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PERSONALITY AND MELANIN-BASED COLOURATION TRAITS IN BARN OWLS, TAWNY OWLS AND KESTRELS

VAN DEN BRINK Valentijn

UNIL | Université de Lausanne
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PERSONALITY AND MELANIN-BASED COLOURATION TRAITS IN BARN OWLS, TAWNY OWLS AND KESTRELS

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Prof. Alexandre Reymond

Voor Anneke

Contents

Résumé

Afin d'assurer leur survie, les individus doivent être adaptés localement à leur environnement. Lorsque les pressions de sélection varient entre différents milieux, on peut observer l'apparition de polymorphismes entre les populations. Parmi eux, le polymorphisme de coloration est le plus remarquable car il se manifeste de façon évidente. Plusieurs pigments interviennent dans la composition des colorations , les plus communs chez les oiseaux étant les pigments mélaniques. Le système des mélanocortines synthétise ces pigments, mais exerce également une action pléiotropique sur de nombreux traits comportementaux et physiologiques. De par ses larges influences, il représente un bon candidat pour expliquer l'apparition et le maintien des polymorphismes de coloration.

Dans cette thèse, j'ai étudié trois espèces de rapaces qui présentent des colorations mélaniques : la Chouette effraie (*Tyto alba*), la Chouette hulotte (*Strix aluco*) et le Faucon crécerelle (*Falco tinnunculus*). La question principale concernait la relation entre la coloration mélanique et les variations individuelles du comportement, l'hypothèse sous-jacente étant que la couleur pourrait être un signal de certains traits adaptatifs. Mon but était ainsi de pouvoir expliquer le maintien de polymorphismes de coloration au niveau évolutif.

Nous avons montré que les poussins de Crécerelles et d'Effraies diffèrent dans leur comportement anti-prédateur, et ceci en fonction de leur coloration mélanique (**chapitres 1 et 2**). Les individus les plus sombres réagissent moins lors de la manipulation par les humains mais chez les Crécerelles, l'aggression et la coloration sont liées à l'inverse de l'Effraie. Les Chouettes effraies les plus rousses dispersent à de plus grandes distances avant leur première reproduction, et cette différence de comportement se retrouve entre parents et enfants du même sexe (**chapitre 3**). Les Chouette hulottes les plus rousses défendent leur nid plus férocement contre les intrus, et souffrent moins de la prédation de ce dernier **(chapitre 4**). Finalement, nous avons montré qu'un polymorphisme dans le gène du récepteur 1 pour la mélanocortine (MC1R), qui est fortement relié à la couleur rousse chez la Chouette effraie, est également lié à la distance de dispersion, ce qui offre une première indication sur les bases génétiques de la relation entre le comportement et la coloration (**chapitre 5**).

Nos résultats démontrent qu'il existe un lien entre la coloration mélanique et les traits de personnalité chez les animaux. Nous avons observé ce lien chez trois espèces différentes, ce qui suggère qu'il provient très vraisemblablement du même mécanisme sous-jacent, qui prend dès lors une portée générale. Différentes pressions de prédation sont responsables de différentes réactions,

et pourraient également expliquer les différences de coloration entre les sexes, des colorations de type mâle ou femelle pouvant signaler des comportements plus ou moins agressifs. Des conditions environnementales changeantes peuvent résulter en un succès reproducteur similaire de différentes stratégies comportementales. Le système des mélanocortines pourrait être le mécanisme sous-jacent à ce phénomène, comme suggéré par les résultats similaires trouvés chez trois espèces dans la présente étude, des relations similaires reportées également chez d'autres espèces, et la relation entre le polymorphisme génétique trouvé dans ce système et le comportement chez la Chouette effraie.

Ma thèse démontre que la coloration est liée aux différences individuelles de comportement et propose pour la première fois un mécanisme sous-jacent à ces relations. Il est dès lors possible d'effectuer une recherche dirigée afin d'étudier plus en profondeur ce mécanisme, et d'en évaluer l'impact au niveau évolutif, par exemple grâce à l'utilisation de techniques d'analyses génétiques quantitatives.

Summary

Individuals need to adapt to their local environment in order to survive. When selection pressures differ in local populations, polymorphism can evolve. Colour polymorphism is one of the most obvious polymorphisms since it is readily observable. Different sources of colouration exist, but melanin-based colouration is one of the most common in birds. The melanocortin system produces this colouration and because the melanocortin system has pleiotropic effects on behavioural and physiological traits, it is a good candidate to be an underlying mechanism to explain the maintenance of colour polymorphism.

In this thesis I studied three different raptors which all display melanin-based colouration; barn owls (*Tyto alba*), tawny owls (*Strix aluco)* and Eurasian kestrels (*Falco tinnunculus*). The main question was if there was a relationship between melanin-based colouration and individual behavioural differences. The underlying hypothesis is that colour could be a signal of certain adaptive traits. Our goal was to find evolutionary explanations for the persistence of colour polymorphism.

I found that nestling kestrels and barn owls differ in anti-predatory behaviour, with respect to their melanic colouration (**chapters 1 and 2**). Darker individuals show less reaction to human handling, but in kestrels aggression and colouration are related in opposite ways than in barn owls. More reddish barn owls travel greater distances in natal dispersal and this behaviour is repeatable between parents and same sex offspring (**chapter 3**). Dark reddish tawny owls defend their nests more intensely against intruders and appear to suffer less from nest predation (**chapter 4**). Finally I show that polymorphism in the Melanocortin 1 receptor gene (*MC1R),* which is strongly correlated with reddish colouration in the barn owl, is related to natal dispersal distance, providing a first indication for a genetic basis of the relation between this behaviour and colouration (**chapter 5**).

My results demonstrate a clear link between melanin-based colouration and animal personality traits. I demonstrated this relation in three different species, which shows there is most likely a general underlying mechanism responsible. Different predation pressures might have shaped the reactions to predation, but also differences in sex-related colouration. Male-like and female-like colouration might signal more or less aggressive behaviour. Fluctuating environmental conditions might cause different individual strategies to produce equal reproductive success. The melanocortin system with its pleiotropic effects might be an underlying mechanism, as suggested by the results from the genetic polymorphism, the similar results found in these three species and by the similar relations reported in other species.

This thesis demonstrates that colouration and individual differences are correlated and it provides the first glimpse of an underlying system. We can now conduct a more directed search for underlying mechanisms and evolutionary explanations with the use of quantitative genetic methods.

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Much has happened in between. Many good moments, both at work and in my private life, but also some very bad ones. When my mother got ill at the beginning of 2010 and passed away later that year, I seriously doubted my decision to come to Lausanne. I thought about quitting for a long time, but I finally decided that I owed it to her to continue and finish it. My mother is no longer here to read my thesis, but I hope she would be proud of what I achieved.

Papa, ik heb altijd alle steun van jou en **Mama** gekregen om te doen waar ik goed in ben en jij en Mama hebben me geholpen om te komen waar ik nu ben. Ik hoop dat Mama trots op mij zou zijn nu ik na alle voor- en tegenspoed toch dit proefschrift heb gemaakt. **Muriel**, ik heb ook aan jou veel steun gehad, en jij hebt me ook overtuigd om toch door te gaan en het af te maken. Ik kan niet genoeg benadrukken hoe belangrijk jouw steun ook voor mij geweest is.

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Shared misery is half misery, is a Dutch saying, and certainly for a bunch of foreigners in Lausanne doing their PhDs that is true. Whenever I needed to complain about bureaucracy, some misunderstanding in the French language or just to have a good time, the "outcasts" were always there. **Fardo, Juan, Fabrice, Dumas, Emma, Olivier, Ricardo, Mikko, Ivan** and **Jose**. Thanks for many good times and all the summer evenings at the lake during the barbeque season.

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Introduction

The development of polymorphism

In a fluctuating environment individuals need to adapt to the local conditions they face. Small differences between these local habitats can exert differential selection pressures, leading to a shift in certain traits, which in turn might eventually lead to speciation if reproductive isolation develops between different morphs. These traits can also signal other traits that help survival in different habitats. Given enough time, we would expect selection to eliminate the morph with the lowest fitness from the population. One of the paradoxes of evolutionary biology is that high levels of genetic variation can persist in populations, despite the eroding effects of natural selection and genetic drift (Lewontin 1974). If individuals in a population begin to differ from each other, a polymorphism in such a trait can develop. Huxley (1955) defined the term he named *morphism* as follows: *"…in which (usually sharply distinct) genetic variants or morphs coexist in temporary or permanent balance within a single interbreeding population in a single spatial region, and in such frequencies that the rarer cannot be due solely to mutation, or to the spread of selectively neutral mutants*". One of the ways in which such a polymorphism might be evolutionary stable is that the selected traits have equal fitness over the long term, if one morph would have the advantage in certain conditions but a disadvantage in other conditions (Fisher 1930, Falconer and McKay 1996).

Maintenance of polymorphism

Several evolutionary mechanisms capable of maintaining high levels of polymorphism (Charlesworth 1987, Barton and Turelli 1989) have been demonstrated theoretically. A first possibility for a polymorphism to persist is heterozygote advantage. The advantage for the heterozygote needs only to be very small, for this to be maintained, with the homozygotes still being produced, by mating of heterozygous individuals (Falconer and McKay 1996). Secondly, there can be frequency dependent selection operating, where a rare morph has an advantage over the more common morph. For

instance in predator-prey interactions, it might not pay off for predators to specialize on the rare morph of prey, or because the rare morph has an advantage in for example foraging or mate choice. In an experiment where Blue jays (*Cyanocitta cristata)* were trained to search for moths on a computer screen, these virtual moths developed cryptic forms, which were more difficult to detect by the jays and consequently had higher reproductive success than moths that were detected (Bond and Kamil 2002). When a rare morph increases in frequency, though, this might let the advantage disappear again (Clarke 1979). The third option is a changing environment in time or geography. When individuals live in a heterogeneous environment, it might pay off to specialize to some extent to this specific type of habitat. A continuous distribution of more or less adapted individuals could develop under these conditions. If the environment changes over time, for instance if harsh and mild winters occur in the same area, different morphs might have better physiological or morphological adaptations, so that one year one morph has an advantage, and the next year another.

Two other possibilities for maintenance are less likely explanations. It can be that the observed polymorphism is in fact not stable, but represents a transition from one allele to another. No more than a small proportion of polymorphisms, however, are likely to be the result of this phenomenon (Fisher 1930, Falconer and McKay 1996). The last option is that it is the result of a neutral mutation, i.e. selection does not act on the polymorphism. It is difficult to make clear inferences about the occurrence and often it will not be possible to distinguish between very small effects of selection and persisting neutral mutations.

