al.* 2005), can modulate numerous physiological and behavioural functions, such as stress response, energy homeostasis, anti-inflammatory response, sexual activity, resistance to oxidative stress and aggressiveness (Cone 1999; Fan *et al.* 2000; Schioth 2001; Tatro & Sinha 2003; Slominski *et al.* 2004; Boswell & Takeuchi 2005; da Silva *et al.* 2005; Fan *et al.* 2005; Bertile & Raclot 2006; Butler 2006; Cone 2006; Hillebrand *et al.* 2006; Maaser *et al.* 2006; Millington 2006; Lin & Fisher 2007; Garruti *et al.* 2008; Page *et al.* 2011). In this context, a fundamental pursuit in the field of evolutionary genetics is to infer the exact genetic mechanisms responsible for variation in specific melanin-based coloration (e.g. nucleotide diversity or patterns of gene expression or still posttranslational modification;

Hoekstra 2006; Mundy 2006; Hoekstra & Coyne 2007).

Hair, skin and feather colour patterns are the result of a mixture of polymers of (grey-black) eumelanin and (reddish-brown) pheomelanin. Genetic pathways leading to the production of both melanins have been intensively studied (Jackson 1994; Mishima 1994; Bennett & Lamoreux 2003; Hoekstra 2006; Mundy 2006; Hoekstra 2010). In epidermal tissues, binding of α-MSH to MC1R, promotes eumelanogenesis (Slominski *et al.* 2004; Pritchard & White 2007; Walker & Gunn 2010). This binding activates indeed the production of intracellular $_c$AMP, a second messenger that up-regulates microphthalmia-associated transcription factor (MITF; Aksan & Goding 1998; Goding 2000; Vachtenheim & Borovansky 2010; Wan *et al.* 2011), and, by extension, the eumelanogenic activity of tyrosinase (TYR) and tyrosinase-related-protein 1 (TYRP1; Kobayashi *et al.* 1995; Barsh *et al.* 2000; Ito *et al.* 2000; Slominski *et al.* 2004; Lin & Fisher 2007; Spencer & Schallreuter 2009) within melanocyte. Rate limiting enzyme tyrosinase catalyses the first two-steps of melanin production: the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) and the subsequent oxidation of 5,6-dihydroxyindole (DHI) to L-dopaquinone (Slominski *et al.* 1991; Ozeki *et al.* 1997; Ito *et al.* 2000; Land & Riley 2000; Park *et al.* 2009; Ito & Wakamatsu 2010; Schallreuter *et al.* 2011). However, binding of inverse agonist and antagonist ASIP to MC1R can block α-MSH binding, leading to the production of pheomelanin at the expense of eumelanin (Suzuki *et al.* 1997; Gantz & Fong 2003; Lin & Fisher 2007). Moreover, the switch between eu- and pheomelanogenesis depends also the presence of thiol groups resulting from cysteine depletion in melanosomes (del Marmol *et al.* 1996; Ito & Wakamatsu 2010). In absence of thiols, dopaquinone undergoes intramolecular cyclization, resulting in the formation of eumelanin, whereas thiol intervention promotes the transformation of dopaquinone in cysteinyldopas, which is further oxidized to produce pheomelanin (Ito *et al.* 2000).

To date, more than 150 genetic loci with well-characterized melanin-based phenotypes were identified in laboratory mice (Bennett & Lamoreux 2003; Hoekstra 2006). Although strongly conserved across vertebrates (Boswell & Takeuchi 2005; Schioth *et al.* 2005), numerous attempts to unravel the genetic architecture of melanin-based coloration revealed nucleotide polymorphisms in the coding sequence of melanogenic genes in non-model organisms (e.g. *MC1R* (Valverde *et al.* 1995;

Theron *et al.* 2001; Mundy & Kelly 2003; Rosenblum *et al.* 2004; Hoekstra *et al.* 2006; Rosenblum *et al.* 2010), *ASIP* (Mundy & Kelly 2006; Nadeau *et al.* 2008; Kingsley *et al.* 2009), *TYRP1* (Gratten *et al.* 2007; Nadeau *et al.* 2007)), based on genetic variations uncovered in model organisms (i.e. candidate gene approach). Whereas some studies failed to detect covariation between nucleotide variation in pigmentation genes and melanin-based coloration (MacDougall-Shackleton *et al.* 2003; Hull *et al.* 2010), others pointed out that alternative patterns of gene expression may account for inter-individual colour variation rather than non-synonymous mutations in the coding sequence of candidate genes (e.g. ASIP gene in deer mice (*Peromyscus* sp.); Linnen *et al.* 2009; Manceau *et al.* 2011). Evo-devo theory also predicts the evolution of alternative phenotypes through different expression or posttranslational modifications of functionally conserved genes or proteins, respectively, rather than key mutation sites in the coding sequence of these genes (Carroll 2005; Hoekstra & Coyne 2007; Carroll 2008). These findings illustrate the complexity and the non-uniform picture of the genetic architecture underlying variation in phenotypic variation among wild organisms.

The colour polymorphic tawny owl (*Strix aluco*) is a promising system to investigate the pleiotropic effects of the melanocortin system. This species exhibits continuous variation in the degree of plumage reddishness (from light to dark), which is mainly due to the deposition of pheomelanin and to a lesser extent to eumelanin (Gasparini *et al.* 2009a). This inter-individual variation in tawny owl plumage coloration is highly heritable (h2 = 0.72-0.93; Brommer *et al.* 2005; Gasparini *et al.* 2009a). Of particular interest, empirical studies highlighted colour-specific norms of reaction to reproductive (Roulin *et al.* 2004; Emaresi *et al.* in prep, ***Chapter 1***) and rearing conditions (Roulin *et al.* 2008b), food supply (Piault *et al.* 2009), pathogens (Galeotti & Sacchi 2003; Gasparini *et al.* 2009a; Karell *et al.* 2011a) and climatic conditions (Galeotti & Cesaris 1996; Karell *et al.* 2011b), leading to differences in recruit production (Brommer *et al.* 2005; Emaresi *et al.* in prep, ***Chapter 1***), probability of skipping reproduction (Roulin *et al.* 2003) and adult survival rate (Brommer *et al.* 2005; Karell *et al.* 2011b). Moreover, we demonstrated that differently coloured female tawny owls adjust their level of circulating POMC prohormone according to the reproductive and environmental conditions (Roulin *et al.* 2011c). Dark melanic females had lower circulating levels of POMC prohormone than light melanic ones when rearing experimentally reduced (but not enlarged) brood and when located in forest

patches with high (but not low) density of beech trees, an environmental feature positively correlated with prey abundance. In this study, we proposed that POMC prohoromone and ACTH levels needed under stressful conditions are colour-specific, suggesting thus colour-specific post-translational modifications of POMC prohormone via alternative patterns of expression of the convertase PC1 and/or PC2. These findings call for an integrative study that establishes the exact genetic mechanism underlying covariation between coloration and physiological reaction norms. A first step is to determine the genetic architecture of tawny owl melanin-based coloration, by identifying single-nucleotide polymorphism (SNP) sites in key melanogenic genes and especially the melanocortin system. A second complementary step is to measure in differently coloured tawny owls the expression levels of melanogenesis-related genes, with a particular focus on prohormone convertases PC1 and PC2 responsible for POMC prohormone processing to ACTH and α-MSH (Roulin *et al.* 2011c). We adopted this two-step approach by collecting developing feather in nestling tawny owls, a life stage characterized by the production of melanin pigments to be stored in feathers.

Given that POMC prohormone regulation was associated with experimentally manipulated brood size in adult tawny owl (Roulin *et al.* 2011c), we replicated a brood size manipulation experiment to modify the level of stress experienced by nestlings. We first screened for SNP sites in the coding sequence of melanocortin genes (i.e. *ASIP*, *MC1R*, *POMC*). Potential source of variation in the downstream molecular cascade also needed to be considered, leading us to screen for SNP sites also in key (i.e. *PC1*, *PC2*, *TYR*, *TYRP1*) or secondary (i.e. *MITF* (Otreba *et al.* 2012) or cystine-glutamate transporter *SLC7A11* (Chintala *et al.* 2005)) regulators of eu- and pheomelanin molecular pathways. Then, we examined how feather bud expression of eumelanogenesis-related (i.e. *MC1R*, *POMC*, *TYRP1* and to a certain extent *TYR*) and pheomelanogenesis-related genes (i.e. *ASIP*), and key regulators of both molecular pathways (i.e. *MITF*, *PC1*, *PC2* and *SLC1A11*), covaries with plumage coloration in interaction with the brood size manipulation experiment.

METHODS

Model species, brood size manipulation and sample collection

The present study was conducted in a 911km^2 study area. In 2010, we monitored an important number of breeding pairs (n = 139). Clutches were composed of 2-7 eggs (mean ± sd: 5.13 ± 0.94), which hatched between February 21 and May 31 (mean ± sd: March 31 ± 13.3 days), leading to 1-7 fledglings per brood (n = 107 successful broods, mean ± sd: 3.92 ± 1.42). On the criteria that clutches were initiated on a similar date, 94 nests were matched into pairs to perform a partial cross-fostering combined with a brood size manipulation experiment (Emaresi *et al.* submitted, ***Chapter 2***). Among pairs of nests, brood sizes were randomly manipulated, leading to an exchange of 1.74 nestlings on average (sd = 0.6) from nest E (experimentally enlarged, n = 47) and placed in nest R (experimentally reduced, n = 47), while 2.74 hatchlings on average (sd = 0.6) were exchanged from nest R to nest E. Nestlings were thus raised by randomly chosen foster parents and experienced different brood size manipulation treatment, disrupting thereby the confounded environmental effect of being born and reared in the same 'genetic' nest. The objective of the brood size manipulation experiment was to investigate whether the relationship between coloration and expression levels of candidate genes is condition-dependent, the experimental addition of one nestling yielding stronger sibling rivalry and hence higher intrinsic stress in the tawny owl (Roulin *et al.* 2008b; Roulin *et al.* 2011c).

When nestlings were 10 days of age, breeding females were captured in the nest box during daylight (8am – 6pm, n = 94), while males were captured at night when provisioning their brood (10pm – 6am, n = 88). Note here that extra-pair offspring are rare in the tawny owl (Saladin *et al.* 2007), and thereby individuals born in a nest are assumed to be sired by the captured male. No differences in adult wing and tarsus lengths were observed between the two brood size treatments (Student's t-tests, $P > 0.42$). To estimate growth rate, nestlings were recaptured every five days until they fledged (mean ± sd: 4.74 ± 1.55 captures per nestling). Upon capture (mean timing of the sampling ± sd: 12:30 ± 2:25; range: [09:00 – 18:00]), each nestling was weighed to the nearest g, their left wing length measured to the nearest 1 mm and left tarsus to the nearest 0.1 mm. Note however that we could not detect an effect of the brood size manipulation experiment on nestling body mass (ANCOVA mixed-model controlling for an effect of age and sex on body mass, $F_{1,41.23} = 0.54$, P =

0.47), probably because food resources were particularly abundant in 2010 (*unpublished data*). At two different ages (Age Class 1: mean ± sd = 11.0 days old ± 2.1; Age Class 2: mean ± sd = 24.7 days old ± 2.9), three developing feathers (i.e. buds) were plucked from their dorsum. These samples were collected in 1.5 ml Eppendorf tubes, immediately frozen in dry ice in the field and transferred at -80°C within 12 hours until later genetic analyses.

Assessment of coloration

Despite its continuous variation in the degree of reddishness, the tawny owl is generally considered as colour polymorphic (Glutz von Blotzheim & Bauer 1980; Galeotti 2001; Brommer *et al.* 2005), leading us to employ the same terminology. Accordingly, each adult was assigned to one of five colour morphs (1 = reddish, 2 = reddish-brown, 3 = brown, 4 = grey-brown, 5 = grey), as described in previous studies (Roulin *et al.* 2003; Roulin *et al.* 2005). This visual scoring method is highly reliable between years (Emaresi *et al.* submitted, ***Chapter 2***) and strongly correlated with brown chroma measured with a spectrophotometer (Emaresi *et al.* submitted, ***Chapter 2***). However, visual colour scores better account for overall plumage coloration compared to brown chroma, which is based on only three back feathers, an approach that does not necessarily reflect entire body coloration (Brommer *et al.* 2005; Emaresi *et al.* submitted, ***Chapter 2***). Here, it is noteworthy that adult plumage coloration was neither associated with hatching date of the first egg (Pearson's correlation, $P = 0.14$), nor with brood size before manipulation (Student's *t*-tests, $P = 0.8$). Moreover, within pairs of experimental nests, foster and biological parents did not resemble each other with respect to plumage colour scores (Pearson's correlations: $-0.16 < r < -0.03$, $P > 0.49$). Pairing with respect to male and female coloration was random in both treatments (enlarged nests: $r = 0.025$, $n = 32$, $P = 0.89$; reduced nests: $r = -0.18$, $n = 28$, $P = 0.34$).

Nestlings are covered in whitish buff down feathers with dark melanic bands. Fledgling feathers (i.e. at approximately 25 days old) have less distinctive colour patterns compared to feathers collected on the same individuals at adulthood. Accordingly these feathers show lower colour variation than in adults, which makes difficult the classification of nestling plumage coloration into one of five morphs. For this reason, nestling plumage coloration was assessed only by spectrometric measurements. We collected three downy feathers from the dorsum of each nestling (when nestling

feathers were more developed at approximately 25 days of age). These feathers were overlaid on black paper to capture reflectance spectra at four distinct positions using the S2000 spectrophotometer (Ocean Optics, Dunedin, FL) and a dual deuterium and halogen 2000 light source (Mikropackan, Mikropack, Ostfildern, Germany). Based on these spectra, a mean brown chroma score was calculated for each nestling as described by Montgomerie (2006). Note here that brown chroma scores were positively correlated with the average plumage coloration of biological parents (Pearson's correlation, r = -0.28, P = 0.002).

RNA extractions

Nestling age at which developing feathers were sampled was not associated with plumage coloration of the biological parents (Pearson's correlation, r = 0.01, P = 0.84). Total RNA samples (n = 325) were extracted from one or two feather buds of 163 nestlings at two different ages (in 52 distinct broods), using the Qiagen Rneasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) with some modifications. In new Eppendorf tube, one bud was frozen with liquid nitrogen to facilitate their grinding with plastic pestles. We added 300 µl of RLT buffer to remove pestles without sample loss. Then we added again 300 µl of RLT with β-mercaptoethanol (β-ME, at a concentration twice as high as mentioned in the RNeasy Mini kit hand book), before heating the samples at 56°C for two minutes. These heated samples were then filtered through a Qiaschredder® (Qiagen, Hombrechtikon, Switzerland) to remove bud residues. At the end of the extraction, we quantified the amount of RNA with the Qubit® 2.0 Fluorometer (Life Technologies, Zug, Switzerland). Prior reverse transcription, total RNA samples were treated with DNase I. One µg of total RNA was incubated in 10 µl with 5U DNase I (Roche diagnostics Ltd, Basel, Switzerland) in 10 mM Tris-HCl pH 8.0, 0.5 mM $MgCl_2$, 1 mM dithiotreitol, 20U RNasin Ribonuclease Inhibitor (Promega, Dübendorf, Switzerland) for 30 minutes at 37°C, followed by 10 minutes at 75°C to inactivate the enzyme. Finally, 1µg of DNase I treated total RNA was reverse-transcribed in a final volume of 20 µl, using 250 ng of Random Hexamer, 40U of RNasin Ribonuclease Inhibitor (Promega, Dübendorf, Switzerland) and 200 U of Superscript III reverse transcriptase (Life Technologies, Zug, Switzerland). $_c$DNA samples were 10-fold diluted in Tris 10 mM pH 8.0 and finally stored at -20°C before further genetic analyses.