Adaptive functions of polymorphism

Let us assume a polymorphism has evolved. Selection could then act either directly on the polymorphic trait, or on another trait with which the polymorphic trait is in linkage disequilibrium. In the latter case, the polymorphic trait can be used as a signal to conspecifics. Whatever the polymorphic trait signals, though, the receiver must be able to receive and interpret the signal

correctly. In addition, the signal needs to be honest. Sexual selection on physical traits can be an honest signal of quality if there are costs in displaying the signal (Zahavi 1975, 1977). An example for direct selection is found in the study species of choice for geneticists, *Drosophila melanogaster.* It displays polymorphism in the Alcohol dehydrogenase enzyme (*Adh*), the enzyme necessary to digest alcohol. In 1974, a study showed that in media where ethanol was added, the F-allele increased in the population. This demonstrated that these individuals had a selective advantage in being able to degrade alcohol and thus not suffer the negative effects (Bijlsma and Van Delden 1974). Another example of direct selection occurs when the polymorphism represents different mating strategies, which are associated with behavioural, physical and colouration differences in male ruffs (*Philomachus pugnax;* van Rhijn 1973, Lank et al. 1995).

More common than direct selection on the polymorphic trait is indirect selection, where the polymorphic trait is linked to another trait and signals different abilities or quality in life history, physiological or behavioural traits such as offspring production, parental care, parental investment, age at first breeding, diet or energy metabolism, physiology or morphology.

Signals can take many different forms. One such form can be auditory signals, such as the courtship song of male crickets (*Teleogryllus oceanicus*), which changes in structure after an immune challenge. Females prefer the songs of males that can mount a high immune challenge (Tregenza et al. 2006). Scent can also play a role in selection for variation in immune system, such as in the different major histocompatibility complex (MHC) genotypes in species as diverse as mice (Singer et al. 1997), sticklebacks (Stearns 1992) and humans (Wedekind et al. 1995).

Colour polymorphism

Colour is an obvious candidate for a signalling function of a polymorphism. Colour polymorphisms are among the most easily observed and therefore widely studied polymorphisms. It has been evident that many colour polymorphisms covary with other traits (Sinervo and Svensson 2002, Roulin

2004b) and that many of these covariations are the result of genetic correlations (see McKinnon and Pierotti (2010) for a review). They may be the result of linkage disequilibrium (non-random association of alleles at two or more loci) or pleiotropy (influence of a single gene on more than one character) (Falconer and McKay 1996). Much is known about genes involved in pigmentation and interactions with other traits, mostly due to research done in laboratory mice (Silvers 1979) for vertebrates, or fruit flies (*Drosophila melanogaster*) for invertebrates. Melanin-based pigmentation is highly conserved in vertebrates and therefore the principles of the system of coat colour genes in mice can easily be translated to other taxa (see Hoekstra (2006) for a review).

Sources for colouration

There are a number of compounds that cause colouration of feathers, skin, hairs or other tissues in vertebrates. A few of the more uncommon ones are the porphyrins, psytacofulvins and pterins (for an overview of the structure and functions of these compounds see McGraw in: Bird colouration (2006)) and also structural differences in feathers, hairs or skin can cause iridisation, reflection and other kinds of colouration (Hill and McGraw 2006). The two main sources of colouration in birds are the carotenoids and melanins.

Carotenoids

Carotenoids usually produce red, orange or yellow hue (McGraw 2006a). Animals do not possess the enzymes needed to produce carotenoids and are therefore relying completely on their diet for the acquisition of carotenoids. They can, however, metabolize them into different forms (Brush 1990). These metabolic changes, along with competition over the source of carotenoids and efficiency in assimilation are thought to be part of the signalling, information content and evolutionary history of carotenoid based colouration. Carotenoids have often been found to be involved in increased

immune capacity and oxidative stress resistance (Bendich and Olson 1989, Blount 2004), which makes it an obvious candidate for sexually selected signals of individual quality (McGraw 2006a).

Melanin

For my thesis I will focus on the most common form of colouration in birds, melanin-based colouration. This type of colouration consists of melanin deposited in feathers and causes dark brown to black colouration in the form of eumelanin and yellow to reddish brown colouration in the form of pheomelanin (Wakamatsu and Ito 2002). The difference with carotenoid colouration is that melanin is produced endogenously by melanocytes, which can switch on and off during production of feathers or hair, allowing the production of precise patterns thought to be involved in crypsis (McGraw 2006b). It would be a misconception to think that the endogenous production of melanic colouration renders it free of costs. There are several factors that are important in the production of melanin and all of these can influence the costs and thereby the function of melanin colouration as a signal of individual quality (McGraw 2006b). Deficiency of amino acid precursors can result in decreased display of melanin based colouration (e.g.: Grau et al. 1989, Yu et al. 2001). Deposits of minerals may help or hinder melanin deposition. For instance, Calcium concentration in feathers of barn owls (*Tyto alba*) was correlated with the amount of melanin based colouration (Niecke et al. 2003). Hormones might also influence the deposit of melanin. Androgens, such as Testosterone (T) are involved in the development of black melanic colour based patches on house sparrows (*Passer domesticus*), both experimentally (Evans et al. 2000) and in natural populations (Gonzalez et al 2001). Injection of corticosterone in nestling barn owls (*Tyto alba*) hindered the development of melanin based colouration during the production of feathers, resulting in a grey area in an otherwise black band on the tail (unpublished data). Finally, deposits of melanin can also have a condition-dependent component (McGraw et al. 2002) and also in kestrels the width of the black tail band (a melanin-based trait; Van den Brink et al. 2012b) can depend on the rank in the nest, which is a measure of individual condition (Piault et al. 2012).

Polymorphism in melanin-based colouration

Polymorphism can occur in melanin-based colouration and this has already been studied for many years. Charles Darwin already studied melanic plumage forms in domestic pigeons (*Columba livia;* Darwin 1859). Another famous example is the polymorphism of industrial melanism in the peppermoth (*Biston betularia*). Following the spread of industrial pollution from the industrial revolution in the 19th century in England, a dark melanistic morph of the moth began to spread throughout the country, together with the deposit of soot from factories on trees. A classical selection experiment showed that each morph had better survival from predation in specific habitats, i.e. the dark morph in dark background and the light morph in a lighter background (Clarke and Sheppard 1966). Such colour polymorphisms often occur in a heterogeneous environment, either spatial or temporal (Hedrick et al. 1976, Hedrick 1986). What maintains such polymorphisms is as yet unknown but it seems that different factors can contribute in different species of birds (Mundy in: Hill and McGraw (2006), also see Lank (2002)). Models have shown that sexual selection alone can be enough to maintain colour polymorphism (Chunco et al. 2007). Correlational selection, where selection acts on one or a suite of traits, which are correlated to colouration is another way that would allow polymorphism to persist (Gray and McKinnon 2006). This requires genetic control of the polymorphism, either directly or pleiotropically, where many genes can influence the expression of the polymorphism.

Pleiotropy in the melanocortin system

Many species display covariation of melanic colouration and physiological, morphological and behavioural traits (Roulin 2004b). In Box 1 I describe the components of the melanocortin system, its mechanisms and some (possible) functions in more detail. Ducrest et al. (2008) proposed that pleiotropic effects of the melanocortin system involved in melanic colouration might be responsible

for these covariations. The melanin-based colouration found in many vertebrates is the result of products of this system, under control of the proopiomelanocortin gene (*pomc*), with several agonistic and protagonistic products that bind to various receptors. The main hypothesis is that the melanocortin system should produce predictable relationships between eumelanic or pheomelanic colouration and the traits under influence of one of the other Melanocortin receptors (MC1R-MC5R; Ducrest et al. 2008). If this link works as proposed, then the amount of melanin-based colouration an individual displays should be an indicator of the traits mentioned earlier. Some examples from barn owls suggest the system works as proposed, thus darker melanic individuals, as a result of activation of MC1R, should be more sexually active, exhibit a lower immune response, are more resistant to oxidative stress, mount a lower corticoid response, and have better energy homeostasis (Roulin and Ducrest 2011). Different strategies might also involve different behaviour, such as boldness, migration tendency. Therefore it would be of interest to investigate if a link between colour polymorphism and behavioural traits exists.

Individual behavioural traits or personalities

Correlations between different behavioural types are commonly found. For instance individuals that are more aggressive towards others are often found to explore faster (Huntingford 1976, Verbeek et al. 1996, Bell 2005, but see Bell et al. 2010). If such behavioural differences are consistent for each individual through time and across different situations, they could be termed *behavioural syndromes* (Sih et al. 2004b).The notion of different individual animal behaviours or personalities came from the field of psychology and was developed further in the last decade since some early attempts (Gosling 2001, Dall et al. 2004). A detailed history of the field, terminology used and the most important current developments are presented in Box 2.

Box 1. Structure, mechanisms, and functions of the melanocortin system

The melanocortin system is present in most vertebrates and it is involved in many neurological, physiological and behavioural traits alongside of the production of eumelanin and pheomelanin, causing colouration of skin, feathers or hair (Gantz and Fong 2003, Cone 2005).

Structure and mechanism of pigmentation

The basis of the system is formed by products of the *proopiomelanocortin* (*pomc*) gene; the melanocortin peptides α-, β-, and γ-melanocyte stimulating hormones (MSH) and adrenocorticotropic hormone (ACTH). These products in turn can bind to five transmembrane melanocortin receptors (MC1R-MC5R) in competition with antagonists agouti and agouti related protein (ARP; Gantz and Fong 2003). A host of knowledge about the functions of the mammalian melanocortin system was derived from studies with mice (see Hoekstra (2006) for a review). The avian melanocortin system differs from this only in details and is functionally equivalent to the mammalian melanocortin system (Boswell and Takeuchi 2005).

MC1R colour production

The main function which the melanocortin system is known for is colouration of skin, hair or feathers. MC1R is involved in melanic colouration in skin, feathers, hair and other tissues. It also plays a role in inflammation and nociception in other tissues (Ducrest et al. 2008). Expression of MC1R genes takes place mostly in tissues close to exterior of the individual. Binding of α-, β-MSH or ACTH to the MC1R receptor induces pigmentation. The final production of melanin (melanogenesis) takes place in the melanocytes, specialised cells located in the epidermis (Lucas and Stettenheim 1972). Melanogenesis (Figure 1.1) starts with the main catalyst tyrosinase, which causes an oxidation step that forms dopaquinone. Dopaquinone acts as an intermediate to form either reddish brown pheomelanin or black brown eumelanin. For the production of pheomelanin, thiols such as glutathione or cysteine are involved. Activation of the MC1R receptor by A-MSH leads to eumelanin production and activation by Agouti signalling protein (ASIP) leads to a switch of the production to pheomelanin (Wakamatsu and Ito 2002, Gantz and Fong 2003). In most mammals melanic colouration consists of a mixture of both eumelanin and pheomelanin and this means that the phenotypically

observed colour depends on the relative proportions of both melanins in hair, skin or feathers (Ozeki et al. 1997). Also in birds it has been shown that, for instance in tawny owls, the observed brown-reddish colouration consists of a mixture of approximately two-thirds pheomelanin and one third eumelanin and other pigments (Gasparini et al. 2009).

Mutations in the MC1R gene can lead to loss of function or gain of function (Robbins et al. 1993, Gantz and Fong 2003). An example in humans is the loss-of function mutation leading to red hear colouration (Valverde et al. 1995). Genetic variation in the MC1R gene suggests that mutations in MC1R are responsible for different phenotypes in many birds, similar to those found in mammals (Boswell and Takeuchi 2005).

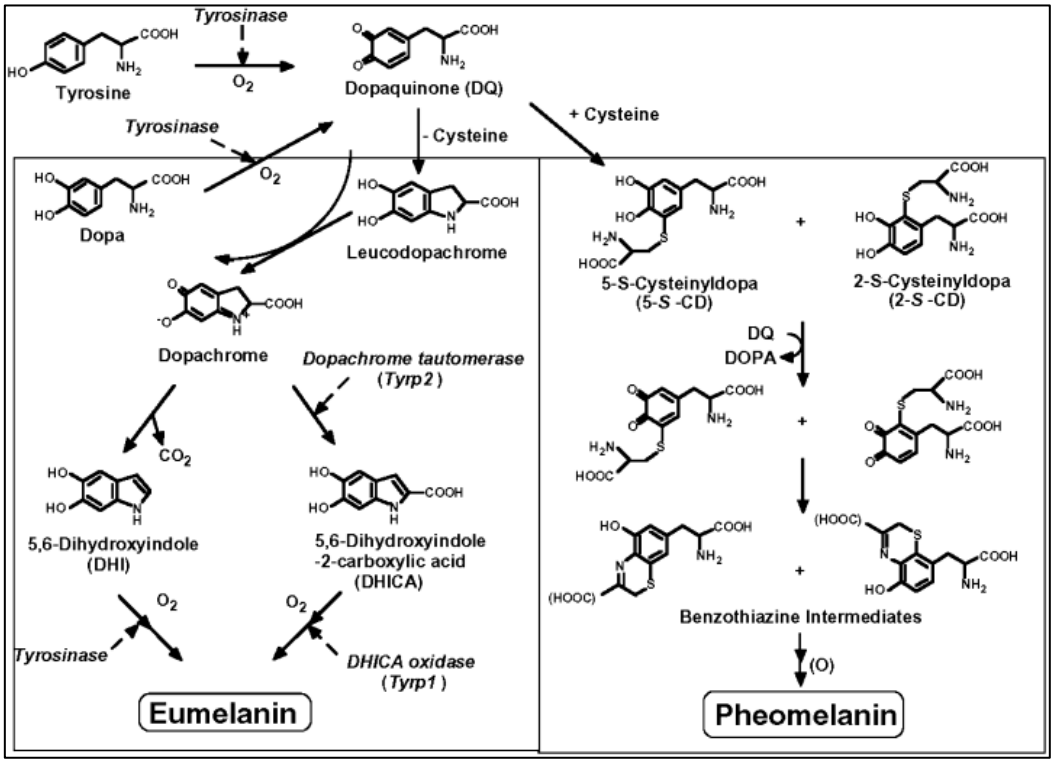


Figure 1.1 Melanogenesis pathway. Reprinted with permission from Wakamatsu and Ito 2002.

Other functions

Expression of the *POMC* gene takes place primarily in the central nervous system (CNS) and in melanocytes. Other areas of expression are adrenal, thyroid and immune system (Gantz and Fong 2003). The main physiological functions of the melanocortin system are in these tissues and influence energy homeostasis, sexual activity, aggression and immune function (Gantz and Fong 2003, Ducrest et al. 2008). A review of the results of manipulations of key components of the melanocortin system on these traits was made by Ducrest et al (2008). Depending on which product binds to the various receptors, positive or negative influences on the associated traits

can be exerted. A schematic overview of some of the most important functions of the melanocortin system and the pleiotropic effects of the melanocortins are presented in figure 1.2.

The pleiotropic effects the melanocortin system exhibits make it a good candidate to study correlated characters, because it could produce predictable relations between traits as diverse as obesity, diabetes, stress response, aggression, sexual activity, skin, hair and feather pigmentation and energy homeostasis (Ducrest et al. 2008)

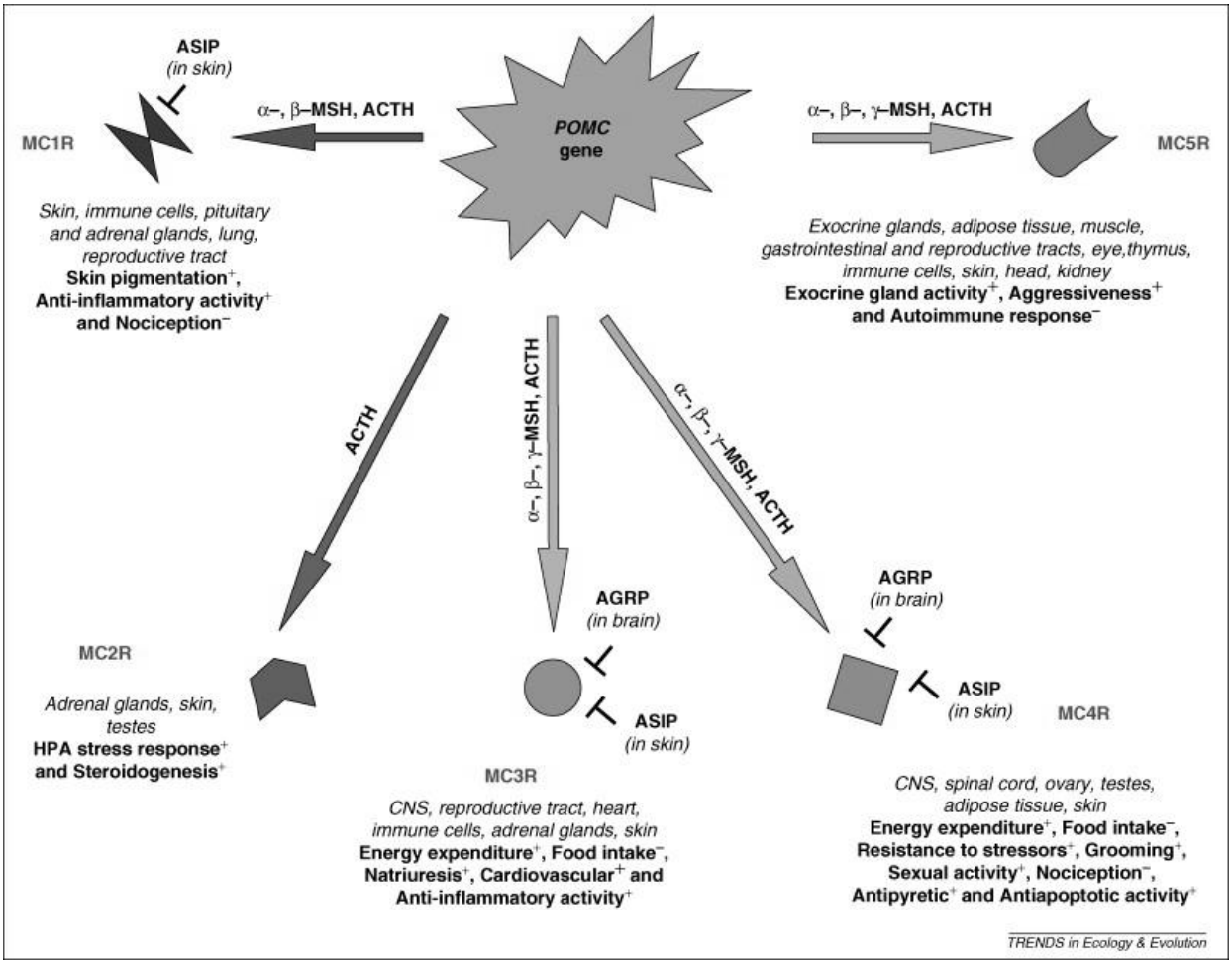


Figure 1.2 Overview of the functions of the melanocortin system. Products from the POMC gene bind to the different receptors which are expressed in the tissues indicated next to each receptor. The effects on the organism are indicated in bold. An increase in function is indicated with a + sign, a decrease with a – sign. Reprinted with permission from Ducrest et al. 2008.

Box 2 Animal personality

Evolution of animal personality research

Biology has seen a gradual shift of focus from description of differences between genera and higher level taxa to species, populations and, since the end of the 1990s, to individuals within populations (Dall et al. 2004). Animal personality has been recognised and studied for many years, but the terminology was mixed and studies were often only interested in differences in one trait. By the end of the 1990s interest of behavioural ecologists began to grow, in part due to some initial attempts to incorporate individual variation in animals, (e.g. the shyness and boldness continuum (Wilson et al. 1994) and correlations between groups of behaviour (Wilson et al. 1998). It has enjoyed a growing interest ever since, until the number of articles about animal personality or one of its synonyms is booming in the present day.

The recognition that personality traits in animals were analogous to human personality traits began in the early 1970s (Huntingford 1976) and has increased since the 1990s (Wilson 1998, Gosling 2001). Individual differences were often treated as (unwanted) variance around population or species means until then (Sih et al. 2004b). Currently the field is growing every year as demonstrated by the increase in number of papers published and has established itself as a fully developed research area within behavioural and evolutionary ecology.

At the beginning of this century first attempts were made to formalise terms and place animal personality in a broader framework and compare it with research on human personality types (Gosling and John 1999, Gosling 2001, Dall et al. 2004, Sih et al. 2004a, Sih et al. 2004b), that helped create more clarity in the myriad of terms and definitions used until then and helped steer the field into more "mainstream" research. By 2007, Réale et al (Reale et al. 2007) finalised the process of capturing the different fields of research in five major axes of personality research (Table 2.1 shows the 5 personality axes and a short description of each). Since then, models (Wolf et al. 2007), new methods of analyses (e.g.: Dochtermann and Roff 2010) and theories about reaction norms, genotype by environment interactions and comparisons between studies (e.g.: Dingemanse et al. 2010) have been formed. Al this contributes to the development to the field.

Currently we are beginning to get a first glimpse of underlying mechanisms, although much remains unclear and new hypotheses of how differences might evolve are frequently proposed (Biro and Stamps 2008, Reale et al. 2010b, Schuett et al. 2010, Stamps and Groothuis 2010, Reddon 2012).

Table 2.1 The five major behavioural "axes" as proposed by Reale et al. (2010). Main ecological behavioural types involved and names of the axes are presented.

Ecological behaviour	Personality Axis
risk taking tameness	**boldness** *docility*
novel habitat novel object new type of food	**exploration**
general activity level	**activity**
social behaviour	**sociability**
aggression competition	**aggressiveness**

Concepts, definitions and terminology

The field of animal personality is teeming with definitions, terminology and methodological questions. In the following section I will deal with some of the most important. Since the field developed, definitions of exactly what it was that researchers were studying were very diverse. From *temperament*, *personality*, *behavioural syndromes* to *coping styles*, with varying definitions of the same terms (Gosling and John 1999, Koolhaas et al. 1999, Sih et al. 2004b, Bell 2007, Reale et al. 2007). Currently most researchers use more or less the same definition. "Consistent individual differences across time and situations" describes what (Sih et al. 2004b) call the "behavioural syndrome". This means that if we were to observe the same individual in different situations and measure for instance activity, this individual should always tend to be more active, in different situations, but also if we measure the same individual more than once in the same situation, it should be more active than others in these instances. Consistency in behaviour is usually measured using so-called repeatability estimates. However, to take into account individual flexibility in reactions when for instance learning or habituation to a stimulus play a role, so-called reaction-norm approaches can be used (Reale et al. 2007). Here it is not specially the responses between consecutive measurements but rather the slopes between them that are compared between individuals.