Sequence analysis

Although well conserved across vertebrates (Boswell & Takeuchi 2005; Schioth *et al.* 2005), we screened for SNPs in the coding sequence of nine melanogenic candidate genes, using $_c$DNA of at least two individuals displaying extreme colour morphs (light and dark melanic). On the basis of mRNA sequences edited in Genbank (e.g. chicken (*Gallus gallus*), Japanese quail (*Coturnix japonica*) and zebra finch (*Taeniopygia guttata*)), we designed our primers on conserved exonic regions of *ASIP*, *MC1R*, *MITF*, *PC1*, *PC2*, *POMC*, *SLC7A11*, *TYR*, *TYRP1* and two reference genes (i.e. *EEF1A1* and *GAPDH*). BLASTN searches were performed to control primer specificity prior being ordered from Microsynth AG (Balgach, Switzerland). Following annealing temperature optimization for each pair of primers (Table 5.1), polymerase chain reaction (PCR) amplifications were performed in a thermal cycler (Biometra TProfessional 96) in 20 µl total volume containing 0.4 U Taq DNA polymerase (Qiagen), 1 X PCR Buffer (Qiagen), 2.5 mM $MgCl_2$ (Qiagen), 200 µM $_d$NTP, 250nM of each primer and 2 µl of $_c$DNA. Cycling parameters were as follows: 95°C for 5 min followed by 35 cycles at 95 °C for 30 s, primer specific temperature for 30 s and 72°C for 30 s to one min, and a final extension step at 72 °C for 5 min. Primers and $_d$NTPs were removed from amplified products using the Promega purification kit (Promega, Dübendorf, Switzerland). In some cases (see Table 5.1), TA cloning was performed using the pGEM-T easy vector system (Promega, Dübendorf, Switzerland). Sequencing of PCR products and plasmid clones were performed on both strands by cycle sequencing using Big Dye® V 3.1 terminator chemistry (Life Technologies, Zug, Switzerland) and reactions were run on ABI3130XL Genetic Analyzer (Life Technologies, Zug, Switzerland). Gene sequences were edited and aligned in CodonCode Aligner (CodonCode Corporations).

Quantitative PCR

Specific qPCR primers were designed on the basis of edited and aligned sequences described above, with the assistance of Primer Express® software 2.0 (Life Technologies, Zug, Switzerland). BLASTN searches were performed to control primer specificity prior being ordered from Microsynth AG (Balgach, Switzerland). Final primer pairs were selected based on amplification efficiency (i.e. slope of standard curve generated by serial dilutions of genomic DNA and R^2), dissociation curve and negative controls (i.e. water sample and RT-PCR negative control). Quantitative PCR reactions were

performed using an ABI Prism® 7900HT Sequence Detection System (Life Technologies, Zug, Switzerland) in a final volume of 10µl containing 5ng of diluted $_c$DNA mixed with optimized primers (Table 5.2.1) and SYBR® Green PCR Master Mix (Life Technologies, Zug, Switzerland). Due to very low *POMC* gene expression in the skin (Slominski *et al.* 2000), we optimized our procedure for this gene as following. First, we increased $_c$DNA concentrations of the diluted samples through an ethanol precipitation with one volume of ammonium acetate 5M. The resulting pellet was resuspended in 15 µl of TE 1x (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). These samples were then pre-amplified using the *POMC*, *GAPDH* and *EEF1A1* primers and TaqMan probes, using the PreAmp Master Mix kit (Life Technologies, Zug, Switzerland). Five µl of concentrated $_c$DNA was preamplified in a thermal cycler (Biometra TProfessional 96 engine) with 14 cycles and then diluted 10 fold with TE 1x. *POMC* specific qPCRs were performed using an ABI Prism® 7900HT Sequence Detection System (Life Technologies, Zug, Switzerland) in a final volume of 10µl containing 5ng of pre-amplified $_c$DNA mixed with optimized primers and probes (Table 5.2.2) and TaqMan Universal PCR Master Mix (Life Technologies, Zug, Switzerland). For each qPCR, cycling conditions were 50°C for 2 min (UNG activation), 95°C for 10 min (enzyme activation), 40 cycles of 95°C for 15 s and 60°C for 1 min, and a final dissociation stage (i.e. dissociation curve analysis). Three technical replicates were performed per $_c$DNA sample. Reactions were performed in 384-well optical reaction plates, assembled with a Tecan Freedom Evo® liquid handler (Tecan group Ltd.). Quantification cycle (hereafter CT) values were recorded with SDS software 2.3. CT values larger than 35 were considered beyond the limit of detection and thus removed from further analyses.

CT scores were imported into qBase^{PLUS} software 1.3 (Biogazelle). Inter-plate covariation was checked according to inter-run calibrators (i.e. IRC). To correct for any variation in $_c$DNA content and enzymatic efficiencies, CT scores of the candidate genes were normalized using the two reference genes: GAPDH and EEF1A1 (showing a variation (CV) of 0.12; (Vandesompele *et al.* 2002)). Technical replicates with less than 0.5 CT variation were at 99.2%, mean RQs (relative quantities) for each sample were calculated and analyzed with the geNorm software 3.4 (Vandesompele *et al.* 2002). These relative scores were finally box-cox transformed before statistical procedures, to enable the use of models with a Gaussian-distributed error.

Statistical procedure

After preliminary statistical exploration, expression levels of the nine melanogenic genes (i.e. *ASIP*, *MC1R*, *MITF*, *PC1*, *PC2*, *POMC*, *SLC7A11*, *TYR*, *TYRP1*) were not significantly affected by two covariates, i.e. time of the day when individuals were sampled (linear mixed-models, $P > 0.20$) and whether nestlings were raised by biological or foster parents (i.e. 'Cross-fostering' factor, alone or in interaction with colour score of biological parents in linear mixed-models, $P > 0.24$). Moreover, nestling residual body mass (corrected for wing length, $t_{1,299} = 28.52$, $P < 0.0001$) was not associated with nestling brown chroma (linear mixed-models, $F_{1,127.7} = 0.002$, $P = 0.96$) or average colour score of biological parents (linear mixed-models, $F_{1,37.75} = 0.29$, $P = 0.59$). Consequently, these three factors were not included in final analyses, in which we considered only nestling sex ('Sex') and the date of the sampling ('Date') as covariates. In preliminary statistical exploration, we considered plumage nestling brown chroma or colour scores of both biological parents (43 fathers and 44 mothers) as independent explanatory variables. The resulting models owned the disadvantage of reduced statistical power, due to either lower colour discrimination in the case of brown chroma or highly complex statistics (i.e. four-way interactions) regarding colour scores of both biological parents. To simplify our statistical models, we considered the most parsimonious models involving the average coloration of both biological parents ('MeanGenetic') as explanatory variable. This approach is justified by the facts that melanin-based coloration is strongly heritable (Brommer *et al.* 2005; Gasparini *et al.* 2009a) and not sexually dimorphic in the tawny owl (Baudvin & Dessolin 1992; Galeotti & Cesaris 1996).

To investigate colour-specific patterns of gene expression, we ran linear mixed-models on the whole dataset (i.e. involving the 325 measurements) by entering each candidate gene as response variable. We included nestling sex (i.e. factor 'Sex') and sampling date (i.e. factor 'Date') as covariates and nestling age classes ('Age Class'), brood size manipulation treatment (hereafter 'BSM') and average colour scores of both biological parents ('MeanGenetic') as explanatory variables, plus all possible interactions between these three variables. Because we sampled twice individuals from the same nest, we included the identity of the biological brood and nestling ring number (nested in biological brood identity) as two random factors. Starting with full models, a backward stepwise procedure was used to sequentially remove non-significant terms ($P > 0.05$), starting with the least

significant higher order interactions, until obtaining best-fitted models. Statistical analyses were performed using JMP IN 8.0.

RESULTS

Gene sequencing

Using $_c$DNA of at least two extreme coloured tawny owls (i.e. light and dark melanic), we genotyped most of the whole coding sequence of nine candidate genes (i.e. *ASIP*, *MC1R*, *MITF*, *PC1*, *PC2*, *POMC*, *SLC7A11*, *TYR*, *TYRP1*) and two reference genes (i.e. *EEF1A1* and *GAPDH*). Edited and aligned sequences revealed no key mutation, potentially affecting the amino acid sequence of candidate peptides or proteins.

Age- and sex-specific patterns of gene expression

All statistics can be found in Table 5.3. Based on the whole dataset, we found strong age-specific patterns of gene expression during the rearing period. Indeed, the factor 'Age Class' was significantly associated with levels of expression of pheomelanogenesis- (i.e. *ASIP* and *SLC7A11* levels of expression were higher at an early rather than an old age) and eumelanogenesis-related genes (*MC1R*, *PC1*, *TYR* and *TYRP1* levels of expression were higher at an old rather than an early age; Figure 5.1), but not with levels of *MITF*, *PC2* and *POMC*.

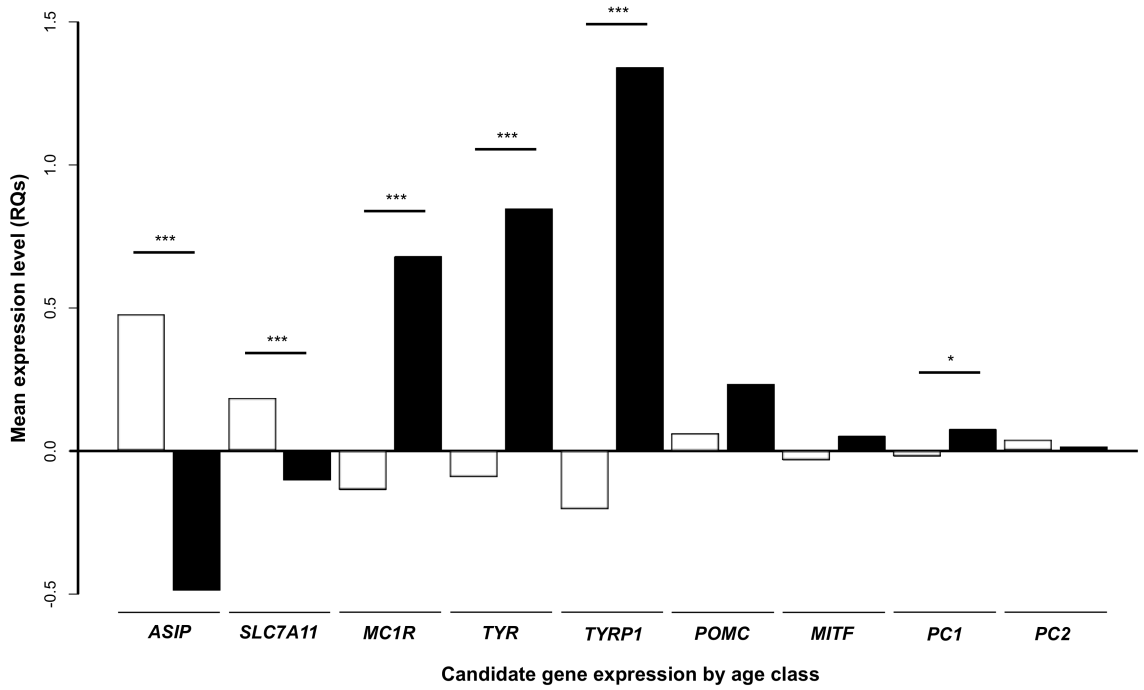

Figure 5.1: Mean expression levels of the nine candidate genes in relation to nestling age class | We investigated age-specific expression levels in pheomelanogenesis-related (i.e. *ASIP*) and eumelanogenesis-related genes (i.e. *MC1R, POMC, TYRP1* and to a certain extent *TYR*) or in key regulators of both molecular pathways (i.e. *MITF, PC1, PC2* and *SLC1A11*). For this graph, but not for the analyses, we calculated mean expression levels (i.e. mean RQs) of the nine candidate genes for each nestling age class. Significant relations (*, ** or *) are derived from Table 5.3.**

Similarly, we found sex-specific patterns of expression for *PC1*, *PC2*, *TYRP1* and to a lesser extent for *ASIP*. Apart from the *ASIP* specific case (females expressing slightly more ASIP than males), males were expressing higher levels of *PC1*, *PC2* and *TYRP1* than females (Figure 5.2). Finally, we also found that *MC1R* and *MITF* levels of expression increased along the season.

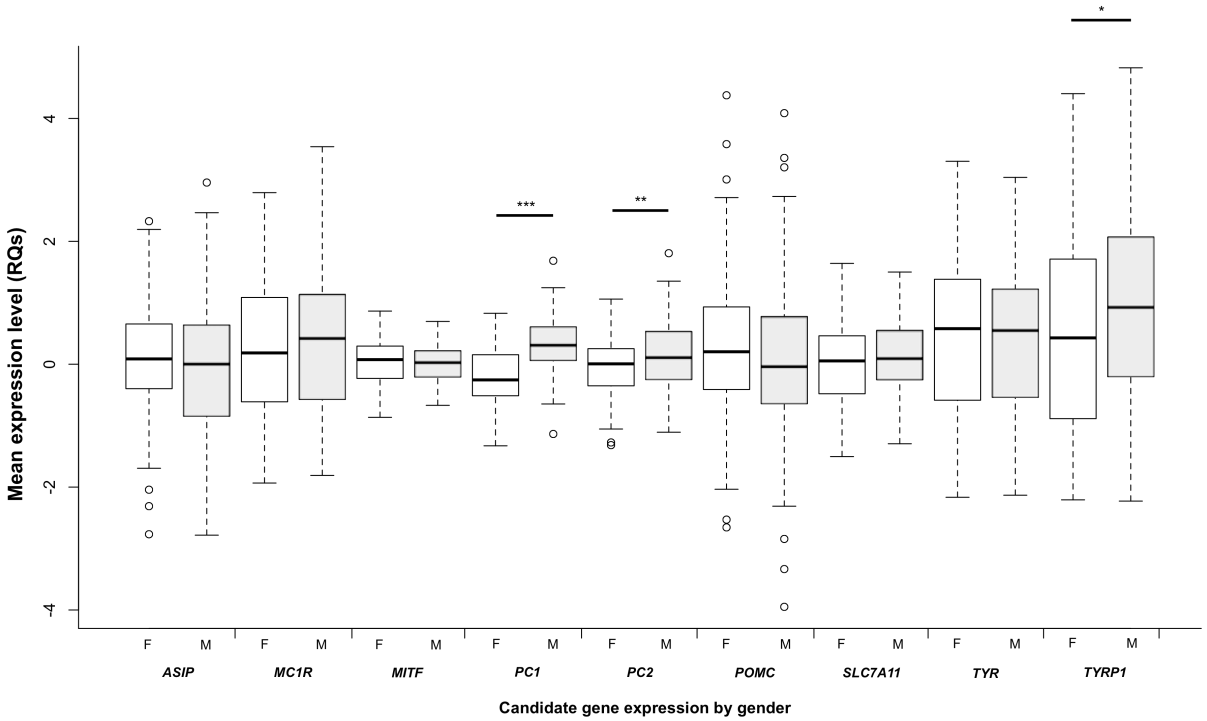

Figure 5.2: Expression levels of the nine candidate genes in relation to nestling sex | Significant relations (*, ** or *) are derived from Table 5.3.**

Colour-specific patterns of gene expression

Complete models performed on the whole dataset (i.e. involving both age classes) are reported in Table 5.3 (same analyses as the ones presented above for age- and sex-specific effects). *MC1R* expression levels were significantly associated with the average colour score of the biological parents (alone or in interaction with either 'Age Class' or 'BSM' factors, Table 5.3).

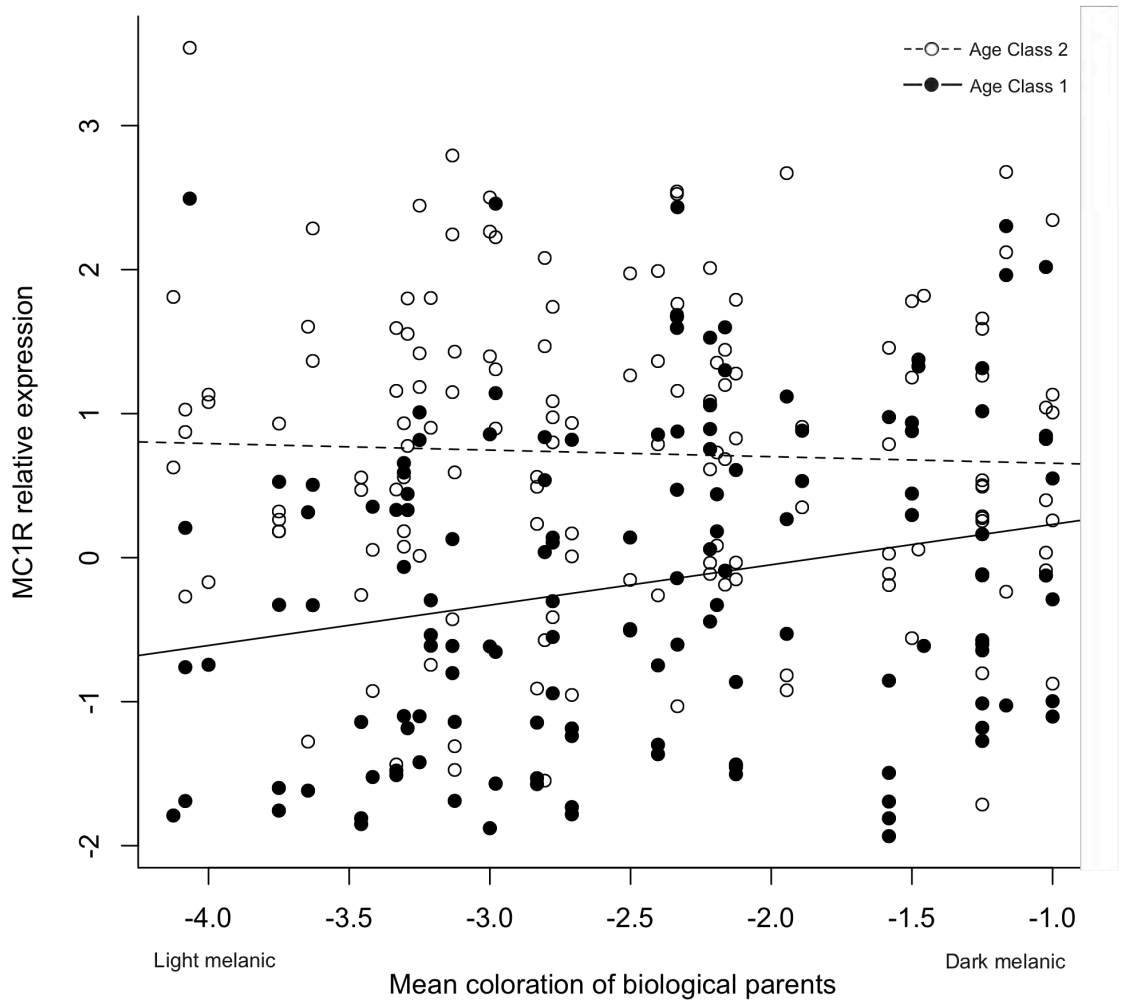

Figure 5.3: Nestling patterns of *MC1R* gene expression in relation to average melanin-based coloration of their biological parents and their age class | The relationship is significant at an early (i.e. Age Class 1: closed circles and straight regression line; Pearson's correlation: r = -0.22, n = 136 nestlings, P = 0.01) rather than older age (i.e. Age Class 2: open circles and dashed regression line; Pearson's correlation: r = 0.04, n = 143 nestlings, P = 0.65).

Offspring of darker melanic biological parents expressed *MC1R* at higher levels than offspring of light melanic biological parents, especially at an early age ($F_{1,42.95}$ = 5.9, P = 0.02, Figure 5.3) or when experiencing an enlarged broods ($F_{1,33.65}$ = 9.3, P = 0.005, Figure 5.4). Interestingly, *PC1* expression levels were also significantly associated with the average colour score of the biological parents (alone or in interaction with 'Age Class', Table 5.3).

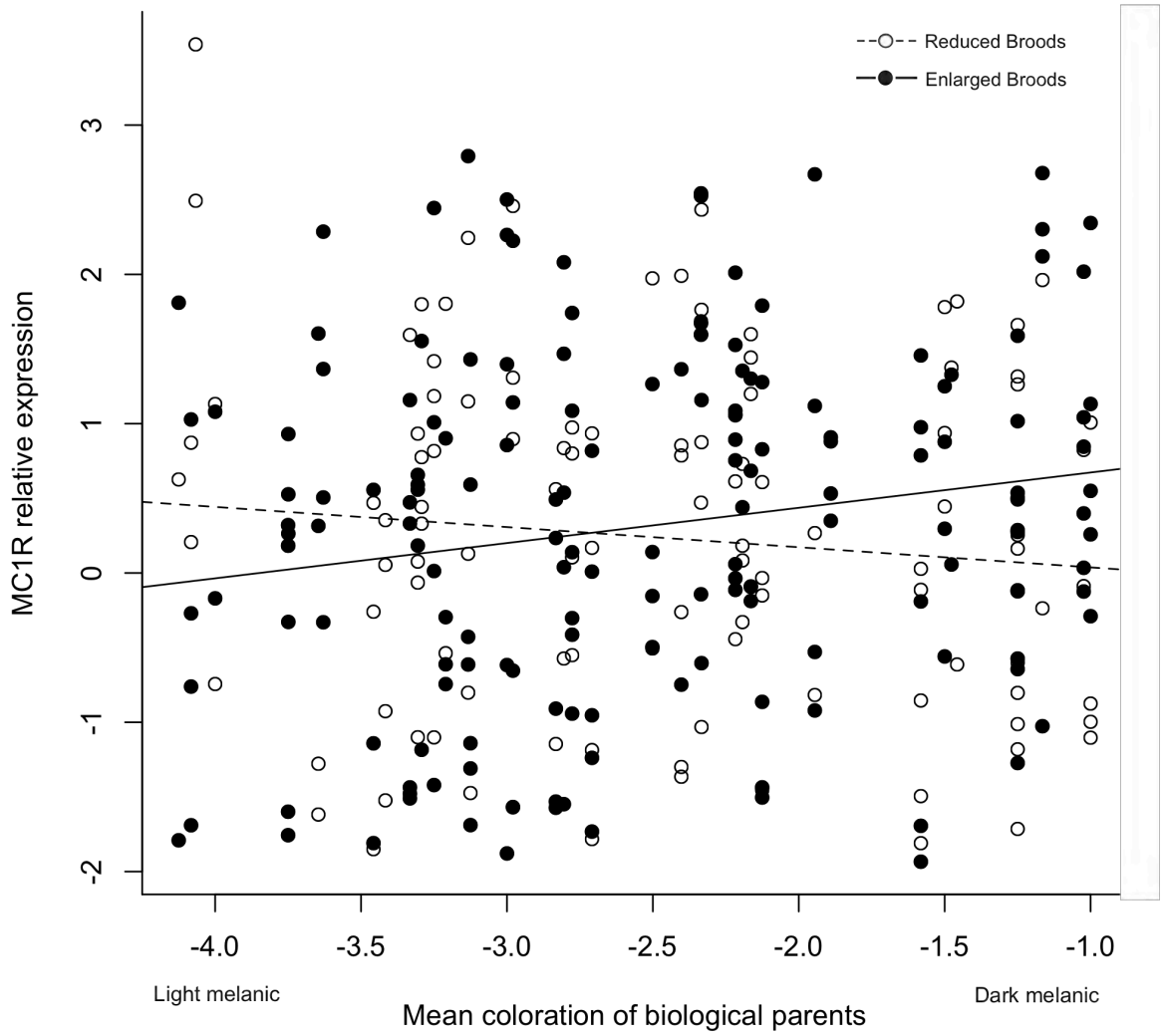

Figure 5.4: Nestling patterns of *MC1R* gene expression in relation to average melanin-based coloration of their biological parents and the brood size manipulation experiment | The relationship is significant in experimentally enlarged broods (closed circles and straight regression line; Pearson's correlation: r = -0.18, n = 167 nestlings, P = 0.02), but not in experimentally reduced broods (open circles and dashed regression line; Pearson's correlation: r = 0.1, n = 112 nestlings, P = 0.28).

In this case, offspring of light melanic biological parents expressed *PC1* at higher levels than offspring of dark melanic biological parents, especially at an early age ($F_{1,46.69}$ = 6.81, P = 0.01, Figure 5.5). Finally, regarding expression patterns of *POMC* and *TYRP1* genes, we also found significant interactions between parental coloration and Age Class or BSM, respectively (Table 5.3). However, post-hoc analyses revealed that, within each age class (i.e. *POMC* gene) or each treatment (i.e. *TYRP1* gene), the relationships between coloration and *POMC* or *TYRP1* expression levels were not significant (P-values > 0.1).

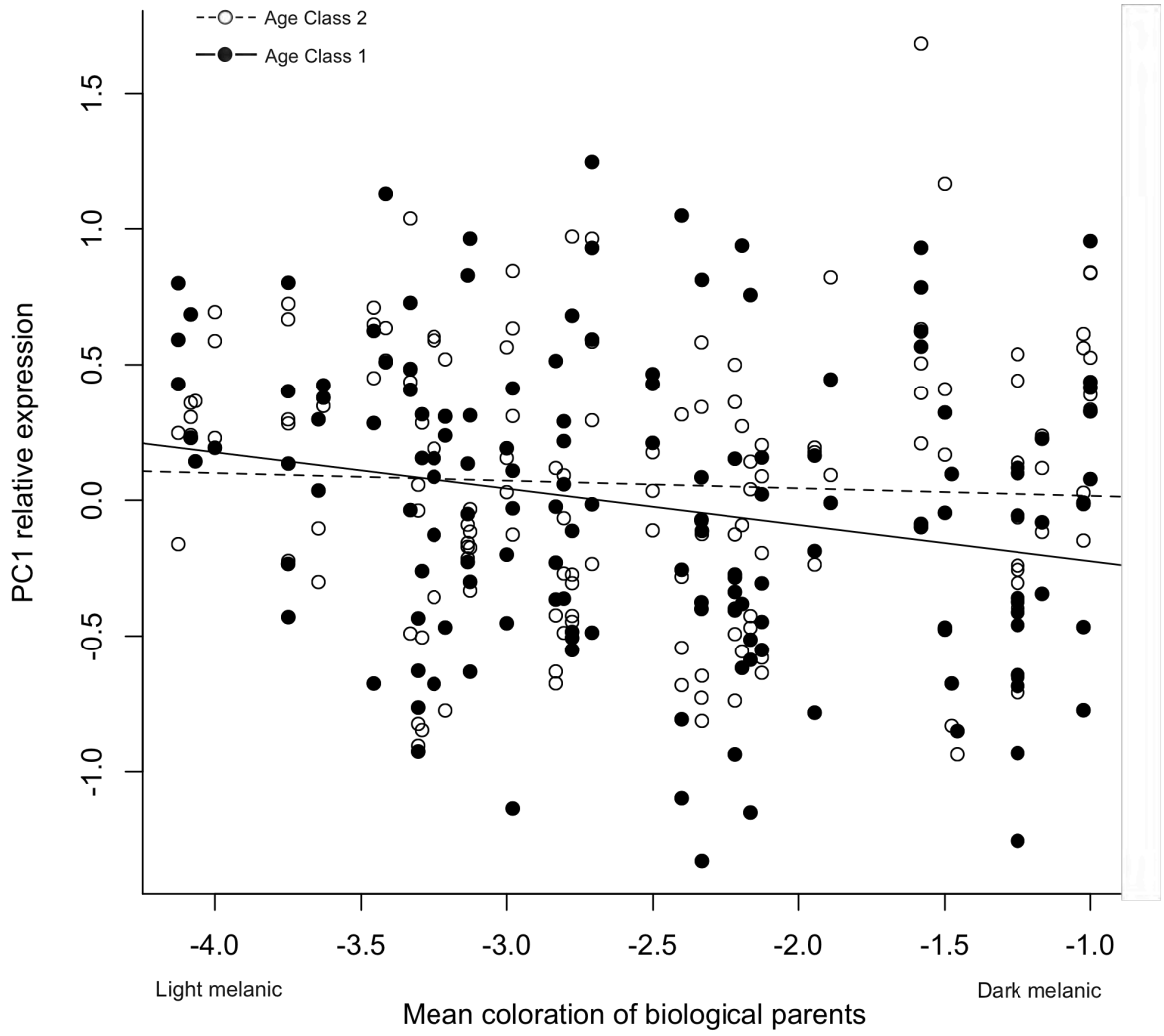

Figure 5.5: Nestling patterns of *PC1* gene expression in relation to average melanin-based coloration of their biological parents and their age class | The relationship is significant at an early (i.e. Age Class 1: closed circles and straight regression line; Pearson's correlation: r = 0.22, n = 146 nestlings, P = 0.008) rather than older age (i.e. Age Class 2: open circles and dashed regression line; Pearson's correlation: r = 0.05, n = 143 nestlings, P = 0.55).

DISCUSSION

In the present study, we explored the genetic architecture of the melanocortin system in the tawny owl, as a potential proximate mechanism underlying the expression of melanin-based coloration and in turn colour-specific reaction norms. To this end, we searched for SNPs in the coding sequence of melanogenic genes (i.e. *ASIP*, *MC1R*, *MITF*, *PC1-2*, *POMC*, *SLC7A11*, *TYR*, *TYRP1*) and for colour-specific patterns of gene expression in growing feathers of nestlings. We failed to detect non-

synonymous mutations in the coding sequence of important melanogenic genes. By cons, we found interesting age-, sex- and colour-specific patterns of gene expression in the growing feather buds. Pheomelanogenesis- and eumelanogenesis-related genes were up- and down-regulated, respectively, at an early age, while the reverse was true at an older age. Moreover, males expressed higher levels of *PC1*, *PC2* and *TYRP1* genes than females. Finally, we demonstrated that *MC1R* and *PC1* patterns of expression in young (rather than old) nestlings covaried with the mean colour score of their biological parents (a proxy of their own coloration given the very strong heritability of this trait). Hereafter, we discuss the key role of *PC1* in processing the *POMC* prohormone and other important prohormones (e.g. proinsulin and proglucagon; (Ugleholdt *et al.* 2006)), a potential proximate mechanism modulating the pleiotropic effects of melanocortins, and potentially leading to colour-specific reaction norms in the tawny owl.

Age- and sex-specific patterns of expression in pheomelanogenesis- and eumelanogenesis-related genes

Animal melanocytes can produce two types of pigments, eu- and pheomelanin. The switch between eu- and pheomelanogenesis is strongly dependent on tyrosinase activity and thiol concentration (mainly through cysteine). Empirical studies emphasized that pheomelanin is likely to occur early in neonatal stage (Lightner 2009), at the expense of eumelanogenesis, due to high thiol concentrations, eumelanin pigments being synthesized once thiol compounds were consumed by pheomelanin synthesis (Ito 1993). In laboratory mice for instance, juvenile tyrosinase activity was low at 3-4 days of age, leading to yellow first coat of hair as a consequence of increased proportion of pheomelanin (Burchill *et al.* 1993). Western immunoblotting also revealed a decreased expression of *TYRP1* in follicular melanocytes of newborn agouti mice, exactly the time at which pheomelanin is produced predominantly (Kobayashi *et al.* 1995). In agreement with these studies, we found that pheomelanogenesis-related genes (i.e. *ASIP* and *SLC7A11*) were up-regulated early in the development of nestling tawny owl compared to eumelanogenesis-related genes (i.e. *MC1R*, *TYR* or *TYRP1*). Interestingly, the reverse was true at an older age, supporting the hypothesis that pheomelanogenesis takes place as long as cysteine is present and that eumelanogenesis is initiated once thiol concentrations is low enough.

Laboratory studies also highlighted that expression of some melanogenic genes can differ between males and females. Although *PC1* and *PC2* genes are not located on sexual chromosomes in mammals (chromosomes 13 and 2 in *Mus musculus*, respectively; Genbank references: NC_000079.6 and NC_000068.7, (Church *et al.* 2009; Church *et al.* 2011)), female mice frequently have higher expression of *PC1* and *PC2* than male mice (Beinfeld *et al.* 2005), potentially as a consequence of *trans*-regulatory elements mapped along mammalian X chromosome, an issue that still need to be tackled further. In birds, *PC1* and *TYRP1* genes are located on sexual Z chromosome (*PC1* Genbank, sequences: AB121969.1, NC_011493.1; *TYRP1* Genbank, sequence: NC_006127.3, (Bellott *et al.* 2010)), while *PC2* is located on chromosome 3 (Genbank, sequence: AB121970.1). Thus, we expected sex-specific patterns of *PC1* (but not *PC2*) and *TYRP1* expression in nestling tawny owls. In line with this prediction, we found that males expressed higher levels of *PC1* and *TYRP1* than females, but also higher levels of *PC2*. This outcome suggests first that nestling male tawny owls have a higher eumelanogenic potential than females (through *TYRP1* gene expression), but also a greater capacity of *POMC* processing (through PC1 and PC2 activities) to give rise to ACTH or α- and γ-MSH (Bell *et al.* 1998; Laurent *et al.* 2004; Helwig *et al.* 2006; Pritchard & White 2007), key peptides of the melanocortin system responsible for numerous pleiotropic effects (Ducrest *et al.* 2008). Although hypothetical, the higher male than female potential to produce ACTH or α- and γ-MSH to bind MC3R, MC4R and MC5R during the breeding season may enable them to increase their energy expenditure, sexual activity and aggressiveness, respectively (Ducrest *et al.* 2008). Accordingly, sex-specific patterns of *PC1*, *PC2* and *TYRP1* genes could be one proximate mechanism underlying the strong partition of reproductive tasks between sexes in the tawny owl (Sasvari *et al.* 2009; Emaresi *et al.* submitted, ***Chapter 2***).