Behavioural "axes"

Animal personality has borrowed five major "axes" of behaviour from human psychology (Gosling and John 1999) to formalise the studied traits and to allow more comparisons. The five major axes are **Boldness** (risk-taking), **Exploration** (new situations, without danger)**, Activity** (more active or less active)**, Sociability** (social interactions with conspecifics) and **Aggression** (competitive interactions) (Reale et al. 2007). The underlying idea is that behaviour from different ecological situations can be placed in an axis, and that these axes and the specific tests used to determine individual differences do not overlap with each other, so that comparisons can be made more easily across studies, species and situations (Reale et al. 2007). In the past, one researcher might measure aggression in a specific situation, and compare it to another researcher's results who had studied it in a slightly different situation. Currently some consensus has been reached,

and comparisons between personality traits can be made, also in a formalised way with meta-analyses (e.g.: Smith and Blumstein 2008, Bell et al. 2009).

Underlying mechanisms

A plethora of papers describing animal personalities in some or other trait exist, but it is essential for the advancement of the field to put the observation sin an evolutionary framework (Bell 2007, Reale et al. 2007). Currently interest lies in underlying proximate mechanisms of individual differences (Bell et al 2007). The problem is that either very high plasticity or very restrained plasticity in behaviour is assumed. Behavioural ecologists need to find the middle-ground between these two extreme viewpoints if they are to adequately the inter-individual and the intra-individual variation that are characteristic of animal personalities (Reale et al. 2010a). Research is focusing on different mechanisms such as neurobiochemical pathways (Coppens et al. 2010), developmental processes (Stamps and Groothuis 2010), and events early in life with carry-over effects (Sih 2011) or metabolism (Careau et al. 2008).

Future challenges

The number of studies reporting one or more animal personality traits, correlated to other traits or not, is increasing rapidly. It appears that individual differences in populations of animals are widespread and it also appears that this phenomenon has been underestimated or understudied for a long period. The current boom in publications can be the result of an increased awareness under researchers. The future challenges are to find mechanisms behind the behaviour that help explain the persistence of individual behavioural differences in populations. Theoretical models have been proposed (Dingemanse and Wolf 2010, Wolf and Weissing 2010) and now confirmation from field studies is needed. A multidisciplinary approach seems called for where the costs and benefits of different behaviour in different situations can be weighed against each other. Aggression in confrontations with competitors or predators can be beneficial, but at the same time it can have negative consequences in social interactions (Sloan Wilson et al. 1994). Evolutionary processes, such as predator pressure and density dependent foraging might help form the correlations between the different behaviours, in such a way that over the life span of an individual, fitness is maximised by exhibiting a certain type of behaviour.

Need for investigating underlying mechanisms

Personality seems universal in many taxa, vertebrate and invertebrate alike. I performed a search for publications with "animal personalit*" as keywords on the ISI Web of Knowledge® in January 2012 and found that between 2000 and 2011 the number increased from 1 to 72 articles published per year. This large increase, especially in the last 5 years, shows the rise in interest in the phenomenon. The underlying proximate mechanisms are however still very poorly understood. Various hypotheses have been proposed and range from mostly environmental control (Sloan Wilson et al. 1994), hormonal influences during development (Stamps and Groothuis 2010) or even parental effects (Reddon 2012). All this illustrates the need to investigate potential mechanisms to better understand personality. The relationship between different components of personality are often under strong genetic control (van Oers et al. 2004), which makes it worth investigating the system behind these correlations, to better understand the maintenance and evolutionary implications of these relationships.

Ducrest et al (2008) proposed a link between melanin based colouration, physiological and behavioural traits, possibly through pleiotropic effects of the melanocortin system. This system is strongly conserved in most vertebrates and it is involved in many behavioural, physiological and morphological characters, making it an excellent candidate to study whether behavioural differences can explain variation in melanic colour polymorphism.

Melanic colouration in relation to individual behaviour

To study if such a link exists, a species with a melanin-based colour polymorphism should be used. In my thesis I studied three long-lived species which all display pheomelanin and eumelanin based plumage colour polymorphism, the barn owl (*Tyto alba*), the tawny owl *(Strix aluco*) and the Eurasian kestrel *(Falco tinnunculus*). A short description of the biology of each of these species is presented in box 3. There are already several studies indicating that physiological traits are related to eumelanin- or pheomelanin-based colouration in a predictable manner. If we can demonstrate a relationship

between such melanic colouration and behavioural (personality) traits we could gain a better understanding of the pleiotropic effects of the melanocortin system. By using three different species, we can demonstrate a possible general nature of these relationships.

Box 3 –Study species

1. The barn owl (*Tyto alba*)

Appearance and colouration

The barn owl is a medium-size owl with a mean body mass of around 295 g ± 26 SD for males and 367 g ± 37 SD for females (females can increase up to 25% in mass shortly before egg laying) and a wing length of around 296 mm ± 6.4 SD for both females and males (Unpublished data from our study area). Females are slightly larger and heavier than males (Glutz von Blotzheim 1987).

The barn owl varies in colouration from a white immaculate ventral side to reddish and heavily covered with black spots (figure 3.5A and B). The dorsal and upper part of the wings is reddish and grey with spots. Barn owls display colour polymorphism from immaculate white to heavily spotted and red in a gradient across Europe (Antoniazza et al. 2010). In our study area in Western Switzerland all morphs occur together, with some sexual colour dimorphism. Females are slightly darker and more spotted than males. The size of the black spots varies between males and females, where females have larger spots than males (Roulin 1999a). Even though there is only moderate difference between male and female plumage, an experiment (Roulin 1999b) showed that males prefer females with larger black spots. Another study showed that selection acts against males with large spots and females with small spots (Roulin et al. 2010).

The barn owl (*Tyto alba*) displays a melanin-based plumage polymorphism, where the number and size of black spots increase with increased eumelanin deposit. The colouration is under strong genetic control, both eumelanin (h^2 = 0.82, Roulin et al. 2010) and pheomelanin (h^2=0.81, Roulin and Dijkstra 2003) colouration. The melanin-based traits are correlated with numerous traits such as parasite resistance (Roulin et al. 2001b), or stress response (Almasi et al. 2010).

Population and range

The barn owl is one of the most widely spread owls in the world. It occurs in all continents with exception from the boreal and arctic areas (Glutz von Blotzheim 1987, Figure 3.1). The worldwide population consists of 5 million individuals. The population trend is stable, but there can be large fluctuations in numbers of breeding pairs from year to year, which can be

explained by the snow cover in the preceding winter (Altwegg et al. 2003). The overall population trend in Europe is moderately declining with a population of 110 000 – 220 000 breeding pairs, while the population in Switzerland is stable and consists of 1000-1500 breeding pairs (Birdlife International 2012).

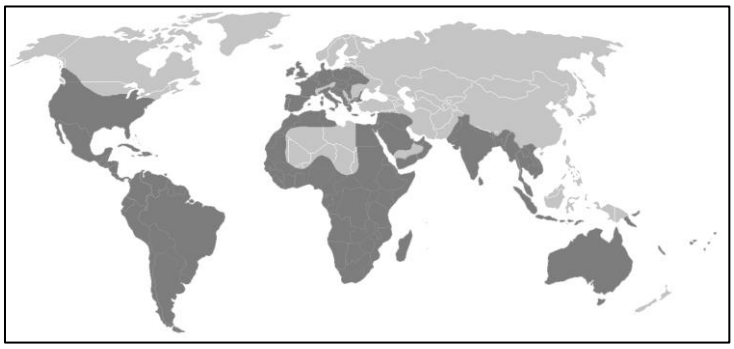


Figure 3.1 geographic map of worldwide distribution of barn owls. Dark grey indicates distribution. Map produced by Achim Raschka.

Feeding habits

Barn owls feed mostly on small rodents such as voles and field mice. (Roulin 2004a) showed that the diet differed between reddish and pale individuals, presumably because they breed in slightly different habitats (woodland versus open terrain). A study by Frey et al. (2011), confirmed that the differently coloured individuals prefer slightly different habitats.

Reproduction

The breeding season is very long, without a clear peak. The earliest clutches can be laid in February, while the last ones might be started as late as August. Around 10% of individuals lay a second clutch (unpublished data). A clutch can contain up to 9 eggs, with a mean of 6.0 ± 0.05 SE eggs (unpublished data). Eggs are laid with intervals of 1 to 2 days. Incubation lasts 32 days and starts after laying of the first or second egg, leading to a pronounced hatching asynchrony. With this asynchrony survival of at least the oldest nestlings in poor food conditions can be ensured. The younger nestlings can be seen as a contingency plan with the potential of producing fledglings in extremely rich food conditions. The mother remains in the nest and continues to brood and feed nestlings until they are thermo-independent at 10-14 days old. The nestlings have a relatively long period in the nest for owls of up to 55-60 days when fledging. After this they will remain in the area for another period where they receive regular feeding from the parents until they disperse and start to search for a suitable territory. Normally pairs produce 3.6 ± 0.06 SE fledglings out of a full clutch (unpublished data).

Life span

Barn owls can reach an age of up to 15 years (unpublished data). First year mortality is high (60-75%, Taylor 1994), and after that yearly mortality drops to 20-30% per year (Taylor

1994). Reported mean life expectancy after fledging ranges between 1.1 and 1.3 years. The most frequent cause of death is traffic, which is mostly caused by the barn owl's typical hunting method of soaring at low altitude of 1-2 metres high along roadsides searching for voles and other small rodents.

Study population

In a 15 X 25 km area in Western Switzerland (46°49" N/ 06°56" E) some 110 nest boxes were placed on the outside of barns. The area consists mainly of agricultural terrain, several villages and small forest patches. A detailed composition of the area can be found in Frey et al. (2011). The study area was expanded in 2006 with an additional 60 nest boxes, placed in an area more to the South of the main study area. The number of breeding pairs fluctuates, presumably as a consequence of weather conditions in the preceding winter (Altwegg et al. 2003). The mean number of breeding pairs is 49 ± 3.2 SE per year (range: 18-70, unpublished data).

2. The Eurasian kestrel (Falco tinnunculus)

Appearance and Colouration

The kestrel is a relatively small diurnal raptor with an average mass of 185 grams for males and 225 grams for females. The length of the wing ranges from 234-256 mm for males to 246-271 mm for females (Glutz von Blotzheim 1987). The kestrel displays sexual dimorphism in size and also in plumage colouration. The main colouration for both sexes is reddish to dark brown on the dorsal side and lighter pinkish cream colour with blackish streaks on the ventral side. The female is usually darker and more heavily streaked with black than the male. The male has a blue-grey head and tail. The female has black bars on a brown tail. Both sexes display a large sub-terminal black band (Figure 3.6A), as well as black spots on the back (Glutz von Blotzheim 1987, Village 1990). Fledglings have a female-like appearance, making it difficult to accurately sex them without the use of molecular markers. Legs and feet are yellow. For juveniles this colouration is slightly paler than for adults (figure 3.6B).