Colour-specific patterns of gene expression

The melanocortin system is implicated in the expression of melanin-based coloration, but also pleiotropically regulate important phenotypic traits, such as stress response, energy homeostasis, anti-inflammatory response, sexual activity, resistance to oxidative stress and aggressiveness (Cone 1999; Fan *et al.* 2000; Schioth 2001; Tatro & Sinha 2003; Slominski *et al.* 2004; Boswell & Takeuchi 2005; da Silva *et al.* 2005; Fan *et al.* 2005; Bertile & Raclot 2006; Butler 2006; Cone 2006; Hillebrand *et al.*

2006; Maaser *et al.* 2006; Lin & Fisher 2007; Page *et al.* 2011). These observations led evolutionary biologists (Ducrest *et al.* 2008; Roulin & Ducrest 2011) to propose that the melanocortin system can be a proximate mechanism underlying the widespread covariations between melanin-based coloration and other important physiological and behavioural traits (Roulin 2004). Due to the complexity of this genetic network across species, a thorough understanding of the mechanism responsible for variation in melanin-based coloration requires insights on genetic variation at both levels, i.e. nucleotide sequences and gene expression. This is a key aspect for understanding the role played by coloration in social interactions (Roulin & Ducrest 2011). In the present study, we adopted such an approach in the colour polymorphic tawny owl, a species characterized by alternative adaptive responses to reproductive conditions (Roulin *et al.* 2004; Roulin *et al.* 2008b; Emaresi *et al.* in prep, ***Chapter 1***), food supply (Piault *et al.* 2009), pathogens (Galeotti & Sacchi 2003; Gasparini *et al.* 2009a; Karell *et al.* 2011a) and climatic conditions (Galeotti & Cesaris 1996; Karell *et al.* 2011b). Of particular interest, we provided experimental evidence that, in breeding tawny owl females, colour morphs differently expressed circulating POMC prohormone according to environmental and reproductive conditions (Roulin *et al.* 2011c), dark melanic individuals having lower levels of blood-circulating prohormone than light melanic ones when rearing experimentally reduced (but not enlarged) brood and when located in forest patches with high (but not low) density of beech trees, an indirect measure of prey abundance (note that these females were not moulting at the time of sampling suggesting that melanogenesis was not taking place). From a proximate point of view, this outcome raised different scenarios in melanocortin regulation, such as an overexpression of *POMC* gene or a down-regulated PC1 activity in red blood cells of adult tawny owls. In this context, a precise understanding of the relationships between melanogenic genes and their relative expression is imperative. To this end, it is particularly relevant to examine patterns of melanogenic gene expression during the production of eu- and pheomelanin, i.e. in nestlings.

We failed to detect consistent SNPs in the coding sequence of nine candidate genes (*ASIP*, *MC1R*, *MITF*, *PC1-2*, *POMC*, *SLC7A11*, *TYR*, *TYRP1*). This is not so surprising since variation in melanin-based coloration in nestling tawny owl is a continuous (rather than discrete) trait, which is hence more likely to result from different levels of gene expression rather than SNPs in nucleotide

sequence. In line with this hypothesis, we demonstrated that *MC1R* and *PC1* patterns of expression in young nestlings (i.e. approximately 10 days of age) covaried with the mean colour score of their biological parents (a proxy of their own coloration). The finding that *MC1R* gene was more expressed in offspring born from dark rather than light melanic tawny owls are not astonishing since this gene is a key regulator of eumelanogenesis pathway. However, the fact that this trend was only significant in nestlings experiencing enlarged broods is more surprising. Although weakly sensitive to environmental factors (Roulin & Dijkstra 2003; Gasparini *et al.* 2009a), a prediction confirmed by non-significant 'BSM' terms in other candidate genes, *MC1R* patterns of expression suggests that part of melanin-based coloration (i.e. eumelanin colour traits) could be condition-dependent. Of particular interest, no colour-specific patterns of *POMC* gene expression were highlighted, while *PC1* gene was more expressed in offspring born from light rather than dark melanic tawny owls. This cleaving enzyme is responsible for the post-translational processing of POMC prohormone into mature ACTH, β-Lipotropin (β-LPH) and N-terminal peptide (N-POMC; Slominski *et al.* 2000; Helwig *et al.* 2006; Pritchard & White 2007; Rousseau *et al.* 2007) and, by extension, related to melanogenesis. Because energy balance-regulating neuropeptides are derived from larger biologically inactive precursors, which need post-translational processing (e.g. POMC prohormone), *PC1* expression is frequently associated with important regulatory effects on energy balance (Jackson *et al.* 1997; Sanchez *et al.* 2004; Helwig *et al.* 2006; Wardlaw 2011). Even though we could not highlight colour-specific patterns of *POMC* gene expression in nestling tawny owls, offspring born from light melanic parents are likely to have a greater capacity of *POMC* processing to ACTH pool through their higher *PC1* gene expression. If true, their larger ACTH pool may enable them by extension a greater processing to α-MSH (Pritchard & White 2007; Rousseau *et al.* 2007), despite the fact that we found no colour-specific patterns of *PC2* gene expression (PC2 cleaving essentially ACTH and not the POMC prohormone). ACTH peptide is a key regulator of the hypothalamic-pituitary-adrenal (HPA) axis, mainly involved in stress response. Upon release into the systemic circulation, ACTH reaches the adrenal gland and binds to MC2R to stimulate the production of stress response effectors, namely glucocorticoids (e.g. corticosterone or cortisol; Slominski *et al.* 2000; Slominski *et al.* 2005), to counteract the effect of stress and buffer tissue damage. Note here that similar functional HPA axis is

likely to play a role in the skin, ACTH production up-regulating cortisol release in epidermal tissues (Ito *et al.* 2005). These corticoids are known to have powerful effects on foetal development (Bolt *et al.* 2001; Simamura *et al.* 2011) and potent anti-inflammatory and immunosuppressive properties (Slominski *et al.* 2000; Franchimont 2004). Of particular interest, glucocorticoids can also strongly influence metabolic traits (e.g. gluconeogenesis or fat break down in adipose tissue; (Macfarlane *et al.* 2008)), potentially explaining why nestlings born from light melanic females converted food less efficiently into body mass when fed *ad libitum* than nestlings born from dark melanic mothers, but suffered less from food restriction (Piault *et al.* 2009). Moreover, binding of ACTH to MC2R can also increase sexual steroid production in adrenal glands, leading to enhanced fertility, female sexual receptivity and male sexual motivation and performance (Shadiack *et al.* 2007). Altogether, our result raise the hypothesis that regulation of *PC1* gene expression may be a potential proximate mechanism modulating the pleiotropic effects of melanocortins in the tawny owl, indirectly regulating important physiological functions such as foetal development, immune system, energy balance and sexual activity.

Conclusion

Tawny owl melanin-based coloration frequently covaries with important physiological and behavioural traits, such as body mass, growth rate, life-history traits, immune system, and oxidative stress. To date, our knowledge of proximate mechanisms leading to the evolution of these covariations was scarce. In this study, we investigated the adaptive function of the melanocortin system in generating the colour-specific reaction norms observed in a wild population of tawny owls. Our candidate gene approach did not reveal colour-specific nucleotide divergence in the coding sequence of melanogenesis-related gene, but showed colour-specific patterns of gene expression in young nestlings. Of particular interest, *PC1* pattern of expression is a promising starting point for further investigation into the developmental basis of melanocortin-based covariations. Additional molecular studies are called to enhance our understanding of the POMC processing through prohormone convertases under different environmental or reproductive conditions. In this context, a central issue is to improve our knowledge of *cis*-acting elements regulating prohormone convertase gene expression, such as 5'UTR or 3'UTR (i.e. untranslated region), located upstream or downstream, respectively, on

the same chromosome. Indeed, their specific nucleotide sequences and structures can dramatically influence $_{m}$RNA half-life and processing, which have in turn important effects on protein translation and therefore melanin production (Rouzaud *et al.* 2010). Other issues that need to be addressed in future studies are the central roles of ACTH and α-MSH in shaping colour-specific norms of reaction in non-model organisms like the tawny owl.

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TABLES

Table 5.1: Specific information on pairs of primers used to sequence the nine candidate loci in the tawny owl. For each locus ('Gene'), Genbank reference sequences (and the corresponding species) used to design primers, the number of individuals sequenced ('N'), the amplification method used prior sequencing (Method), the forward and reverse primer sequences (5' -> 3'), the annealing temperature used in the corresponding PCR ('Ta'), the amplicon size ('Length') and its position in the coding sequence ('Coding sequence') are given.

					Primer				
Gene	**Reference species**	**Genbank references**	**N**	**Methods**	**Forward (5' - 3')**	**Reverse (5' - 3')**	**Ta**	**Length**	**Coding sequence**
ASIP	*Gallus gallus*	NM_001115079.1; BU337545. ; BU206868.1	15	Cloning	AGG TTT TGA CCG ACT TTG G	TTA GCA CTT TGG GTT TAA CAT T	50	440	exons 2-4
	Coturnix japonica	AB304510.1; AB304509.1; AB304511.1							
EEF1A1	*Gallus gallus*	NM_204157.2; NM_204157.2	1	PCR	TTG TGC TGT CCT GAT TGT TGC T	ATT CTG TGA CAG ATT TTT GGT CAA	55	1300	exons 3-6
	Xenopus Laevis	NM_001087442	2	Cloning					
	Taeniopygia guttata	XM_002190770							
GAPDH	*Gallus gallus*	NM-204305	3	Cloning	AGT CGG AGT CAA CGG ATT TG	CAT CAA AGG TGG AGG AAT GG	55	875	Data of prof. N. Mundy
	Taeniopygia guttata	AF255390							
MITF	*Gallus gallus*	NM_205029, XM_00219313	4	Cloning	TGA CCT CAC GAA TCC TGC TAC G	AAC GTA TTT GCC ATT TGC AAG G	55	600	exons 2-4
	Coturnix Japonica	AB005229	3	Cloning	CCG GCT CTG AAT ACC CAC TC	CTC CGC CTG CTA CTC GTT TT	55	900	exons 3-9
	Taeniopygia guttata	XM_002193131	2	PCR					
PC1	*Gallus gallus*	XR_026679, ENSGALT00000023658	8	PCR	ATG GCT TGG AGT GGA ATC AC	TTC CAT CTT TTG GGA TCA GC	55	869	exons 4-10
	Taeniopygia guttata	XM_002187938, ENSTGUG00000006229	2	Cloning					
PC2	*Gallus gallus*	XM_419332	2	PCR	GCC GTC TAC ACC AAC CAG TTC T	GCC ATC TTA ACC ATT GCT CCT G	55	1210	exon 2-10
	Taeniopygia guttata	XM_002197408							
POMC	*Gallus gallus*	NM_001031098	12	PCR	TCC TAC TCC ATG GAG CAT TTC C	CTG ACC CTT CTT GTA GGC GC	58	340	exon 3
			2	Cloning	ATG CCG CTG TGG AGC AGC C	TTT TGA TGA TGG CGT TTT TGA AG	65	750	exons 2-3
			3	Cloning	GAG TTA AAT CTG GCT GCT TGG GAA AGA G	GGC CAA ACT TGT TCC AGC GAA AGT GG	65	2000	exons 2-3
SLC7A11	*Gallus gallus*	XM_426289	11	PCR	CTT CAT CTC TCC CAA AGG CAT C	GCA GGA ACT CCA GTC AGG GTT A	55	1203	exons 1-11
	Taeniopygia guttata	XM_002191554_1	4	PCR	TGT GCC CAC GGT CTC TAA TG	AAG CAG GTA ATG GGC CAA AG	57	352	exons 1-2
			2	Cloning	GCT GGC ATC TTC ATC TCT CC	ATG ACA GCT GGC AGA GGA GT	57	932	exons 1-9
			2	Cloning	GCC TTT TCA GGG AAT GAT GC	AGG CAG GAA CTC CAG TCA GG	55	721	exons 5-11
TYR	*Gallus gallus*	NM_204160.1, AB023291	2	Cloning	TTG CTG AGG AAR GAG TGC T	CCA CAG CAG CAG AAA AGC ACG AT	55	533	exon 1
	Coturnix japonica	AB024278	2	Cloning	GTC CTG CCA GAA CAT CCT TC	GGC TTG CTC AAG GTA GGG TA	57	1256	exons 1-5
TYRP1	*Gallus gallus*		1	Cloning	CTC AGT TCC CTC GCC AGT	TGC TGG CTA CAG GTA GGT C	55	302	exon 1
			2	Cloning	CTG AGG AGC GGC ATG TGT T	ACT GGT CAG TAA GAA GAG GCT GA	57	1450	exons 1-6
MC1R	*Gallus gallus*	AB201629.1; AB2016630.1; AB2016631.1; NM_001031462.1	2	PCR	CTT GTT GAC GCA GGG ACC ATG T	CTA CCA GGA GCA CAA CAC CAC CT	59	970	exon 1

Table 5.2.1: Specific information on qPCR pair of primers used with SYBR SYBR® Green PCR Master Mix. For each locus ('Gene'), the sequence of forward and reverse primers (5' -> 3'), their relative concentrations ('Conc Fw' and 'Conc Rev'), the amplicon size ('Length'), the slope of standard curve generated by serial dilutions of genomic DNA or specific plasmids ('Slope'), the related coefficient of determination ('R^2') and the calculated efficiency of the qPCR ('E') are given.

Gene	Sequence Fw (5'-3')	Sequence Rev (5'-3')	Conc Fw	Conc Rev	Length	Slope	R^2	E
ASIP	GCT CCT TGA CAG GTT GCA TTC	GTT TGC TCA GAG CTG CCA ATT	150	300	67	-3.12	0.99	1.09
EEF1A1	GAT TGA TCG TCG TTC TGG CA	GCA GCA TCT CCG GAT TTC AG	300	300	66	-3.05	0.99	1.12
GAPDH	TTT TCC AGA CGG CAG GTC A	TGG AAT GGC TTT CCG TGT G	300	300	66	-3.2	0.91	1.05
MC1R	GTC CGG CAC ATG GAC AAT G	GAA GGA GAG GGA GGA CAC GAC	300	150	87	-3.46	0.99	0.95
MITF	GCC TTT GCT CCC CTG ATA TG	GGT TGC AGT TGT CCA GCA CA	200	200	66	-3.45	0.99	0.95
PC1	ACG GGC TGG AAA TTC AGG AT	AAC CCA AAT CGG CTG TTG AC	300	150	66	-3.28	0.95	1.02
PC2	GCT GGG ATA CAC AGG GAA GG	CAT AGC TGG CTT TGG CAT TG	200	200	103	-3.48	0.99	0.94
SLC7A11	TGT GCT TGC GGA CAT GAA TC	TCT TCG TTG CAT CCC GTG A	300	300	66	-3.36	0.99	0.98
TYR	AAA GAA CAC CCC TAG CCA GGA	GGG TTG GAG CCA TTG TTC AT	300	150	72	-3.24	0.99	1.03
TYRP1	TTT GTC CTC CTG TCC CAT TCA	ACG ACC CAG CAG TTC GAA GT	300	150	66	-3.31	0.987	1.01

Table 5.2.2: Specific information on qPCR pair of primers and TaqMan probes used with PreAmp Master Mix kit. For each locus ('Gene'), the sequence of forward and reverse primers and TaqMan probe (5' -> 3'), their relative concentrations ('Conc Fw', 'Conc Rev' and 'Conc Probe'), the amplicon size ('Length'), the slope of standard curve generated by serial dilutions of genomic DNA or specific plasmids ('Slope'), the related coefficient of determination ('R^2') and the calculated efficiency of the qPCR ('E') are given.

Gene	Sequence Fw (5'-3')	Sequence Rev (5'-3')	TaqMan Probe (5'-3')	Conc Fw	Conc Rev	Conc Probe	Length	Slope	R2	E
POMC	TTC ATG ACC TCG GAG CAC AG	TCG AGA CTT TTG ATG ATG GCG	CCA GAC CCC TCT AGT GAC TCT CTT CAA	900	900	300	71	-3.36	0.99	0.98
EEF1A1	CTC CTC TCG GTC GTT TTG	CAG CCT TCT TGT CAA CTG	ATG ACA CCA ACA GCA ACC GT	900	900	300	100	-3.32	0.99	1.00
GAPDH	TGC CAA CCC CCA ATG TCT C	AGC AGC CTT CAC TAC CCT C	GTG GAC TTG ACC TGC CGT CTG GAA AAA	900	900	300	81	-3.23	0.99	1.04

Table 5.3: Results of linear mixed-models investigating the effects of the brood size manipulation ('BSM'), nestling age ('Age Class') and average plumage coloration of their biological parents coloration ('MorphGenetic') on nestling expression levels of nine candidate genes, plus all possible interactions between these three variables. In these models, we considered nestling sex ('Sex') and the date of the sampling ('Date') as covariates. Since several individuals of the same nest were sampled at two different ages, we included the identity of the biological brood and nestling ring number (nested in biological brood identity) as two random factors.

Source of variation	*ASIP*					*PC1*					*SLC7A11*			
	n	*df*	*F*	*P*		n	*df*	*F*	*P*		n	df	*F*	*P*
Age Class	**293**	**1,149.6**	**110.77**	**<.0001**	**Sex**	**289**	**1,136**	**65.49**	**<.0001**	**Age Class**	**293**	**1,144.7**	**60.91**	**<.0001**
					Age Class		**1,143.8**	**3.85**	**0.05**					
Sex		1,147.6	3.51	0.06	**MorphGenetic**		**1,45.01**	**4.34**	**0.04**	BSM		1,121.4	3.02	0.09
BSM		1,132.6	0.84	0.36	**Age Class x MorphGenetic**		**1,146.6**	**5.66**	**0.02**	Date		1,88.04	0.72	0.40
Date		1,63.1	0.79	0.38						Sex		1,132.1	0.68	0.41
MorphGenetic		1,43.52	0.26	0.62	BSM		1,121	0.65	0.42	MorphGenetic		1,45.21	0.41	0.53
Age Class x BSM		1,147.4	0.45	0.50	Age Class x BSM		1,143	3.32	0.07	Age Class x MorphGenetic		1,143	1.55	0.22
BSM x MorphGenetic		1,129.3	0.75	0.39	Date		1,66.28	2.91	0.09	Age Class x BSM		1,141	1.19	0.28
Age Class x MorphGenetic		1,148.8	0.11	0.75	BSM x MorphGenetic		1,123.7	0.0001	0.99	BSM x MorphGenetic		1,120.4	0.0003	0.99
Age Class x BSM x MorphGenetic		1,148.6	0.50	0.48	Age Class x BSM x MorphGenetic		1,145	0.14	0.71	Age Class x BSM x MorphGenetic		1,141.4	0.16	0.69
	MC1R					*PC2*					*TYR*			
	n	*df*	*F*	*P*		n	*df*	*F*	*P*		n	*df*	*F*	*P*
Date	**279**	**1,57.72**	**4.27**	**0.04**	**Sex**	**266**	**1,128.3**	**7.42**	**0.007**	**Age Class**	**283**	**1,145.6**	**49.52**	**<.0001**
Age Class		**1,182.3**	**16.67**	**<.0001**										
BSM		**1,251.2**	**0.09**	**0.76**	MorphGenetic		1,43.93	1.89	0.18	MorphGenetic		1,40.64	2.25	0.14
Age Class x BSM		**1,139.9**	**0.87**	**0.35**	Date		1,144.4	0.06	0.81	Age Class x MorphGenetic		1,143.2	3.50	0.06
MorphGenetic		**1,91.3**	**5.78**	**0.02**	Age Class		1,178.2	0.24	0.63	Date		1,56.82	2.10	0.15
Age Class x MorphGenetic		**1,142.7**	**5.28**	**0.02**	BSM		1,114	0.05	0.82	BSM		1,125.9	0.03	0.87
BSM x MorphGenetic		**1,120.5**	**5.51**	**0.02**	BSM x MorphGenetic		1,113.6	2.50	0.12	Sex labdet		1,139	0.01	0.92
					Age Class x BSM		1,138.3	0.20	0.66	BSM x MorphGenetic		1,124.8	2.88	0.09
Sex		1,127.2	0.001	0.98	Age Class x MorphGenetic		1,138	0.06	0.81	Age Class x BSM		1,141.3	0.91	0.34
Age Class x BSM x MorphGenetic		1,142.7	0.02	0.88	Age Class x BSM x MorphGenetic		1,136.8	0.07	0.79	Age Class x BSM x MorphGenetic		1,141.4	0.002	0.97
	MITF					*POMC*					*TYRP1*			
	n	*df*	*F*	*P*		n	*df*	*F*	*P*		n	*df*	*F*	*P*
Date	**293**	**205.3**	**9.35**	**0.003**	**Age Class**	**258**	**1,123.8**	**1.03**	**0.31**	**Sex**	**271**	**1,134.1**	**5.44**	**0.02**
					MorphGenetic		**1,46.95**	**0.25**	**0.62**	**Age Class**		**1,139**	**105.11**	**<.0001**
Sex		1,134.6	3.24	0.07	**Age Class x MorphGenetic**		**1,126.6**	**5.82**	**0.02**	**BSM**		**1,111.4**	**0.05**	**0.83**
BSM		1,118.6	2.38	0.13						**MorphGenetic**		**1,38.05**	**0.01**	**0.91**
MorphGenetic		1,39.7	1.17	0.29	Date		1,69.05	0.61	0.44	**BSM x MorphGenetic**		**1,114.2**	**5.33**	**0.02**
Age Class		1,130.4	0.05	0.82	Sex		1,121.8	0.06	0.80					
Age Class x MorphGenetic		1,147	2.00	0.16	BSM		1,109.9	0.10	0.75	Date		1,52.12	0.74	0.39
BSM x MorphGenetic		1,118.4	0.43	0.51	Age Class x BSM		1,123	1.07	0.30	Age Class x MorphGenetic		1,138.8	1.70	0.19
Age Class x BSM		1,143.9	0.23	0.63	BSM x MorphGenetic		1,110.2	0.00	0.98	Age Class x BSM		1,136.1	0.20	0.66
Age Class x BSM x MorphGenetic		1,145.4	0.06	0.81	Age Class x BSM x MorphGenetic		1,123.8	0.14	0.70	Age Class x BSM x MorphGenetic		1,137.7	0.02	0.88

ANNEXE 1

Melanin-based coloration signals nest defence behaviour in tawny owls (*Strix aluco*)

Arnaud Da Silva[*#1,2], Valentijn van den Brink[*2], Guillaume Emaresi[*2], Ester Luzio[2], Pierre Bize[2] and Alexandre Roulin[2]

*These authors contributed equally

1) Université de Bourgogne, UMR CNRS 5561 Biogéosciences, Equipe Ecologie Evolutive, 6 Boulevard Gabriel, 21000 Dijon, France

2) Department of Ecology and Evolution, University of Lausanne, Biophore building, CH-1015, Lausanne, Switzerland

 # current address: Behavioural Ecology and Evolutionary Genetics, Eberhard-Gwinner-Straße, 82319 Seewiesen, Germany

ABSTRACT

Nest defence is risky and jeopardize future reproduction. Here, we investigated individual variation in nest defence behaviour in the tawny owl (*Strix aluco*) because adults defend their nest vigorously against any potential predator including humans. Females are larger than males but on average similarly coloured, with birds varying from dark to light reddish melanic. Since dark and light melanic individuals evolved alternative life history strategies, we propose the hypothesis that coloration also signals nest defence behaviour. We examined several predictions using both correlative and experimental approaches. When the offspring were old enough so that their parents were naturally sleeping outside the nest, we found that during daylight hours our presence induced females to fly around us, eventually attacking us, more often when they were dark than light reddish, while we hardly saw males. Furthermore, a playback experiment during daylight hours showed that darker females defended their nest more intensely when we broadcasted territorial calls of a male tawny owl. A similar playback experiment performed at night the following year showed that the stronger nest defence by dark than light reddish birds is not restricted to females but also applies to males. Finally, irrespectively of their colour breeding females responded more vigorously to dark than light reddish stuffed tawny owl placed beside their nest. We conclude that dark reddish individuals are more aggressive and also represent a greater threat than light coloured individuals. The bolder behaviour of dark than light reddish individuals is likely to increase their current reproductive success, which is in agreement with our long-term dataset on tawny owl breeding biology showing that dark males suffer lower nest predation rates than light males. In this polymorphic bird, melanin-based coloration therefore signals anti-predator strategies and is used in social interactions.

INTRODUCTION

Anti-predator behaviour is critical to organisms because predation is one of the major causes of reproductive failure in many species (Orians & Janzen 1974), including birds (Martin 1995). Parental defence behaviour is therefore likely to be linked with key life-history trade-offs between offspring quality vs. number and between current offspring vs. future reproduction (Wolf *et al.* 2007). In Ural owls (*Strix uralensis*) for instance, increased nest defence by parents was associated with higher recruitment rate of their offspring (Hayssen *et al.* 2002). This adaptive, but risky behaviour against predators or competitors is likely to evolve in parents that invest more energy in the production of high quality offspring. In this case, the direct costs incurred by this defence behaviour through higher adult mortality caused by predators (Montgomerie & Weatherhead 1988) and indirect costs due to aggressive behaviour, such as the cost of increased testosterone levels (Marler & Moore 1988) should be lower than the benefits generated by rearing high-quality offspring. The cost/benefit ratio of anti-predator behaviour is likely to differ between individuals (Montgomerie & Weatherhead 1988) and therefore we could expect that individuals signal investment in anti-predator behaviour to conspecifics of the same (intraspecific sexual selection) or different sex (interspecific sexual selection; e.g. Reyer *et al.* 1998).

Life history theory proposes that individuals optimally allocate resources among energy-demanding and time-consuming life history traits (Stearns 1992). Some of the best known life history trade-offs are resource allocation in reproduction vs. maintenance, in offspring number vs. quality, and in current vs. future reproduction (Stearns 1989). If for instance past investment in reproduction was made at the expense of maintenance, it will reduce adult survival and also negatively affect the value of current offspring (Williams 1966; Roff 1992). Assuming that information about the competitors intentions is available, several evolutionary stable strategies to resolve such trade-offs can theoretically coexist in single populations, for instance through a hawk-dove game (Johnstone 2001), where it pays off to always be a hawk or a dove. Different behavioural types may also evolve through slight differences in investment in current or future reproduction (Wolf *et al.* 2007). The latter model was designed to understand how different degrees of boldness in front of a predator can coexist in a single population. It revealed that differences in risk taking behaviour evolved since individuals with

higher future expectations have more to lose and are thus more risk-avoiding. Only few studies report natural populations exhibiting such strategies, though . Examples are colour-specific mating strategies in male ruffs (*Philomachus pugnax*; Rhijn 1973; Lank *et al.* 1995) or reproductive strategies in the white-throated sparrow (*Zonotrichia albicollis*; Tuttle 2003).

Melanin-based coloration is an appropriate phenotypic marker of potential genetic strategies in trade-off resolutions in life history traits within a single population. Indeed, inherited variation in the deposition of melanin pigments (grey-black eumelanin and reddish-brown pheomelanin) is frequently reported to covary with morphological, physiological, behavioural or life history traits (Roulin 2004), such as resistance to stress, sexual activity and aggression (Ducrest *et al.* 2008). In the common buzzard (*Buteo buteo*) for instance, melanin-based coloration covaries with aggressiveness (against both predators and conspecific competitors) and fitness components (Boerner & Kruger 2009). Such covariations may be due to pleiotropic effects of key regulators of the melanogenesis (Ducrest *et al.* 2008). Melanocortins derived from the proopiomelanocortin gene (*POMC*) can induce eumelanogenesis by binding to the MC1 receptor and promote aggressiveness by binding to the MC5 and MC4 receptors (Morgan *et al.* 2004). Melanin-based coloration can thus be associated with alternative behavioural (*e.g.* boldness, aggressiveness) and life history strategies, involved in specific trade-off resolutions such as investment in current vs. future reproduction.

In long-lived species, conspecifics are likely to differ in the resolution of fundamental life history trade-offs (Amat *et al.* 1996), because they have the choice to invest in the current offspring or decide to wait for another breeding season. Therefore, we decided to investigate whether melanin-based coloration is associated with differences in nest defence behaviour in the tawny owl (*Strix aluco*). This owl displays large inter-individual variation in the deposition of melanin pigments, which was found to covary with physiological, behavioural and life history traits in several European populations (Galeotti & Sacchi 2003; Roulin *et al.* 2003; Roulin *et al.* 2004; Brommer *et al.* 2005; Roulin *et al.* 2005; Roulin *et al.* 2008b; Gasparini *et al.* 2009a; Piault *et al.* 2009; Karell *et al.* 2011a; Roulin *et al.* 2011c). Light coloured females breed less often than dark reddish ones, skipping sometimes one breeding season, but compensate this cost by producing more offspring during prime breeding seasons (Emaresi *et al.* in prep, ***Chapter 1***). In contrast, reddish individuals seem less

flexible, adopting a constant reproductive investment (Piault *et al.* 2009; Roulin *et al.* 2011c) and produce fewer but higher quality offspring (Becker 1984; Roulin *et al.* 2008b; Emaresi *et al.* in prep, ***Chapter 1***). Accordingly, darker reddish individuals may be more aggressive and bolder during nest defence to increase the survival of their offspring. To test this prediction, we began with an analysis of nest defence in a long-term dataset with records of nest visits. The tawny owl is a paradigmatic species for nest defence as it can attack humans who approach their nest too closely (e.g. Leifert *et al.* 2004). We thus predict that dark reddish individuals display bolder behaviour than light coloured ones. In addition, we manipulated parental workload during these years, by performing a brood size manipulation experiment. We were thus able to study whether differently coloured individuals experiencing experimentally reduced or enlarged broods (i.e. current reproduction) responded in different ways regarding nest defence.

Male and female tawny owls have distinct reproductive tasks: males deliver food to their offspring and partner (Sunde 2008; Sasvari *et al.* 2009), while females distribute the prey items among the progeny. Because of their 5-10% larger size and 20-25% greater body mass (Glutz von Blotzheim & Bauer 1980), females are also responsible for nest defence (Wallin 1987), whereas, in territorial disputes, males and females were found to be equally active (Sunde & Bolstad 2004). Since tawny owls remain territorial during the breeding season (Glutz von Blotzheim & Bauer 1980), we performed playback experiments in two consecutive breeding seasons, during daylight and at night respectively. In the second experimental year we simulated a tawny owl intrusion in different territories by combining the playback experiment with the presence of mounted model of stuffed owls (either dark or light reddish), a procedure that enabled us to test whether tawny owl response differed according to the colour of intruder (i.e. stuffed bird) or defender. Previous studies already reported associations between anti-predator behaviour and melanin-based coloration in related species, i.e. marsh harriers (*Circus aeruginosus*; Sternalski & Bretagnolle 2010), common buzzards (*Buteo buteo*; Boerner & Kruger 2009), barn owls (*Tyto alba*; Van den Brink *et al.* 2012) and European kestrels (*Falco tinnunculus*; Van den Brink et al. in press). Based on the hypothesis that reddish coloration acts as a signal of boldness, a darker reddish stuffed owl might be considered a greater threat to a breeding pair and elicit a stronger defensive response.

METHODS

Study species and site

The tawny owl is one of the most common owl species in Eurasia. It is a long-living, mostly monogamous and philopatric species that can live up to 20 years (Glutz von Blotzheim & Bauer 1980; Galeotti 2001). Brood sizes vary between one and eight eggs, which hatch between February and the end of May (Galeotti 2001). Nestlings leave the nest-box as soon as they are able to fly, at an age of 25-30 days but parents continue to feed and protect them until they are about two months old (Sunde 2008). The reddish coloration is independent of age and sex and is highly heritable (h2 = 0.72-0.80, Brommer *et al.* 2005; Gasparini *et al.* 2009a; Karell *et al.* 2011b). The variance in coloration is explained by a mix of pheomelanin (68%) and eumelanin (21%), feathers of darker reddish individuals containing more pheomelanin and eumelanin; no carotenoids have been recovered in the feathers (Gasparini *et al.* 2009a). Our study was carried out in a forested area of 911 km^2 situated in western Switzerland, at a mean altitude of 672 m (range 400-950 m). In this area, 377 nest-boxes were set up within forest patches of at least 4000 m^2. The mean distance between two nest-boxes was 627 m, with a minimal distance of 500 m (Roulin *et al.* 2011c).