There appears to be sexual selection for this dimorphism as shown by an aviary experiment (Palokangas et al. 1994) where females preferred males with bright colour over those with dull colour and where brighter males produced more offspring. The melanin colouration can also provide information about individual quality (Parejo et al. 2011).

In the black and grey feathers, the colouration consists almost completely of eumelanin and in reddish feathers the ratio of eumelanin to pheomelanin is approximately 0.5. (Van den Brink et al. accepted). No carotenoids were found in feathers (Van den Brink et al. accepted). The width of the subterminal black tail band thus reflects eumelanin deposit during the development of the feathers.

Population and range

The population size fluctuates between years but is estimated at five million individuals. About 20% of this population lives in Western Europe. The population has experienced a slight decline since 1980, but is still listed as "Least Concern" with the IUCN. The European population consists of 330 000 – 500 000 breeding pairs and shows a moderate decline. The Swiss population consists of 3000 – 5000 breeding pairs and is stable (Birdlife International 2012).

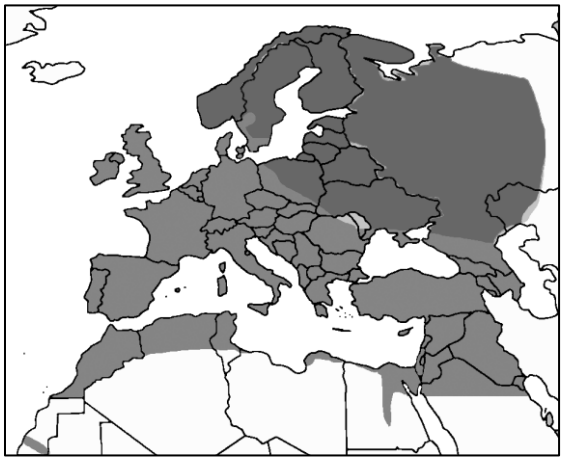


Figure 3.2 Geographic map of distribution of Eurasian kestrels. In light grey resident area; in dark grey breeding area. Map produced by Andreas Trepte.

Feeding habits

Kestrels feed mostly on small rodents, such as voles and mice. Insects, small birds or other small animals such as frogs or lizards can also be part of its diet. Voles are usually the most important part of the diet (Village 1990). The kestrel has a characteristic hovering hunting technique, where it will search while using the lift of the wind to stand still in the air. This is not the only method used, however. Perching and flying lowly over the ground are also commonly employed.

The kestrel is widespread throughout Europe and parts of Asia and Africa (Figure 3.2). Open areas for hunting and nesting possibilities in trees, rock faces or man-made structures are the only habitat requirements, which explain the broad geographic distribution of the kestrel. Kestrels from areas with permanent winter snow are all migrants, but kestrels from areas with milder winters are resident. There is some overlap with birds from the north overwintering in areas where the birds migrated south

Reproduction

Kestrels lay up to 7 eggs (Glutz von Blotzheim 1987), but most clutches consist of 4 to 6 eggs (Village 1990; unpublished data). Incubation starts after laying of the last egg or shortly before completion of the clutch and takes between 28 and 32 days (Glutz von Blotzheim 1987).

Many nesting sites used, from ledges on buildings to used stick nests of other species. Kestrels are very flexible in nest site choice, a

factor that has undoubtedly contributed to their wide distribution. The main criterion appears to be the availability of hunting areas nearby (Village 1990), although placement of nest boxes appear to result in higher breeding success (Hasenclever et al. 1989). In our Swiss study area, breeding occurs mostly in barn owl nest boxes. There is some competition over nest sites between the two species, but the larger and heavier barn owl will usually expel the small kestrel. Physical confrontations can occur and even result in death of kestrels (pers. obs.). One case of breeding in the same nest box was observed where a female kestrel was incubating eggs near the entrance of a nest box where a barn owl pair was feeding nestlings of up to 14 days old. Both nests failed. (Personal observation).

Egg laying starts in April and eggs hatch from the end of May until the beginning of July. Breeding starts as soon as the second egg is laid, and nestlings hatch partially synchronised, with the first few hatching on the same day and the others with small intervals. Eggs hatch after 28-32 days of incubation and nestlings remain in the nest until fledging at approximately 30 days. The mother broods and feeds the nestlings until they become thermo-independent and can feed themselves between 10 and 14 days old. After fledging nestlings will remain in the vicinity of the nest box for the next 2-3 weeks where they receive regular feedings (Glutz von Blotzheim 1987, Village 1990).

Life span

Kestrels have an average reported life span of only 2.4 years (Glutz von Blotzheim 1987). Mortality is highest in juvenile birds, especially in poor vole years and after harsh winters. Adult mortality is lower, but with a 60-70% survival rate each year, the expected average lifespan is still relatively short. Most individuals will only have one breeding attempt in their lifespan. The main cause of death is starvation, followed closely by collisions with road traffic (Village 1990).

Study population

The kestrels in our study population breed in the same nest boxes as those used for the barn owls.

3. The tawny owl (*Strix aluco*)

Appearance and colouration

Tawny owls are sexually dimorphic in size. The females are heavier and larger than males (body mass females: 584 g ± 2.6 SE; males: 409 g ± 2.5 SE, unpublished data from our population). The tawny owl displays heritable melanin-based colour polymorphism (Gasparini et al. 2009). The colouration consists of a mix of eumelanin and pheomelanin, which leads individuals to display plumage colours from greyish brown to dark reddish brown (Figure 3.4A and B). The colour is largely determined by the relative

amounts of eumelanin (68%) and pheomelanin (21%) deposited in the feathers and is under strong genetic control with a heritability h^2 of 0.72-0.80 (Brommer et al. 2005, Gasparini et al. 2009, Karell et al. 2011b). The amounts of pheomelanin and eumelanin are correlated, resulting in dark reddish individuals displaying the most of both pigments and greyish brown individuals the least. There is no sexual colour dimorphism (Student's t-test based on colour morph scores as described in (Roulin et al. 2003): t_{530} = 0.86, P = 0.39), nor is there assortative mating for colour in our study population (Pearson's correlation, r = 0.046, P = 0.39). No information is available on occurrence and variation in colouration in different populations.

Population and range

The tawny owl is Europe's most common owl. It lives in forested areas and is a resident breeding bird (Figure 3.3). It can withstand cold better than the barn owl, which has much poorer feather isolation (Glutz von Blotzheim 1987). The population is fairly large, with an estimated number of between 1.4 and 3 million individuals in Europe and a stable population trend. In Switzerland the breeding population is between 5000 and 6000 pairs (Birdlife International 2012).

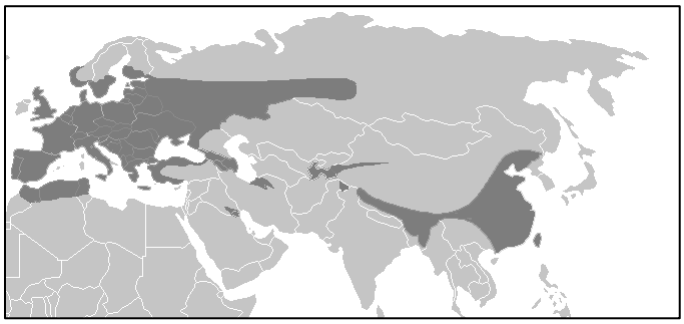


Figure 3.3 geographic map of distribution of tawny owls in Eurasia, with dark grey indicating distribution. Map produced by Achim Raschka.

Feeding habits

The tawny owl hunts mostly wood mice (A*podemus sylvaticus*) and bank voles (*Clethrionomys glareolus)*. Occasionally it will also hunt small passerine birds, frogs or earthworms. The tawny owl is a mostly perching hunter. It will regularly sit on a branch of a tree and listen for movements of small rodents in the undergrowth and then strike swiftly.

Reproduction

The tawny owl can form stable pair bonds, with females breeding in the same location, with the same male, for several years (Glutz von Blotzheim 1987). Breeding begins early in spring, with the first clutches laid as early as the end of January and the first young appearing after 30 days of incubation at the end of February. The breeding season is relatively synchronised and usually lasts until the end of May Breeding densities and clutch sizes are influenced by prey availability, which

can be predicted based on their numbers in the preceding autumn (Pucek et al. 1993). Second clutches occur almost never, with the exception of clutches lost very early in incubation (Glutz von Blotzheim 1987).

Incubation of the 1-9 (mean: 3.7 ± 0.05 SE eggs, study population) lasts for 30 days. Incubation begins after the second or third egg is laid, which produces hatching asynchrony, with the oldest nestlings in a typical clutch of 5 being usually between 5 days and one week older than the youngest of the clutch. Nestlings remain in the nest for approximately 30-35 days after which they fledge. After fledging, nestlings remain in the vicinity of the nest where they are still being fed and protected by the parents for up to two months (Sunde 2008).

Life span

Average yearly mortality 24 % per year, after the first year, where mortality is much higher and can reach 70%. The mean expected life span is approximately 3.5 years and the maximum life span can be up to 15 years (Study population).

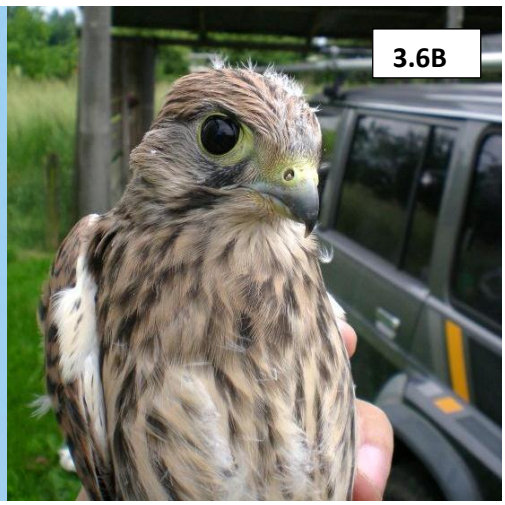
Study population

The study population is situated in western Switzerland, roughly between the cities of Yverdon-les-Bains and Lausanne. A total of 377 nest boxes are placed in forested areas greater than 4000 m^2 with a total area size of 911 km^2. The average distance between the boxes is 627 m, the minimal distance 500 m (Roulin et al. 2011b). The mean altitude of the study area is 672 m above sea level, with a range of 400-950 m. The number of breeding pairs varies between 57 and 149 breeding pairs each year, with fluctuations, most likely due to the cyclic nature of prey availability.

Figure 3.4 displays the extremes of tawny owl colour morphs. **Figure 3.4 A** shows a light reddish (grey) individual, and **figure 3.4B** shows a dark reddish pheomelanic individual. (Photos © C. Humphries)

Figure 3.5 Two extreme examples of colour morphs of barn owls. **3.5A,** a white, almost spotless individual and **3.5B,** a dark reddish pheomelanic and heavily spotted individual.