Colour measurements

Males and females were measured, weighed and assigned to one of five colour morphs (1 = dark reddish brown, 2 = reddish-brown, 3 = brown, 4 = brown-grey, 5 = grey Roulin *et al.* 2003; Roulin *et al.* 2005) providing a good estimation of overall coloration (Roulin *et al.* 2005). Between- and within-year visual assessments of individual coloration were found to be significantly repeatable for both females (280 individuals, $r = 0.86 \pm 0.023$ SE, $F_{279,613} = 11.37$, $P < 0.0001$) and males (226 individuals, $r = 0.88 \pm 0.025$ SE, $F_{225,354} = 12.59$ $P < 0.0001$; Lessells & Boag 1987).

Brood size manipulation experiment

Based on the criterion that clutches were laid on similar dates ($r = 0.92$, $P < 0.0001$), between 2005 and 2011 we matched 388 out of 545 successfully hatched pairs of nests to decrease (R) or increase (E) parental investment of breeding pairs. This resulted in a reduced body mass in E parents compared to R parents, demonstrating the increase in clutch size represents an increased parental effort (Roulin *et al.* 2011c). Brood sizes were manipulated by exchanging on average 2 hatchlings or

eggs from a nest E to a nest R and on average 3 hatchlings or eggs from a nest R to a nest E. Pairs of nests were randomly selected and colour characteristics of the biological and foster parents were not correlated to each other, neither same sex nor opposite sex (all *P*-values > 0.19). Adult plumage coloration was not associated with clutch size and brood size before or after the manipulation (Pearson's correlations; *P*-values > 0.11). We found no evidence for assortative mating within breeding pairs (r = 0.05, n = 383, P = 0.28). Note that sample sizes might differ from total nest numbers, since we were not always able to capture both partners.

Long-term analyses of female nest-defence during daylight hours

Between 2005 and 2010 we examined whether plumage coloration of the parents was associated with their anti-predator behaviour. We considered 1555 brood visits performed in 318 nests during daylight hours when nestlings were old enough to be thermo-independent (mean ± SE = 20.2 ± 0.2 days of age). At this time, parents do not rest in the nest anymore (Glutz von Blotzheim & Bauer 1980), but usually in a tree nearby, so that they can detect and react to potential predatory threats. We only used those observations where no parent was captured inside the nest-box and we further restricted our analyses to females (1039 observations), because males were rarely seen (357 times). Moreover, preliminary analysis in males revealed no significant effect of colour on presence (P = 0.39), as expected if nest defence is the main responsibility of females (Wallin 1987). We then proceeded to focus on females. We determined sex either visually based on the sexual dimorphism in size (Baudvin & Dessolin 1992) or the type of alarm calls (Galeotti & Pavan 1991).

Nest predation

During this period we also recorded breeding success or failure during both egg and nestling phase. Main avian predators are goshawk (*Accipiter nisus)*, eagle owl (*Bubo* bubo) and buzzard (*Buteo buteo*; Mikkola 1976). Mammalian predation also occurs, mostly by mustelids, and after fledging by red foxes (*Vulpes vulpes*; Sunde *et al.* 2003a). We distinguished between abandoned nests, successful fledging and predation. During the incubation phase, if eggs were missing, or we found shell remains in the nest, this was considered predation; cold eggs left in a nest without the parents being present were considered abandoned. In the nestling phase, if the nestlings were missing long before they should have fledged at 30-35 days, this was also considered predation. If non-injured nestlings were

found dead in the nest, we considered this abandonment by the parents. Predation and abandonment in the egg phase are difficult to distinguish and therefore we limited our analysis to the nestling phase.

Playback experiment during daylight hours in 2010

In 2010, after the size of broods had been manipulated, we studied 26 enlarged and 25 reduced broods of the same mean hatching date (Student's t-test, $t_{49} = 0.56$, $P = 0.52$). Foster and biological parent's colour was not correlated with each other (Pearson's correlations, all *P*-values > 0.30). Between April 21 and May 18, when nestlings were 26 ± 0.6 SE days old, we performed a playback experiment during daylight hours (range: 8h50 – 15h50). Male and female coloration was not significantly associated with time of the day and date of playback (Pearson's correlations, *P*-values > 0.08). To this end, we placed a stereo CD player at 10 m from the nest-box and played territorial calls of a male tawny owl during five minutes. This recording was a series of male hoots (3 per minute) from a single male originating from a breeding population in the UK, unknown to all owls in our local population. During the playback we retreated to approximately 20 meters from the nest, visually recording tawny owl presence or absence and a proxy of tawny owl presence, specifically passerine alarm calls. Passerines are known to actively mob raptors (Hogstad 1995) and in particular owls as passerines are a possible prey to them (McPherson & Brown 1981). Passerine alarm is therefore an efficient proxy of movement of tawny owls, because usually during the day tawny owls are not moving, but hiding in order to avoid being mobbed by passerines. We saw the breeding female in 24% of the playback experiments but we heard passerines alarming in 55% of cases. When we saw a tawny owl, we also heard passerines 10 out of the 13 times (77%).

Playback experiment at night in 2011

In 2011, we performed a playback experiment at night when nestlings were 17.6 ± 0.4 days old and a second time when they were 21.1 ± 0.4 days old, with an average of 3.2 ± 0.3 days between visits. The playback experiment took place at night (mean time 23:15h ± 12 min, range: 21h15 – 01h15)), since we expect the owls to be more active at this time and hence we could obtain a measure of the intensity of their alarm calls. We obtained data of 31 nests that were visited twice between April 19 and May 23. We placed a recorder (Marantz PMD 661) at 10 metres from the front of the nest box, and the microphone (Beyerdynamic MC 930) at 10 meters from the recorder. The researchers retreated

to a location at 10 meters away, perpendicularly from the microphone. Because longer playbacks are believed to increase owl response (Redpath 1994), we played 19 (instead of five as in 2010) minutes of the same male tawny owl territorial call as used in the 2010 experiment (3 hoots per min). Eight nest boxes that were occupied in 2010 were also used in 2011, 5 by the same pair, 2 by the same female and 1 by the same male. A loudspeaker was placed close to the nest box and we started a playback sequence of eight minutes (three minutes of calls followed by five minutes of silence) after an initial three minutes of silence as an acclimation period. We repeated the sequence twice. The same recording was used for all nests, to have a uniform treatment for all nests, which allowed us to eliminate variance caused by the specific recording used (McGraw *et al.* 2003). Note that coloration of both parents was unknown at the time of the experiment. In addition, we presented next to the loudspeakers a stuffed owl of either dark or light reddish morph. The stuffed owl was placed next to the loudspeaker when setting up the speakers and was covered under a black sheet until we broadcasted calls. A single mount was randomly allocated to each nest to simulate an intruder. The colour of parents did not differ between nests assigned the two different mounted owls (Student's *t*-tests: males, $t_{28} = 1.68$, $P = 0.1$; females, $t_{29} = 0.02$, $P = 0.99$).

We recorded a number of variables in breeding males and females, all related to nest defence and aggression; males and females were distinguished by the type of calls. These were the time before a response was given after we started to broadcast calls (latency in seconds) and frequency of the response calls during the entire 18-minutes long playback experiments, the distance (metres) between the location of the mounted owl and each breeding owl when it emitted its first response call as well as the minimal distance (metres) of all responses, and the number of flights an individual was seen making during the entire 18-minutes long playback experiments. Because the experiment was carried out during the night, we were not always able to see individual birds and therefore we recorded the distance and the number of flights by listening to the calls or the sound of leaves made during movement.

Statistical procedure

All analyses were performed using JMP 9.0.2 and SAS 9.1. Final models were obtained by eliminating non-significant variables, non-significant interactions first. All tests were two-tailed and P-values smaller than 0.05 are considered significant, values are reported as means ± standard error.

Long-term analyses of female nest-defence during daylight hours

We used a generalized linear mixed model (GLMM) with a logit link function and a binomial response variable for whether or not the female was seen during our nest visits. We incorporated female identity, and year as two random factors to account for individuals breeding in more than one year (only 20 out of 212 females were only seen breeding in one year) and for more than one visit to the same nest in the same year. As independent variables we introduced female colour morph, brood size manipulation treatment (enlarged or reduced), time of the day (mean: 14h05 ± 35min, range: 6h-21h), date (i.e. number of days after the 1st of January; range: 4 Feb-24 June) and initial unmanipulated brood size (mean: 3.9 ± 0.04, range: 1-7 nestlings). In the initial model, we incorporated two-way interactions between female coloration and time of the day, date, brood size and nestling age.

Nest predation

To investigate possible differences in predation risk between individuals of different colour that might be associated with different nest defence strategies, we compared brood predation rates during the nestling rearing stage between 2005 and 2010. We performed a generalized linear mixed model with a logit link function and presence/absence of predation on nestlings as binomial response variable. We included year and nest identity as random variables. Factors included in the model as explanatory variables were adult coloration, sex, brood size manipulation, hatching date and two-way interactions between adult coloration and brood size manipulation.

Playback experiment during daylight hours in 2010

We used passerine alarm calls as a proxy for the presence of an adult tawny owl and with this we constructed a logistic regression model with passerine alarm as a binomial response variable (i.e. presence or absence). The variables included in the initial model were date, age of the nestlings, brood

size manipulation treatment, colour morph of the mother and all two-way interactions with colour morph.

Playback experiment at night in 2011

Compared to the statistical models for the data collected in 2010, we considered a number of extra parameters: frequency of alarm calls, distance to the stuffed owl at first response, minimum distance to the stuffed owl during response, number of times moved and the latency until the first response. Since these parameters were often correlated with each other (Pearson's correlations, $0.77 > |r| > 0.33$, $P < 0.027$), we performed a principal components analysis (PCA) on the correlation matrix. Males and females were analysed separately because they differed in their responses (females responded faster (Mann-Whitney U test, $z = -3.21$, $P = 0.005$), more often (71% vs. 27%, chi-square test: $\chi^2 = 21.8$, $P < 0.001$) and with a higher call frequency ($z = -5.36$, $P = 0.005$) compared to males. We thus decided to analyse the response of each gender in separate models. For both females and males we retained only those two principal components with eigenvalues larger than one (Quinn & Keough 2002). Both components explained 46.3% and 20.9% of the variation in female response, with eigenvalues of 2.32 and 1.04, and 48.7% and 24.6% of the variation in male response, with eigenvalues of 2.44 and 1.23, respectively. After inspection of the loadings for the females, we found that alarm frequency and number of flights contributed negatively to PC1 (respectively -0.78 and -0.65), whereas log latency of response, the distance to the stuffed owl of first response and minimum distance contributed positively (respectively 0.50, 0.60 and 0.83). Thus, a negative value for PC1 indicates a stronger reaction to intrusion as mimicked by our playback experiment and stuffed owl placed beside the nest. For PC2, log latency of response (-0.70), distance to the stuffed owl at the first response (0.49) and the minimum distance (0.44) contributed importantly. The alarm frequency contributes (0.35) and number of flights (0.07) had weaker loadings. Therefore, we conclude that a higher value for PC2 indicates a quicker and more intense response to our playback experiment and stuffed owl.

In males, a higher value of PC1 indicates a greater distance of first response (loading: 0.56) and minimum distance to the mounted owl (0.95), while it also indicates fewer alarm calls (-0.57) and fewer flights (-0.93). The second component is only influenced strongly by the latency (0.98), with

weaker contributions for the distance at first response (-0.38) and frequency of calls (0.28). Thus, the most intense reactions are found for negative values of PC1 and positive values of PC2.

PC1 and PC2 were used as response variables in separate general linear mixed models for males and females. This resulted in four different models. As random variables we included individual identity and individual nested in replicate to account for repeated measures. Fixed effects were the colour morph of the individual, date, time of the night, clutch size, age of the nestlings, colour of the stuffed owl used and brood size manipulation treatment. We included two-way interactions of the colour morph of the breeding individual with clutch size, brood size manipulation and the model owl that was used.

Three males and 16 females responded in both replicates, therefore we could only assess change in response and repeatability of behaviour in female individuals. The chance of obtaining a response did not increase for females between the first and the second replicate (chi-square test, d.f = 1, $\chi^2 = 0.08$, $P = 0.78$). We tested repeatability by using one-way Analyses of variance on the PC1 and PC2 values with individual identity as factor and if there was a significant effect of individual identity by then calculating repeatability values (Becker 1984; Lessells & Boag 1987).

RESULTS

Long-term analyses of female nest-defence during daylight hours

Female tawny owls were more likely to fly around us, vocalise or attack us when they were dark rather than light reddish ($F_{1,1270} = 4.80$, $P = 0.029$), when the number of nestlings was large rather than small ($F_{1,1270} = 17.06$, $P < 0.0001$), in the morning rather than afternoon ($F_{1,1270} = 14.50$, $P < 0.0001$) and late rather than early in the season ($F_{1,1270} = 92.06$, $P < 0.0001$).

Predation

We found that sex ($F_{1,80} = 7.2$, $P = 0.009$) and the interaction between sex and colour ($F_{1,80} = 3.96$, $P = 0.049$) showed a significant relationship with predation at the nestling stage. Closer inspection revealed a strong trend for lower predation in darker reddish males ($F_{1,20} = 4.02$, $P = 0.06$). For females no significant relation was found between female plumage coloration and predation ($F_{1,9} = 0.48$, $P = 0.5$).

Playback experiment during daylight hours in 2010

The probability that passerines alarmed was significantly associated with female plumage coloration in interaction with the brood size manipulation experiment (logistic regression analysis: female coloration: $\chi_1 = 3.02$, $P = 0.08$; brood size manipulation: $\chi_1 = 3.25$, $P = 0.07$; interaction: $\chi_1 = 10.2$, $P = 0.001$). Upon closer inspection we can see that passerines were more likely to alarm when the female was dark reddish and rearing a reduced ($\chi_1 = 9.79$, $P = 0.002$) rather than an enlarged brood ($\chi_1 = 1.43$, $P = 0.23$; Figure A.1).

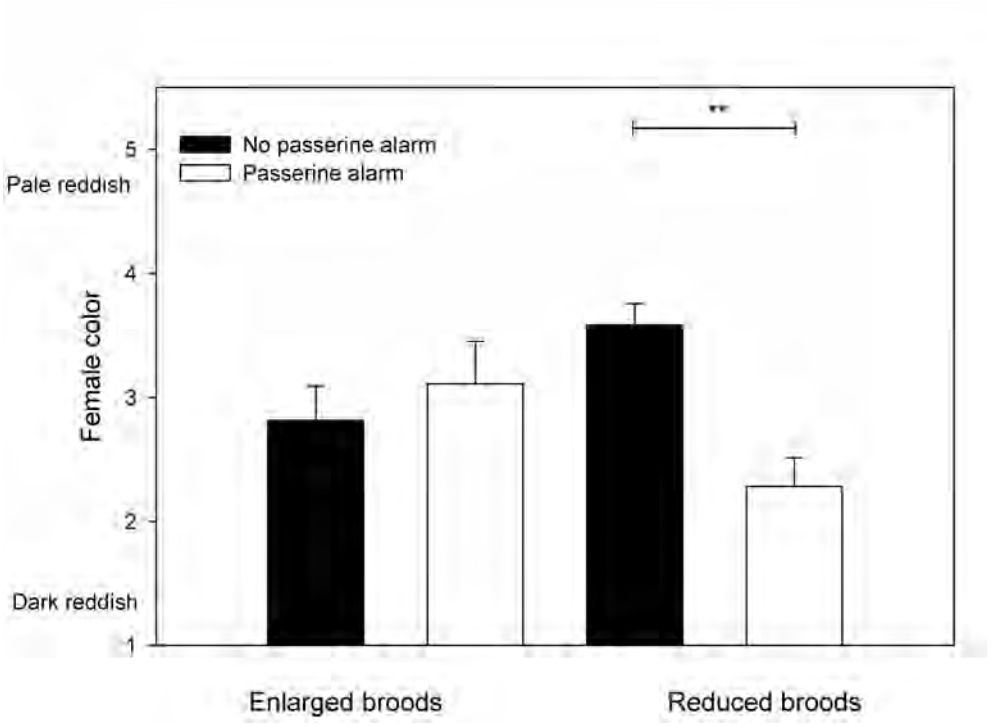

Figure A.1: Relation between passerine alarm and colour of female tawny owls depending on brood size manipulation. Data from the playback experiment carried out in 2010. Stars indicate significant differences.

Playback experiment at night in 2011

Response of breeding females

The final model for PC1 (i.e. reaction to intrusion) showed no significant influence of any of the variables on the intensity of the response. The final model for PC2 (i.e. intensity of the response to intrusion) showed that females responded more intensely to a dark than light reddish mounted owl

($F_{1,28.05}$ = 7, P = 0.01; Figure A.2). In this model, the reaction of females rearing a reduced brood tended to be more intense than that of females with an enlarged brood ($F_{1,24.59}$ = 3.6, P = 0.07).

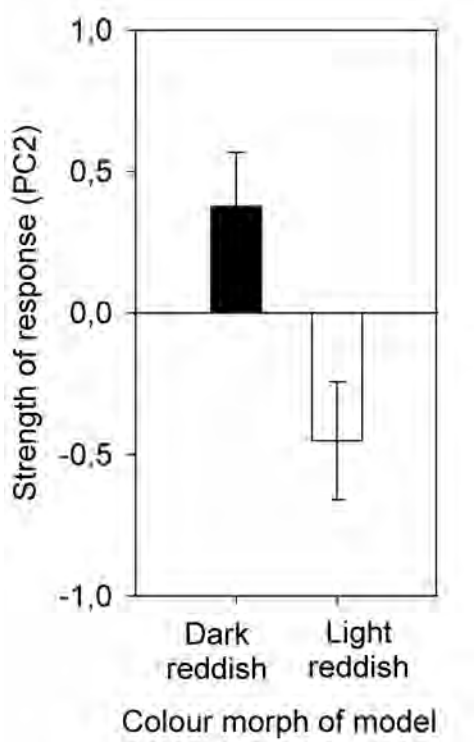

Figure A.2: Strength of the response of female individuals (measured as latency to respond and frequency of alarm calls in principal component 2 of a principal components analysis) to a dark or light reddish mounted tawny owl.

Both PC1 and PC2 were not repeatable within individuals (ANOVA, with individual as factor, all p-values > 0.34). However, if we look at the individual variables, the latency ($F_{25,\ 40}$ = 3.4, P = 0.007, r = 0.57 ± 0.02) and frequency of calls ($F_{25,\ 40}$ = 3.8, P = 0.005, r = 0.50 ±0.04) were repeatable, whereas minimum distance, distance at first response and number of flights were not significantly repeatable (P-values > 0.65).

Response of breeding males

The final model for males with PC1 (i.e. distance of first response, minimum distance and calls) showed that the intensity of the response was higher in darker reddish males ($F_{1,10}$ = 21.65, P = 0.0009; Figure A.3). Males showed a stronger response when rearing fewer offspring ($F_{1,10}$ = 5.56, P = 0.04) and later in the season ($F_{1,10}$ = 14.1, P = 0.004). The model with PC2 (i.e. latency time) showed no significant effects (all P-values > 0.26).

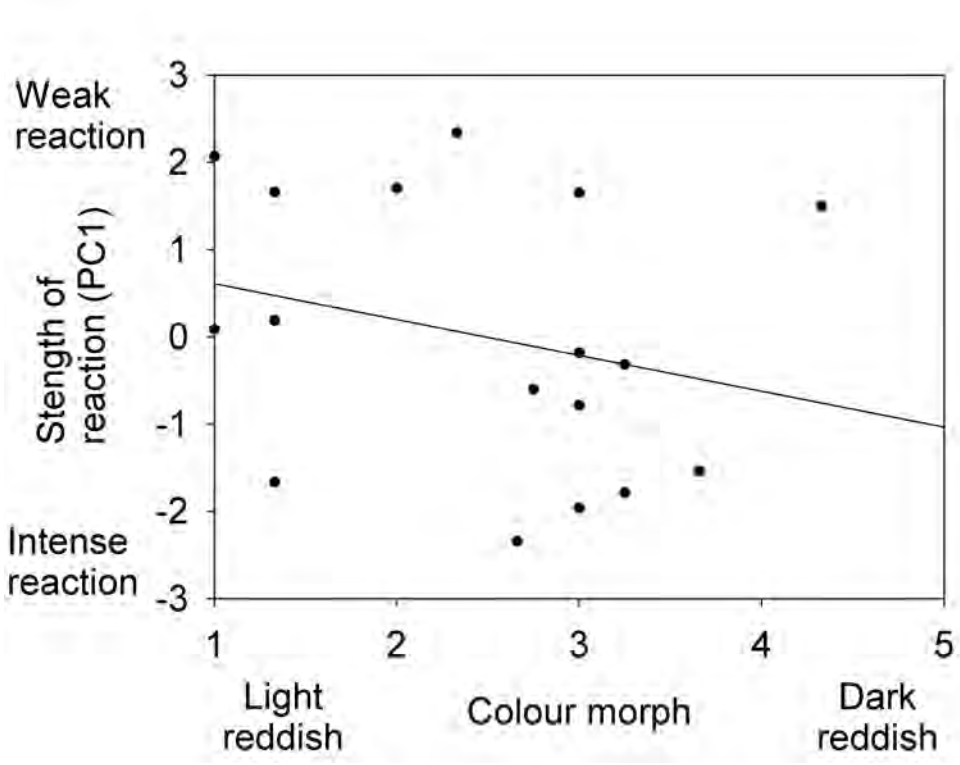

Figure A.3: strength of reaction by male individuals in relation to their colour morph, measured as principal component 1 (PC1; call frequency, latency to respond, number of lights and distance to the intruder) in a general linear model analysis. A negative value for PC1 indicates a stronger reaction.

DISCUSSION

Our results show that differently coloured tawny owls differ in their response to intruders. The long-term dataset revealed that compared to light reddish females, darker ones are present more often when a human intruder approaches their nest site. The daytime playback experiment has produced a similar result, with the extra merit of a brood size manipulation experiment that allowed us to manipulate the number of offspring to protect against potential predators. This experiment showed that passerines alarmed more frequently in situations where the tawny owl female was dark than light reddish and when rearing an experimentally reduced brood; in contrast light reddish females induced a less intense response in passerines whatever the brood size treatment. This result suggests that our presence around nests and broadcasted male calls induce owls to move from their hiding place particularly when the female is dark rather than light reddish and mainly when brood size is small. It also demonstrates that (reddish) parents invest more in defending few, presumably high quality offspring, rather than many, lower quality offspring. The long-term dataset indicates the reverse,

however, with larger broods being defended more intensely. An experimentally increased brood might not reflect the quality of offspring accurately, but rather the response to parents to an increased workload. In a natural situation parents that rear many young are better able to do so than parents rearing only few nestlings and might then also have better quality nestlings to defend.

A similar playback experiment carried out this time at night has brought complementary information on the association between melanin-based colour morphs and nest defence. First, in response to broadcasted male calls, darker reddish individuals were more aggressive. Second, a dark reddish tawny owl mount induced more intense nest protection than a light one. Altogether, our results demonstrate that dark reddish males and females invest more effort to protect their nest than light coloured conspecifics and that a darker reddish intruder represents a higher threat (at least for females) than a light reddish intruder. This is consistent with the finding of our long-term survey of the breeding biology of owls in the same area showing that light reddish owls tend to suffer more predation than dark reddish conspecifics.

The use of a single male for our broadcast calls could have biased our results, as it could be the reaction to this specific male (colour, size, or other information contained in its song) that is measured. All our data, both experimental and observational are congruent with each other, however, making us confident that our results are robust and biologically relevant.

Colour, reproductive success and survival

Colour is not selectively neutral in tawny owls as results from a Finnish population showed that light reddish individuals of both sexes produce about 33% more offspring during their lifetime than dark reddish ones and have a longer breeding career (Brommer *et al.* 2005). The lighter reddish owls from that population also recruit more than twice as much (Brommer *et al.* 2005) and have a higher survival in cold winters (Karell *et al.* 2011b). Even though the selective pressures might not necessarily be the same for the Finnish and our Swiss population, selection pressures in different parts of the tawny owl range appear to select for alternative colour-specific behavioural, physiological and life history strategies. Dark reddish individuals are affected by parasites more often in Italy (Galeotti & Sacchi 2003), although not at the cost of reproduction. In Finland, occurrence of parasites is similar in light and dark coloured owls, but dark reddish females lose more body mass during breeding if

infected (Karell *et al.* 2011a). In Switzerland, light reddish individuals invest more in the number of offspring in good years, whereas darker individuals produce fewer, higher quality offspring in these years (Emaresi *et al.* in prep, ***Chapter 1***). Furthermore, lighter reddish individuals lose less weight after an immune challenge (Gasparini *et al.* 2009a) and skip more often reproduction during bad years (Roulin *et al.* 2003), therefore saving energy. Given the fewer but higher quality offspring of darker reddish individuals, it would pay off for them to invest more in nest defence, as each nestling represents a greater value to them than the lower quality nestlings of a lighter reddish individual do. The trend for higher nest predation in lighter reddish males is in line with our observations of less intense nest defence and suggest that colour might serve as a signal of the reproductive strategies of individual tawny owls. We find no assortative mating for colour in our population (similar to results found in a population in Finland, Brommer *et al.* 2005), which could mean that partners do not select a mate with necessarily similar or dissimilar nest defence or parental care strategies.

Aggression and melanin

Nest defence is a costly and risky behaviour, particularly in terms of survival and injuries (Wallin 1987), which is why the larger and heavier female might be more active in this part of parental care (Wiklund & Stigh 1983). However, it might allow dark reddish individuals to recruit more of their offspring into the population, by being bolder and more aggressive, since nest predation usually ends in all nestlings of a clutch being killed. The survival of nestlings in the first year is one of the most important traits for fitness (Francis & Saurola 2002) and this might be improved by increased nest defence of the parents, possibly at the cost of their own survival.

The more aggressive behaviour we observed from darker reddish individuals and of all individuals towards dark reddish individuals (in the form of the stuffed owls) could occur because they might also be perceived as more dangerous by breeding females. In the buzzard (*Buteo buteo*) different coloured individuals are more aggressive towards a lure that has the same colour (Boerner & Kruger 2009). In marsh hariers (*Circus aeruginosis*, Sternalski & Bretagnolle 2010) colour morphs react differently to predators. Colour might then signal such an agonistic behaviour, like the black badges in the siskin (*Carduelis spinus*, Senar & Camerino 1998), the house sparrow (*Passer domesticus*, Moller 1987) or Gouldian finch (*Erythruria gouldiae*, Pryke & Griffith 2009).

Coloration as signal of personality

Bold individuals habitually form routines quickly and can have a selective advantage in stable environments, whereas shy individuals are better able to adapt quickly to a changing environment (Reale *et al.* 2007). Such a relation between melanic coloration and proactivity/reactivity has already been demonstrated in other species such as the Herman's tortoise (*Testudo hermanni*, Mafli *et al.* 2011). There is also evidence for an association between melanin-based coloration and nest defence intensity in great tits (*Parus major*, Quesada & Senar 2007), male American Robins (*Turdus migratorius*, Row & Weatherhead 2011) and house sparrows (Klvanova *et al.* 2011).

The differences in individual behaviour we observed lead us to think that light reddish individuals are more flexible and reactive, whereas darker reddish individuals are more proactive and may form fixed routines, in line with the active/proactive or bold/shy behavioural types (Tuttle 2003), normally measured in traits such as aggression, risk-taking, fear, exploration and reaction to environmental changes (Sih *et al.* 2004). The Pace of Life Syndrome (Reale *et al.* 2010) could explain most of the trade-offs observed in our tawny owl population, where the inflexible, aggressive reddish individuals might choose different reproductive strategies than the flexible, less aggressive light reddish individuals. The direction and underlying mechanisms for relationships between physiology, behaviour and life history traits do not have to be similar in all species (Reale *et al.* 2010).

Melanocortin system as potential proximate explanation

For selection to act on the colour as signal of boldness, a genetic basis for both antipredator behaviour and plumage coloration need to be present, as well as genetic correlation between those two traits via pleiotropic genes or genetic linkages. Although evidence is accumulating that adult antipredator behaviour (Bize *et al.* 2012) and melanin-based coloration can be inherited (e.g. Bize *et al.* 2006; Gasparini *et al.* 2009a), we still know very little about the existence and nature of genetic correlation between those two traits. A candidate is the melanocortin system. In this system, pheomelanic red coloration is caused by the binding of a melanocortin antagonist (*i.e.* the Agouti signalling protein, ASIP) to the receptor responsible for skin pigmentation, which then switches from producing eumelanin to pheomelanin. In tawny owls, eumelanin and pheomelanin both play a role in reddish coloration (Gasparini *et al.* 2009a) and thus an increase in reddish coloration coincides with an

increase in eumelanin, which is associated with aggression (Ducrest *et al.* 2008). Thus, our observation of increased nest defence, a measure of aggression, is consistent with predictions.

When experimentally experiencing stressful (enlarged brood) conditions, light reddish tawny owls were able to reduce circulating levels of POMC (proopiomelanocortin, a part of the melanocortin system) prohormone in their blood, whereas dark reddish individuals have a more constant level (Roulin *et al.* 2011c). Given the effects the melanocortin system has on various physiological and behavioural traits (Ducrest *et al.* 2008) this might help explain the different levels of aggression found in differently coloured individuals. We could have expected light reddish individuals to have higher levels of aggression than dark reddish individuals in relaxed conditions, as the level of POMC in their blood were higher. We do not know how the level in the blood translates into a link with aggression and thus it is clear that much of the mechanisms of interplay between the melanocortin system and behaviour remain to be unravelled. There is however some evidence of a link between the melanocortin system and aggressive behaviour in deer mice (*Peromyscus maniculatus*) and rats (*Rattus norvegicus*) where the most pheomelanic individuals were found the most aggressive, the most difficult to handle and the most active (Hayssen 1997).

Although the precise mechanisms remain to be determined, the melanocortin system could thus be involved in both coloration and aggression, here measured in nest defence against predators and competitors. Future work needs to demonstrate if the melanocortin system is causing the relation between coloration and behavioural traits, as a proximate mechanism underlying the maintenance of polymorphism in tawny owls and perhaps, given the results in deer mice, other vertebrates.

SYNTHESIS AND CONCLUSION

SUMMARY OF MAIN RESULTS

The emergence and maintenance of variation in physical attributes, such as melanin-based coloration, is a long-standing debate (Darwin 1872; Wallace 1913; Galeotti *et al.* 2003; Roulin 2004; Bond 2007; Gray & McKinnon 2007). Whereas the evolution of carotenoid-based coloration seems obvious according to the handicap principle (Zahavi 1975) and the idea that these colour traits can honestly signal individual qualities in wild populations (Baker & Parker 1979; Hill 1990, 1991; Olson & Owens 1998; Faivre *et al.* 2003; Peters *et al.* 2004; Preault *et al.* 2005; Hill 2006), the adaptive significance of melanin-based coloration remains largely unsolved. Although the commonest pigmentation system used in animal kingdom (Majerus 1998), its colour patterns are under strong genetic control (Chapter 5; Mundy & Kelly 2003; Roulin & Dijkstra 2003; Bize *et al.* 2006; Hoekstra 2006) and weakly sensitive to environmental conditions (Chapter 3; Roulin *et al.* 1998; Gonzalez *et al.* 1999; McGraw & Hill 2000; McGraw *et al.* 2002). But the finding that melanin-based coloration covaries with morphological, physiological, behavioural or life history traits (Galeotti *et al.* 2003; Jawor & Breitwisch 2003; Roulin 2004) raised the idea that melanin-based colour patterns can signal alternative strategies and, by extensions, individual qualities. Recently, a review of genetic and pharmacological studies proposed that pleiotropic effects of the melanocortin system could account for the observed covariations between melanin-based traits and other important phenotypic traits (Ducrest *et al.* 2008). The melanocortin system regulated numerous physiological functions, such as melanin-based coloration, HPA axis and resistance to stressors, sexual behaviour and aggressiveness, energy balance, resistance to oxidative stress and immunity (Ducrest *et al.* 2008). As a consequence of the co-regulation of these traits by the same set of peptides, the expression of these traits according to a specific melanic colour trait is likely to be predictable. Dark melanic individuals are expected to be more resistant to stressful factors (i.e. inducing a glucocorticoid response) or to oxidative stress, have a reduced inflammatory responses and higher proliferation of B cells and regulatory T cells, better regulated the balance between food intake and energy expenditure and are sexually more active (Roulin & Ducrest 2011). In this context, experimental studies are needed to further test these colour-specific predictions, while laboratory studies need to investigate the proximate mechanisms underlying

these covariations (e.g. nucleotide diversity, patterns of gene expression, posttranslational modification; Hoekstra 2006; Mundy 2006; Hoekstra & Coyne 2007).