Figure 3.6A displays a male kestrel in hovering in flight, demonstrating the black eumelanic subterminal tail band. On figure **3.6B** a nestling kestrel, displaying the female-like colouration. (Photograph 3.2a © B. Holm)



Predictions about relationship between colouration and behaviour

Based on our knowledge of the melanocortin system and the effects the different melanocortins have on various traits we can make predictions of the direction of the influence of the specific melanocortin involved. Increased production of melanin stimulating hormone (MSH) results in increased production of eumelanin, while increased production of agouti signalling protein (ASIP) results in the MC1R switching to pheomelanin production. This allows us to use the melanic colouration of an individual as a predictor of its behaviour. Ducrest et al. (2008) predicted that darker melanic individuals should be, among other things, more aggressive (in contact with conspecifics), show less strong stress response in the glucocorticoid system and be more sexually active. Roulin and Ducrest (2011) described several cases in barn owls where these predictions were met.

The barn owl, and also the kestrel possess distinct eumelanic traits; the size and number of black spots and the black stripe of the tail, respectively. The barn owl and the kestrel also display clearly pheomelanic traits in the brown reddish plumage colouration on the sides and the back.

In tawny owls, where the basis of the colouration is depending more on the relative proportions of eumelanin and pheomelanin in the same feathers (Gasparini et al. 2009), predictions are perhaps not as straightforward to make, but since eumelanin and pheomelanin are strongly positively correlated in tawny owls (Gasparini et al. 2009), an increase in pheomelanic colouration, means that more eumelanin is present. Thus, darker reddish individuals should show comparable responses with darker eumelanic barn owls or kestrels.

The sexual dimorphism in colour in barn owls (females are more eumelanic and pheomelanic) and kestrels (males are more eumelanic) might also impact behaviour in typical male or female traits such as aggression, if certain behavioural traits are related to melanic colouration.

The main research for this thesis focused on whether or not a relationship between individual melanin based colouration and individual behavioural differences exists. The underlying thought is that such a relationship might help explain local adaptation to a fluctuating environment. This might

then help to explain the maintenance of colour polymorphism in the populations of barn owls, tawny owls and kestrels.

Thesis outline

We first need to demonstrate that a link exists between the behaviour and the melanic colouration. Once that has been established, we can try to search for explanations. I will describe relationships between several personality traits (mostly from the boldness/shyness, exploration, and aggression axes) and melanin based colouration in the various chapters of this thesis. I demonstrate relationships of these personality traits with melanin based colouration in three different raptor species. First, in ***chapter one*** I describe together with my co-authors how a relationship between nestling anti predator behaviour and melanin colouration exists in barn owls.

In ***chapter two***, a similar study but this time performed on nestling kestrels confirmed the most important results found in the barn owl. The differences we found between the two species will be discussed with regard to different predator type or – pressures each might face.

Chapter three deals with dispersal in recruits and adult barn owls from our study population. We describe a relationship between natal dispersal and pheomelanin colouration and show that this behaviour is heritable from parent to same sex offspring.

In ***chapter four***, nest defence in adult tawny owls is studied. Here we study the defence of the nest towards conspecific and human intruders, both during the day and during the night. Both types of nest defence are related to reddish pheomelanic colouration. We discuss this in relation to different breeding strategies, life span and lifetime reproductive success of the different colour morphs of the tawny owl.

C*hapter five* provides a first glimpse on the genetic background of the found relationships between colouration and individual behaviour. We studied the influence of a polymorphism in the MC1 receptor on dispersal behaviour of recruits with known colour. The MC1R gene was found to be

polymorphic, with one locus and two alleles. This polymorphism has been shown to be related to pheomelanin colouration. I will further describe how we could proceed to analyse this data with available genotypic data. This would allow us to elucidate the nature of the relationships between the melanocortin system, the genes, gene products, colouration and behaviour.

In the ***discussion*** I will summarize the main conclusions and place the results in the context of maintenance of colour polymorphism. I will describe the type of evidence found in my thesis, how much our proposed underlying mechanism of the melanocortin system can explain and if our findings can be generalized to other vertebrate species. In addition I will explore several future lines of research that could help us to explain evolutionary relationships between colouration and behavioural traits.

1. Melanic color-dependent antipredator behavior strategies in barn owl nestlings

Valentijn van den Brink[1]*, Vassilissa Dolivo[1]*, Xavier Falourd[2], Amélie N. Dreiss[1] & Alexandre Roulin[1]

1 Department of Ecology and Evolution, University of Lausanne, CH-1015 Lausanne, Switzerland

2 Laboratory of Electromagnetics and Acoustics, École Polytechnique Fédérale Lausanne, station 11, CH-1015 Lausanne, Switzerland

*These authors contributed equally to this work.

Key words: melanin, natural selection, predation, sexual selection, animal personalities

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Personal contribution: I performed the behavioural experiments with the exception of the recordings of hissing. I performed the analyses and writing of the paper in close collaboration with the co-authors.

Abstract

The arms race between predators and prey has led to morphological and behavioral adaptations. Different antipredator strategies can coexist within a population if each strategy is the result of a trade-off with competing demands. Antipredator behavior can be associated with morphological traits, like color patterns, either because in the context of sexual selection coloration signals the ability to avoid predators or because coloration is a naturally selected trait useful in avoiding predators. Because in the barn owl (*Tyto alba*) heritable eumelanic plumage coloration is associated with the glucocorticoid-dependent response to stress, we tested whether antipredator behavior is also related to this trait. Compared to small-spotted nestlings, individuals displaying larger black spots hissed more intensely in the presence of humans, feigned death longer, had a lower breathing rate under stress and were more docile when handled. Cross-fostering experiments showed that the covariation between spot size and the duration of feigning death was inherited from the biological mother, whereas covariation between spot size and docility was inherited from the biological father. Our results confirm that melanin-based coloration is associated with suites of behavioral traits, which are under both genetic and environmental influence. Coloration can thus evolve as a direct or indirect response to predation, but it can also be a signal of antipredator strategies to potential mates.

Introduction

An important factor in the evolution of morphology and behavior is the arms race between predators and their prey. While predators evolve more efficient foraging techniques, their prey is in turn selected for more refined adaptive strategies to escape them (Dawkins and Krebs 1979). These adaptations can involve specific color patterns (Stuart-Fox et al. 2004) or shapes (Hoso and Hori 2008) to enhance camouflage. They can also concern behavior including hissing calls in insects (Davis and Heslop 2004) and lizards (Labra et al. 2007), aimed at scaring predators, or feigning death in the presence of predators that do not eat or react to dead animals (Miyatake et al. 2004), or even feigning of injuries by parents to redirect the attention of predators toward themselves rather than toward their vulnerable offspring (Grimes 1936). Although a wide variety of antipredator strategies have evolved in many species, only in the last decade with the development of the field of behavioral syndromes or "animal personality" (Gosling 2001, Sih et al. 2004b) has interest been devoted to possible individual differences in antipredator strategies (Bell 2005). A growing body of literature supports the proposition that individuals can differ in their response to predators (e.g.: Cockrem 2007, Thaker et al. 2009, Jones and Godin 2010).

Different mechanisms could favor the coexistence of several antipredator strategies within a population. Polymorphism in the way individuals escape from predators can be maintained if the balance between the costs and benefits of alternative strategies to avoid predation is the same (Roff 1996). For instance, morphs may exploit habitats where different predators are found, requiring alternative strategies to escape them, including specific colorations that allow genotypes to be cryptic in alternative habitats (Hoekstra et al. 2005), or they may adopt different antipredator behavior specifically directed to the size or type of predators (Seyfarth et al. 1980, Templeton et al. 2005). Another mechanism favoring strategy diversity is negative frequency-dependent selection, where predators are used to a prey having certain characteristics, leading to a disproportionate consumption of the most common type of prey, thereby favoring rare alternative antipredator strategies (Punzalan et al. 2005, Bond and Kamil 2006).

In several species, alternative antipredator strategies have been shown to be associated with morphological traits such as coloration, probably because viability selection mediated by predators can favor certain combinations of antipredator behavior and color patterns. In the pygmy grasshopper (*Tetrix subulata*), experimentally altered combinations of camouflage color patterns and behavior changed survival probability when confronted with a predator (Forsman and Appelqvist 1998). In the eastern red-backed salamander (*Plethodon cinereus*), a conspicuous red morph displays different antipredator behavior than a cryptic lead-colored morph (Venesky and Anthony 2007). In Hermann's tortoises (*Eurotestudo boettgeri*) darker eumelanic individuals are bolder in the presence of humans suggesting that the behavior toward predators is color specific (Thomas 2002, Mafli et al. 2011). Further proof for this proposition comes from a recent study performed in the marsh harrier (*Circus aeruginosus*) which shows that 2 different color morphs of male harriers display different antipredator behavioral strategies. Gray individuals mobbed intruders less and recruited fewer helpers for mobbing than did brown males (Sternalski and Bretagnolle 2010). Finally, artificial selection for high and low stress responsiveness in the rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) showed that heavily spotted individuals display a lower physiological and behavioral stress response than lightly spotted individuals (Kittilsen et al. 2009). Thus, differently colored fish may respond differently to predation risk due to a link between the hypothalamic-pituitary-adrenal (HPA) stress response and eumelanic coloration. Such a link could be mediated by the melanocortin system with products of the proopiomelanocortin (*POMC*) gene binding not only to the MC1R (melanocortin 1 receptor), resulting in melanin-based coloration, but also to 4 other receptors (MC2R-MC5R) which regulate other traits such as the HPA-axis, aggressiveness, physical activity and immune function (Boswell and Takeuchi 2005, Ducrest et al. 2008). This can result in a covariance between these traits and melanin-based coloration (McKinnon and Pierotti 2010).

Barn owls have been studied intensively to understand the adaptive function and maintenance of variation in their plumage color. Both within and among populations, barn owls vary in the size of

eumelanic black spots, a heritable sexually dimorphic trait with females displaying on average larger spots than males, and variation in spot diameter is due to both sex-linked and autosomal genes (Roulin et al. 2010). This trait is associated with many physiological and behavioral functions (Roulin and Ducrest 2011). Of particular interest is the finding of Almasi et al. (2010), who showed that the size of black spots of barn owls is associated with the (HPA) stress response as in the rainbow trout and Atlantic salmon (Kittilsen et al. 2009). In their study, barn owl nestlings born from a mother with larger spots mounted a lower corticosterone stress response and had lower levels of corticosterone after implanting corticosterone-releasing pellets. As shown experimentally in other species, the presence of a predator induces a rise in blood circulating corticosterone (Cockrem 2007, Muller et al. 2009), and hence we predict spot diameter to be associated with personality and more specifically with individual differences in the reaction to predation risk.