In my PhD thesis, I first experimentally tested whether the degree of tawny owl melanism was associated with life history or physiological strategies, which may confer fitness-related benefits, but also entail costs to differently coloured individuals in different environments. Accordingly, I performed brood size manipulation treatments to induce changes in the levels of parental workload and/or in the levels of sibling competition for prey items. In both cases, I modified the levels of stress experienced by the parents or the nestlings. In ***Chapter 1***, I specifically investigated whether tawny owl colour morphs have a different pace of life. Based on capture-recapture data collected during eight consecutive years, I showed that light melanic owls have lower survival compared to dark melanic conspecifics. This finding suggests that the former individuals have a slower pace of life than the latter. If this hypothesis holds, life-history theory states that individuals have to allocate optimally resources among life-history traits (MacArthur & Wilson 1967; Stearns 1992), a decision rule that can differ between individuals (Pianka 1970; Mcleod *et al.* 1981). Concordant with previously published results, I demonstrate that dark melanic males had a slow-pace of life, characterized by the production of few high quality offspring that were more often recruited in the local breeding population than offspring of light melanic males. In line with this hypothesis, I also showed, in ***Annexe 1***, that dark reddish individuals are bolder and more aggressive in terms of nest defence compared to light reddish conspecifics, potentially explaining why they suffered lower predation rate in our population. This result demonstrates that reddish parents invest more in defending few, presumably high quality offspring, rather than many, lower quality offspring.

Because oxidative stress is closely associated with individual pace of life, and specifically its reproductive strategy (Cohen *et al.* 2008; Monaghan *et al.* 2009; Metcalfe & Alonso-Alvarez 2010; Isaksson *et al.* 2011), I tested, in ***Chapter 2***, whether these colour-specific trade-off resolutions between offspring number and quality induce, in turn, alternative trade-off resolutions between the production of deleterious reactive oxygen species (ROS) and antioxidant defence mechanisms, such as glutathione (GSH), a molecule that potentially plays a role also in pheomelanogenesis. In agreement with previous studies, I revealed that the adaptation of light melanic tawny owls to stressful

reproductive conditions (i.e. brood enlargement) leads them to overproduce ROS, but which was compensated by larger levels of $_{t}$GSH. This outcome advocates for a larger consumption of GSH by light melanic tawny owls to counter the detrimental effects of their ROS overproduction. This hypothesis points out the central issue of GSH expression and expenditure. The global pool of GSH ($_{t}$GSH) being composed of two forms, namely $_{red}$GSH (amount of available antioxidants) and $_{ox}$GSH (amount of $_{t}$GSH recently used), the percentage of each fraction may indeed covary with melanin-based coloration. Accordingly, and because GSH can be used either as an antioxidant to counter by-products of metabolism (Reddy *et al.* 1982; Halliwell & Gutteridge 1989) or as a source of cysteine in pheomelanogenesis (Benedetto *et al.* 1981; Ozeki *et al.* 1997), I specifically focused, in ***Chapter 3***, on the relationships between GSH levels and melanin-based coloration in breeding adults (and their colour-specific reproductive strategy) and growing nestlings (that potentially need cysteine to produce pheomelanin pigments). Based on the idea of GSH dependence of pheomelanin-based colour traits, we expected a greater consumption of GSH (i.e. higher $_{ox}$GSH levels) in dark melanic nestlings. This was however not the case. In contrast, we found that in adults, which were not moulting and hence not in a state of intense melanisation, melanin-based coloration was negatively correlated with $_{t}$GSH and $_{red}$GSH levels, light melanic individuals showing higher $_{t}$GSH and $_{red}$GSH concentrations. Because of their energy-demanding life history strategies (***Chapter 1***) and their adaptation to stressful environmental and reproductive conditions (Roulin *et al.* 2003; 2004; 2008b; Piault *et al.* 2009; Emaresi *et al.* 2011), these individuals are more likely to face stronger metabolic constraints (e.g. ROS production in ***Chapter 2***), forcing them to develop a stronger antioxidant response (i.e. higher $_{t}$GSH and $_{red}$GSH concentrations).

In a proximate perspective, the second main objective of my PhD thesis was to infer the exact genetic mechanism leading to the covariations between melanin-based coloration and other important phenotypic traits. Based on the assumption that *POMC* gene plays a key role in the observed colour-specific reaction norms (Roulin *et al.* 2008b; Piault *et al.* 2009), I measured, in ***Chapter 4***, the levels of circulating POMC prohormone in breeding females rearing manipulated broods (i.e. different parental workload). Interestingly, I found that light melanic females decreased their level of plasma POMC prohormone when experiencing a higher level of stress (i.e. experimentally enlarged broods),

whereas dark melanic females produced POMC prohormone independently of the brood size manipulation treatment. One plausible scenario is that, under stressful conditions, high levels of ACTH are necessary, which imply stronger processing of plasma POMC prohormone (Myers *et al.* 2005), a constraint that can lead light melanic owls to reduce plasma POMC prohormone in such conditions. In this context, a thorough understanding of the mechanism responsible for variation in melanin-based coloration (and by extension covariations with other physiological traits) requires insights on genetic variation at both levels, i.e. nucleotide sequences and gene expression. To this end, the study of melanogenic gene expression in nestling tawny owls (for which tissue sampling is easier than in adults) was of particular interest (***Chapter 5***), especially genes related to POMC prohormone processing (i.e. genes encoded prohormone convertases PC1 and PC2). Although I could not detect nucleotide polymorphism in the coding sequence of nine candidate genes (*ASIP*, *MC1R*, *MITF*, *PC1-2*, *POMC*, *SLC7A11*, *TYR*, *TYRP1*), I demonstrated that *MC1R* and *PC1* patterns of expression in young nestlings (i.e. approximately 10 days of age) covaried with the mean colour score of their biological parents (a proxy of their own coloration). Because no colour-specific patterns of *POMC* gene expression were highlighted, the finding that *PC1* gene was more expressed in offspring born from light rather than dark melanic tawny owls suggests that owlets born from light melanic parents are likely to have a greater capacity of *POMC* processing to ACTH, a key regulator of the hypothalamic-pituitary-adrenal (HPA) axis, mainly involved in stress response. This result raises the hypothesis that regulation of *PC1* gene expression may be a potential proximate mechanism modulating the pleiotropic effects of melanocortins in the tawny owl, indirectly regulating important physiological functions such as foetal development, immune system, energy balance and sexual activity for instance.

CONCLUSION

In a general background, the main questioning of my PhD thesis was to understand why different colour morphs coexist in our local population of tawny owl. What are the main selective forces enabling the maintenance of these alternative colour variants? Similarly, what are the proximate mechanisms responsible for colour polymorphism in this species?

From an ultimate point of view, variation in genetically-inherited melanin-based coloration can be maintained under different non-mutually exclusive scenario (see also Box 3 in Introduction). First, under frequency-dependent selection, the adaptive value of a given phenotype depends on its frequency. Under negative frequency-dependent selection, a rare genotype is relatively favoured by selection and will increase in frequency. As it becomes more common, its fitness decreases and may be no longer favoured. At that point, the adaptive values of alternative genotypes are equal and natural selection enables them to stay at equilibrium, maintaining, in turn, genetic polymorphism in the population (Ridley 2004). Under apostatic selection for instance (Clarke 1962), individuals displaying a new coloration enjoy the advantage of being less rapidly detected by preys or predators compared to other colour morphs (Bond 2007). A classical example is the emergence and maintenance of the melanic colour morph in Moths *Biston betularia* (Grant 2004). Due to the industrial revolution in Britain during the last 150 years, smoke and soot produced by industrial factories caused a darkening of tree trunks in urban areas. While trunks were originally covered with light-coloured lichens, favouring initially (light-coloured) peppered moths rather than rare melanic ones, the darkening of trunks drastically increased the adaptive value of melanic moths, since they were less exposed to predation. Another well-known example is the maintenance of colour polymorphism in the pea aphid (*Acyrthosiphon pisum*; Losey *et al.* 1997). Green colour variant suffers from stronger parasite load than red colour variant, but which suffers from higher predation rate compared to green conspecifics. Therefore, colour morphs adopt alternative life-history strategies, both being maintained through balancing density and/or frequency selections. A second scenario is called ’heterosis’, ‘heterozygote advantage’, or ‘heterotic balancing selection’. In this case, individuals known or assumed to be heterozygote at a specific locus enjoy greater fitness benefits than those known or assumed to be homozygote at the same locus. Two examples are the maintenance of colour polymorphism in a wild

population of butterfly *Danaus chrysippus* (Smith 1980) or in the common buzzard *Buteo buteo* (Kruger & Lindstrom 2001). A third scenario is linked to heterogeneous and fluctuating habitats due to stochasticity in environmental conditions. Because natural selection often varies in space (i.e. heterogeneous habitats), such divergent selection enables genetically-inherited phenotypes (e.g. colour morphs) to interact with their environment. Genotype x environment (GxE) interactions lead local population (or deme) to evolve traits that provide fitness advantage under local environmental conditions (Kawecki & Ebert 2004). This local adaptation process can occur within a single population (Kassen 2002; Galeotti *et al.* 2003; Roulin 2004; Sgro & Hoffmann 2004; Byers 2005; Chunco *et al.* 2007). Since environmental conditions can also fluctuate over time, the GxE interactions developed by locally adapted phenotypes can be favoured at different period of time (e.g. seasonality). Indeed, models including age- or stage-specific selective regimes revealed that temporal fluctuations in environmental conditions can promote the maintenance of genetic variants (Ellner & Hairston 1994). When the strategy of one morph is favoured during a specific period of time, the strategy of the other morph still enables them to persist until a more favourable period. According to the observed covariations between melanin-based coloration and important behavioural, physiological or life history traits, which selective force can be involved in the maintenance of variation in melanin-based coloration in the tawny owl?

Although absence of evidence does not mean evidence of absence (*pers. comm.* Dr. Romain Piault), it appears that evolution and maintenance of alternative colour variants in our population does not necessarily involve direct selection on melanic colour traits, since we were not able to identify adaptive function of coloration itself in our population. Although this questioning was out of the scope of this PhD thesis, preliminary results reveal that colour morphs are randomly distributed across environmental variables (types of forest, vegetation covert, undergrowth density, altitude, latitude or temperatures; Master projects of Adrian Moriette and César Metzger, *unpublished results*), unlike other studies performed on larger and less heterogeneous study areas (Galeotti & Cesaris 1996; Karell *et al.* 2011b). These trends suggest that colour morphs do not derive specific advantages in terms of crypsis by exploiting territories that enable them to blend into the environmental background or conceal their shape (Endler 1988; Bond 2007). Moreover, the finding that variation in melanin-based

coloration was not associated with altitudinal or latitudinal gradients in our study area suggests that dark melanic individuals do not benefit from specific advantages in terms of thermoregulation. Altogether, these results also suggest that colour morphs are unlikely to be locally adapted in our study area. Nevertheless, and despite the fact that we did not find assortative mating in our local population, it is worth noting that melanin-based coloration can still play a direct signalling function in social interactions (Rohwer 1975; 1977; see also *Chapter 4*). Additional studies are thus needed to clearly demonstrate whether or not melanin-based coloration is directly under selective regimes.

In this context, the emergence and maintenance of colour variants in the tawny owl (at least in our population) is more likely to involve selective process on traits that are correlated to melanin-based coloration. Covariations between melanic coloration and aspects of individual quality due to pleiotropy suggest that colour morphs may undergo correlational selection (Sinervo & Svensson 2002). This force of selection favour particular combinations of traits expressed together, a process favouring in turn covariance due to linkage disequilibrium or pleiotropy (Lande 1980). In our highly heterogeneous landscape, characterized by important environmental stochasticity within and between years (e.g. food availability), selected suites of traits resulting in colour-specific strategies may be favoured at different periods of time. Light melanic individuals are selected to perform particularly well under stressful conditions, as suggested by their capacity to produce large number of fledgling when conditions permit (*Chapter 1*) or nestling ability to resist to food deprivation (Piault *et al.* 2009) or brood enlargement (Roulin *et al.* 2008b). This strategy is likely to induce some physiological constrains, such as ROS overproduction (*Chapter 2*), forcing them to produce larger antioxidant response (*Chapters 2 & 3*). In these circumstances, light melanic tawny owls need to be more flexible in their decisions, as suggested by limited offspring ability to gain body mass in rich environments (Roulin *et al.* 2008b; Piault *et al.* 2009), short-lasting immune response in adults (Gasparini *et al.* 2009a), lower investment in specific life history traits (e.g. skipping some reproductive events, producing fledglings of lower quality (*Chapter 1*) or reducing nest defence behaviour (*Annexe 1*)) or fluctuating levels of POMC prohormone according to the environment (*Chapter 4*). Conversely, dark reddish individuals are selected to perform particularly well under relaxed conditions, probably because they allocate resources equally among the different activities, such as immune response

(Gasparini *et al.* 2009a; Gasparini *et al.* 2009b), offspring ability to gain body mass in rich environments (Roulin *et al.* 2008b; Piault *et al.* 2009), life history traits (i.e. adult survival, production of few but high quality offspring (*Chapter 1*) or nest defence (*Annexe 1*)), GSH production and consumption (*Chapters 2 & 3*), or constant production of circulating POMC prohormone independently of changes in the environment (*Chapter 4*). Although speculative, one can temptingly define dark melanic owls as adopting a '*specialist*' strategy, whereas lighter coloured owls adopt a more '*generalist*' strategy (Wilson & Yoshimura 1994). *Specialist* morphs (i.e. dark reddish owls) are characterized by good performance under relaxed environmental conditions (e.g. reduced broods), precisely because these conditions enable them to invest optimally in the whole suites of behavioural, physiological, or life history traits. Conversely, stress-resistant *generalist* morphs (i.e. light reddish owl) adopt a versatile strategy that enables them to perform particularly well in harsher environmental conditions. These theoretical models (Wilson & Yoshimura 1994) emphasized that temporally varying environments such as seasonal fluctuations and stochastic variation are thought to favour *generalists*, since they are more flexible in their decisions. Nevertheless, the same models also demonstrated that both strategists can coexist by the adaptive switching behaviour (or strategy) of *generalists*, which can temporally reduces variation in habitat conditions experienced by *specialists*. For instance, the skipping behaviour of *generalist* (i.e. light melanic) tawny owls leads to lower competition for resources during poor breeding seasons. Thus, *specialist* (i.e. dark melanic) tawny owls are likely to face less stressful habitats during these breeding seasons, a situation that enables them to allocate resources equally among the different activities.

Mechanistically, my PhD thesis provides important insights in the central role of the melanocortin system in modulating covariations between melanin-based coloration and important phenotypic traits in the tawny owl. Indeed, the capacity of light melanic adult tawny owl to modulate the levels of circulating POMC prohormone (*Chapter 4*) and the finding that offspring born from light melanic parents expressed higher levels of prohormone convertase 1 (PC1; *Chapter 5*) supported the hypothesis that POMC gene expression, and its processing by prohormone convertases, are potential proximate mechanisms underlying the observed colour-specific reaction norms. Through the regulation of POMC prohormone and PC1, light melanic individuals can physiologically better cope

with stressful conditions since they are likely to produce higher levels of ACTH, a key component of the HPA axis and thus a central regulator of organism response to biological stress. As previously mentioned, *POMC* gene expression and the activity of prohormone convertases in species displaying variation in melanin-based coloration are promising starting points for further investigation into developmental basis of melanocortin-based covariations. Of particular interest, future studies need to address the central issue of POMC prohormone processing into α-, β-, γ-MSH and ACTH. A first step would be the measurement of *POMC*, *PC1* and *PC2* gene expressions in different tissues, to test whether patterns of expression are proportional across tissues. Although complex to measure, a particularly interesting step would be the assessment of melanocortin levels (especially α-MSH and ACTH peptides) in different tissues (i.e. organs vs. skin) of non-model species (i.e. wild populations), especially under different environmental or reproductive conditions, since different colour variants display alternative physiological or life history strategies. This approach would provide strong insights in the proximate mechanism underlying the adaptive function of melanin-based coloration. Nowadays, recent advances in the development of next generation sequencing techniques allow us to quickly identify regions of the genome associated with particular phenotypic traits. A genome wide association study (GWAS) could thus help us to identify QTLs involved in melanic colour patterns and behavioural, physiological or life history traits.

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