In a free-living Swiss population of barn owls, we examined 3 antipredator behaviors in relation to melanin-based coloration. 1) When a predator is close to a brood, nestlings often start to hiss loudly, probably to scare the predator. In various animals, this behavior is also observed (Sibley 1955, Apel and Weise 1986, Labra et al. 2007), with the apparent goal to make the predator mistake the identity of the prey with another predator so that it is scared away (Dobkin 1979). We thus simulated a predator intrusion by opening nest-boxes and recorded the intensity of hissing calls. 2) Because nestling barn owls can become aggressive and agitated once captured by humans, probably in an attempt to escape, we recorded the degree of agitation when handled. 3) We carried out the tonic immobility test to measure the extent to which nestling owls feign death. Animals are put on their back and the time taken to turn back and stand on the legs again is measured. Tonic immobility is commonly observed in a wide variety of taxa in response to external stimuli, and it is considered an adaptive defense mechanism against predators (Boissy 1995). Because the way individuals react in the presence of a predator is probably due to the ability to cope with stressful events, we also measured the breathing rate after capture, a measure of stress and fear (Carere and van Oers 2004,

Fucikova et al. 2009). As larger spotted individuals show a lower glucocorticoid stress response, we predict larger spotted nestlings to breath at a lower rate. To investigate whether the relationships between antipredator behavior and coloration are environmentally mediated or genetically inherited, we allocated nestlings randomly among environments by swapping hatchlings between randomly chosen pairs of nests.

Methods

Study species

The worldwide-distributed barn owl is nocturnal and hunts mainly small mammals in the open landscape. In Switzerland (46°49' N, 06°56' E), where the study was performed, most barn owls breed in nest-boxes (1.0 X 0.6 X 0.5 m) and lay between 2 and 11 eggs (mean ± standard error [SE]: 6.1 ± 1.6) between the end of February and August (mean laying date: 29 April ± 24 days). Eggs are incubated for 32 days and hatch asynchronously on average every 2.5 days implying that in large broods the first-hatched nestling can be up to 3 weeks older than its last-hatched sibling. During the first 2 weeks of life, the father forages, whereas the mother broods and distributes food to the nestlings. Once the offspring can eat without maternal help, the mother participates in foraging. From this time until fledging at around 55 days of age, the offspring are in their nest without their parents who sleep in barns located sometimes several kilometers away from the nest. At night, nestlings produce not only begging calls in the presence of their parents but also several hundreds of calls during their prolonged absence to pacifically resolve conflicts over the single indivisible food item brought at each parental feeding visit, a sib-sib communication process referred to as "sibling negotiation" (Roulin et al. 2000). These calls are noisy and hence may attract mammalian predators (Roulin 2001, McDonald et al. 2009), which may explain why barn owls evolved several antipredator strategies. This includes aggressive behavior toward predators using their claws and beak, extremely noisy hissing calls that may frighten predators (such as mustelids, cats and humans, personal observation AR and VD) and feigning death by staying immobile.

General methods

A single person measured the diameter of black spots located at the tip of feathers of the ventral body side of adults and their offspring aged approximately 50 days. This was done on the breast, belly, flanks and undersides of the wings to the nearest 0.1 mm using a caliper. A representative number of spots were measured on each body part and then a mean value was calculated. The mean of both flanks (and both wings) was calculated, and finally a mean value over the 4 body parts to be used in the statistical analyses. The assessment of this plumage trait is reliable (Roulin 1999b, 2004c). From 2008 to 2010 we carried out partial cross-fostering experiments by exchanging approximately half of the hatchlings between pairs of nests with the criterion that the matched broods had a similar hatching date. To recognize nestlings we marked them with nontoxic color paint until we could ring them with a numbered aluminum ring. We collected a blood sample to determine nestling sex from blood cell DNA using sex-specific molecular markers.

Hissing behavior

In 2009, we studied hissing behavior in 15 broods containing 2-6 (3.9 ± 0.3) nestlings aged 40 ± 0.9 days. At the beginning of the night (mean time: 22h13 ± 12 min) a recorder (Marantz Professional Audio PMD-670) was placed at 10 m from the nest-boxes. The top of the nest-box was opened, a microphone (Beyerdynamic M69N) installed inside of it, the nestlings touched and briefly illuminated with a flashlight to mimic the presence of a human predator, and the top of the nest-box was closed. We then retreated to 10 m away from the nest-box and did not make any noise while recording nestling behavior. This procedure lasted approximately 30 s. We recorded until 10 min after hissing had ended or in those cases where no hissing was induced, for 10 min. To obtain a reliable mean estimate of hissing behavior we repeated this procedure 6.7 ± 0.6 times (range: 2-12) in a row and induced hissing in the nestlings in 95 of 103 cases. The number of times our presence induced hissing behavior was not associated with mean nestling spot diameter (Pearson's correlation: $r = 0.21$, $n = 15$

nests, P = 0.45). The beginning of each hissing period was defined as the moment when the top of the nest-box was closed after having disturbed the brood. It ended when the last nestling stopped hissing and either siblings resumed negotiation calls (68 cases) or did not produce any hissing call in the next 10 min (35 cases).

In order to quantify the acoustic sound levels of hissing calls, a first calibration procedure of the acoustic device was done in the lab so that every recording was free of distortion and digitalized with a good quality and an adequate quantification. Because in situ acoustic calibration before each recording was not possible, we assumed all nest-boxes as closed boxes with the same volume and a microphone placed at equal distance to the nestlings during all experiments. Even though these assumptions could induce some minor changes of acoustic levels of the recordings, the analysis of the recordings validates the acoustic protocol permitting then to post-process the whole acoustic dataset. For each recording sequence selected with Adobe Audition 3.0 software (Adobe systems, Inc. 2007), the energy contained in hissing calls was quantified using a script written in Matlab R2008b (Mathworks 2008).

A mean value per brood was calculated over all the measurements. Because we measured hissing behavior of entire broods and not individual nestlings, we could not investigate whether this behavior was associated with spot diameter measured in the biological and foster parents (every brood contained nestlings of 2 origins since we performed a partial cross-fostering experiment) but only with the mean spot diameter measured in the nestlings themselves. Before calculating mean nest-mates' spot diameter, we also had to remove variation explained by sex for the entire brood because females have on average larger spots than males (Roulin 1999b). We did this by extracting residuals from a one-way analysis of variance with nestling spot diameter as dependent variable and nestling sex as a factor. Then, for each brood we calculated a mean residual value. To analyze the relationship between residual nestling spot diameter and hissing behavior we incorporated date and number of nestlings as covariates.

Because of the short time interval, the number of inductions within the same night cannot be considered as independent events. Ideally, to obtain reliable repeatability estimates of hissing behavior we should have repeated the measurements on several independent visits. We therefore calculated repeatability over the consecutive measurements made on the same night to decide whether we can calculate a mean hissing value.

Tonic immobility

In 2009, we carried out the tonic immobility test in 37 cross-fostered nestlings from 13 origins and in 32 non cross-fostered nestlings from 10 origins. Each individual was tested between 1 and 3 times (2.0 ± 0.1) on different days at a mean age of 27.4 ± 1.5 days. Tonic immobility was recorded at the beginning of the night (mean time: 22h24 ± 5 min) a few days before or after we assessed hissing behavior but never on the same day. Using the methods described by Jones and Faure (1981) and Jones (1986), a single person put each individual on its back on a flat illuminated surface and restrained it for 10 s with a hand on its breast. The hand was then removed and the time until the nestling moved to turn and stand again was measured. The same person stayed nearby within sight of the nestling until the end of the test. In this test, individuals differ not only in the duration, but also in the ease with which the immobile state can be induced. In addition, often the duration of tonic immobility is negatively correlated with the number of attempts needed to induce it (Hennig 1978, Mills and Faure 1991). Following the most common way to measure this tendency (Jones 1986), we obtained a measure of the motivation to not stay on their back by repeating the tonic immobility test up to 3 times. If an individual stayed on its back more than 15 s on the first occasion, we did not repeat the test; if it stayed less than 15 s we tested it again and if at the second trial it again stayed less than 15 s, we carried out a third and final trial. Thus, each individual was tested between 1 and 3 times. The mean number of times an individual was tested thus indicated the tendency or repeatability of an individual to stand up quickly after having been put on its back. Over the 1 to 3 trials, we considered the longest duration this individual stayed on its back as another

measure of tonic immobility. If an individual stayed longer than 120 s, we stopped the test and hence 120 s was the maximum duration. The tests were done without prior knowledge of plumage traits because at that time color traits were not yet developed and a different person measured spot diameter without being aware of the results of the tonic immobility test.

Breathing rate

The number of times an individual breathes in 1 min, as measured by counting the number of breast movements is an indication of response to handling stress (Carere and van Oers 2004, Fucikova et al. 2009). On the same day when the tonic immobility test was performed, we assessed breathing rate in 48 nestlings (29 cross-fostered and 19 noncross-fostered) immediately after being taken out of their nest-box. Because we recorded breathing rate in more than one nestling per visit, for each individual we recorded the time between the moment when we opened the nest-box for the first time and when we started to count breast movements; however, this measure was not associated with breathing rate ($r = 0.08$, $n = 48$, $P = 0.61$). Ambient temperature was recorded but was also not significantly correlated with breathing rate ($r = 0.23$, $n = 48$, $P = 0.10$). The test was performed on 1-3 different days (2.7 ± 0.2) to allow calculation of repeatability. Unfortunately, we could not calculate heritability for breathing rate because we only started to measure this trait in 2009 in nestlings (but not in adults), and hence we do not have yet enough data.

Docility

Between 2008 and 2010 a single person handled 448 nestlings and assessed aggressiveness toward the handler and the degree of agitation around the age of fledging (mean age: 48 ± 0.28 days). Score 0 was assigned to nestlings that did not express any aggressive behavior, score 1 when they tried to bite once or a few times, score 2 when they frequently scratched, attacked or pinched with the beak, and score 3 when they were extremely aggressive by grabbing with their bill and claws and when trying to catch the bird to be handled they were on their back with claws raised. Assessments of

agitation were given according to a similar index (0-3), the minimal score (0) being assigned to nestlings that did not move or hiss during manipulation, whereas the maximal score (3) corresponds to nestlings that were struggling, flapping their wings and/or hissing all the time. The values for aggression and agitation were summed to get an index of docility. The higher the value, the less docile we considered the individual to be. We only started to record docility in 2008, and although we measured this trait in both nestlings and adults, currently we cannot yet calculate heritability, as we need to compare nestling docility with parental docility measured at the same life history stage. Nestlings are much less docile than adults and in adults docility depends on several variables such as reproductive stage (unpublished data). Thus, although it would be very interesting to have heritability estimates, the data are currently not yet available to do so.

Covariation of the measured behavioral traits

If the different behavioral traits are correlated with each other, this could indicate that differences between individuals are part of different antipredator strategies. To investigate possible covariation between the measured behavioral traits we calculated mean values per individual for each behavioral trait (breathing rate, tonic immobility duration and number of attempts and docility score). Not all traits were normally distributed and because transformations did not improve normality, we used nonparametric Spearman's rank correlations.

Statistical methods

Statistical analyses were performed using the software programs JMP 7.1 and SAS v9.1 (SAS Institute Inc.). To analyze tonic immobility test in relation to spot diameter we considered only the 37 cross-fostered nestlings and calculated the mean of the 1-3 recorded values. In a mixed model analysis of covariance (ANCOVA) we entered the nest of rearing as a random variable, nestling sex as a factor and nestling age and body mass as well as spot diameter of the biological and foster mothers and fathers as 6 covariates, and the interaction between sex and spot diameter was also included. A

similar procedure was applied to breathing rate. In these tests we were able to enter sex as a factor in the model because contrary to the hissing experiment, here we obtained a behavioral value for each individual nestling.

We analyzed nestling docility with a general linear mixed model. Separate models were built to assess the association between docility and spot diameter of cross-fostered nestlings or of the biological and foster parents. We analyzed cross-fostered nestlings separately so that we could assess the influence of color traits of the biological and foster parents on the behavior. Other factors in the models were nestling sex, nestling age, brood size, spot diameter of the nestlings or of the parents and two-way interactions. For the model including nestling spot diameter we added nestling nested in nest of rearing as a random factor to account for multiple measurements of the same individual. In addition to this for the models with parental spot diameter, year and identity of the parent were added as random factors to account for multiple nestlings and multiple breeding seasons of the same individual. All statistical analyses are two-tailed and P values smaller than 0.05 considered significant. Nonsignificant variables were removed one after the other starting with the least significant interactions. Means are quoted ± SE.

Results

Hissing behavior

The total amount of energy contained in hissing calls was measured several times in a row on the same night in each site and was found to be significantly repeatable ($r = 0.50 \pm 0.04$; $F_{14,76} = 6.43$, $P < 0.0001$). The mean amount of energy per brood contained in hissing calls increased with mean residual nestling spot diameter (stepwise linear regression analysis on mean brood values: $F_{1,11} = 5.72$, $P = 0.036$; Figure 1.1) after controlling for brood size ($F_{1,11} = 10.04$, $P = 0.009$; more energy was invested in hissing by larger broods) and date ($F_{1,11} = 10.40$, $P = 0.008$; owls hissed more at the end than beginning of the season); brood sex ratio and nestling age were not significant ($P > 0.60$) and so we removed these 2 variables from the model. Note that in a preliminary model the interaction

between brood size and mean residual nestling spot diameter was not significant ($F_{1,10}$ = 0.19, P = 0.67) indicating that the higher hissing response to stress by large- than small-spotted nestlings was probably not mediated by social interactions associated with brood size. This result is not confounded by habituation because the positive relationship between the amount of energy contained in hissing calls and mean residual nestling spot diameter was also detected when considering only hissing calls produced the very first time we disturbed nestlings (r = 0.59, n = 13 nests, P = 0.032).

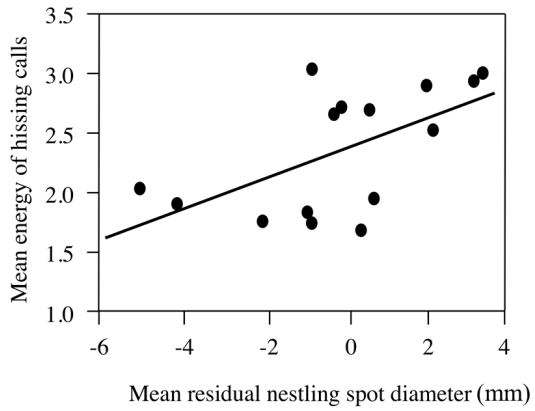

Figure 1.1 Energy contained in hissing calls (log + 1 transformed) in relation to residual nestling spot diameter in nestling barn owls. Mean values per nest were calculated so that each nest appears only once in the figure. We extracted residual nestling spot diameter to remove variation explained by sex. Regression line is drawn for illustrative purpose. Least squares (LS) values from the statistical model presented in the results are presented.

Tonic immobility

When put on their back nestlings took on average 62.8 ± 4.1 s to turn over and stand again on their feet. The mean speed with which nestlings turned back on their feet was significantly repeatable between days (r = 0.19 ± 0.07; $F_{68,101}$ = 1.58, P = 0.018). In a mixed model ANCOVA with the nest of origin entered as a random variable, nestlings stood up on their feet quicker when their biological mother displayed smaller than large black spots ($F_{1,6.9}$ = 13.24, P = 0.0085; Figure 1.2A); spot diameter of the biological father and of the 2 foster parents were not significant (P values > 0.21). When replacing maternal spot diameter by nestling spot diameter, the relationship was no longer significant ($F_{1,30.43}$ = 0.43, P = 0.51). The interaction terms, nestling sex and age were never significant and hence removed from the final models.

The mean number of times we tested nestlings, that is, the other measure of the nestling's motivation to turn over and stand again on their feet, was 1.70 ± 0.08. As we carried out the tonic immobility tests 2-3 times on different days, we could assess whether this second measure of tonic immobility was repeatable. This was the case (r = 0.15 ± 0.07; $F_{68,101}$ = 1.44, P = 0.047). In contrast to the previous measure of tonic immobility, this second measure was not significantly associated with spot diameter of the biological and foster parents (mixed model ANCOVA with nest of rearing as random factor: P values > 0.25; the interaction of nestling spot diameter and sex, nestling sex and age were also not significant, P values > 0.15) but with spot size measured in the nestlings themselves (another mixed model ANCOVA: $F_{1,8.3}$ = 13.78, P = 0.0056). When all nestlings (cross-fostered and noncross-fostered) were considered simultaneously, qualitatively similar results were obtained ($F_{1,56.4}$ = 6.27, P = 0.015). Nestlings displaying larger black spots were tested less often than individuals with smaller spots (Figure 1.2B) indicating that individuals with larger black spots were more prone to stay longer on their back. Again, nestling sex and age were never significant and hence removed from the final models.

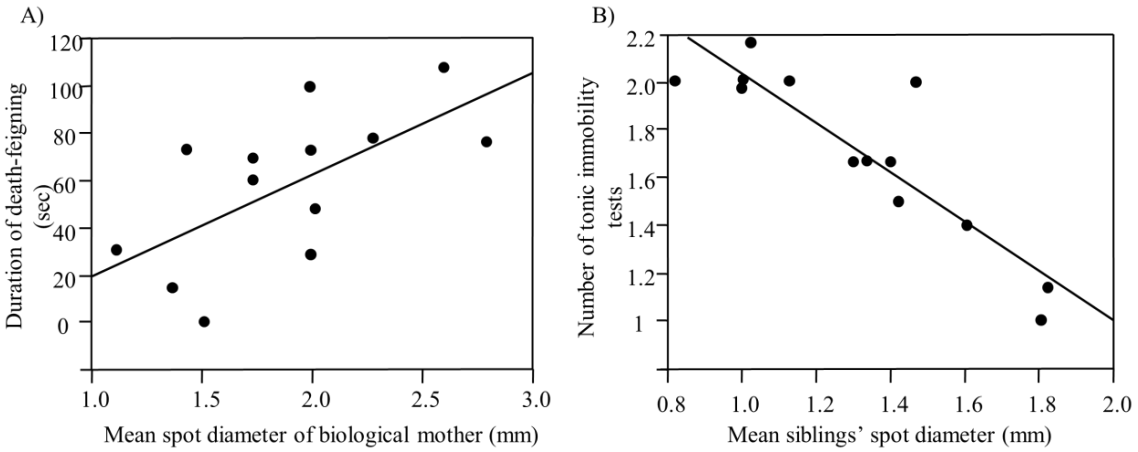

Figure 1.2 Tonic immobility in nestling barn owls raised by foster parents in relation to the size of black plumage spots. A) Mean amount of time siblings took to feign death (i.e. amount of time between the moment when a nestling was put on its back and turned to stand back on its legs) in relation to the size of black spots of their biological mother. Pearson's correlation is r = 0.64, n = 13 nests, P = 0.018. B) Mean number of attempts needed for nestlings to stay longer than 15 s on their back before turning back and standing again on their legs in relation to the size of black spots measured in the nestling themselves. Pearson's correlation is r = -0.88, n = 13 nests, P = 0.0002. In the two panels mean sibling values were calculated so that each origin appears only once. Regression lines are drawn for illustrative purpose.

Breathing rate

Breathing rate was repeatable within nestlings in consecutive visits (r = 0.32 ± 0.06; $F_{47,93}$= 2.305, P = 0.0027). When considering only the 29 cross-fostered nestlings for which we measured breathing rate, this variable was not associated with spot diameter measured in the biological and foster parents (mixed model ANCOVA, P values > 0.10). In a similar model using spot diameter measured in the nestlings themselves instead of in parents, individuals displaying larger black spots made fewer breathing movements per minute (Figure 1.3; mixed model ANCOVA: $F_{1,14.51}$ = 5.90, P = 0.029; nestling sex and age were not significant). When measured in all nestlings, including noncross-fostered ones this was also significant ($F_{1,40.1}$ = 4.56, P = 0.04).

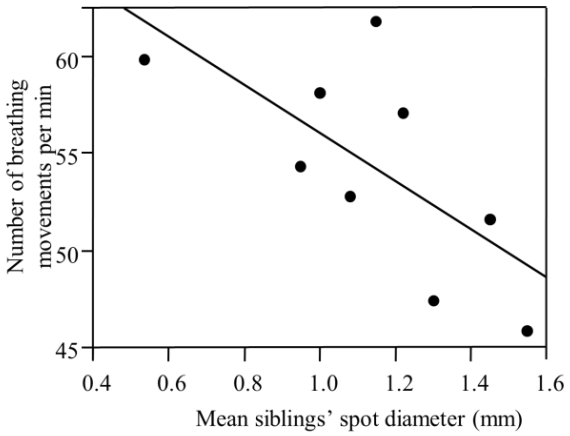

Figure 1.3 Number of breathing movements per minute recorded as result of handling stress in relation to siblings' spot diameter in the barn owl. We calculated mean values so that each nest only appears once. Pearson's correlation is r = -0.68, n = 9 nests, P = 0.04. Regression line is drawn for illustrative purpose.

Docility

In 284 nestlings, we recorded docility on different days (3.9 ± 0.09 times) which proved repeatable (r = 0.27 ± 0.066 $F_{1,494}$ = 1.992, P < 0.0001). After correcting for nestling age ($F_{1,333}$ = 6.9, P = 0.014; with age individuals became less docile), nestlings were less docile when their biological father displayed small than large black spots ($F_{1,88}$ = 5.64, P = 0.02; figure 1.4). Nestling sex and brood size had no effect on docility (for all traits P > 0.30). For the nestlings themselves, only a positive association with age was found ($F_{1,445}$ = 4.64, P = 0.032). When all nestlings (cross-fostered and noncross-fostered) were in the model, the results remain qualitatively the same ($F_{1,1307}$ = 34.8, P <0.0001). In the models

investigating the effects of mean spot diameter of nestlings and mean spot diameters of biological mother and foster parents no relations with color traits were found (all P values > 0.41).

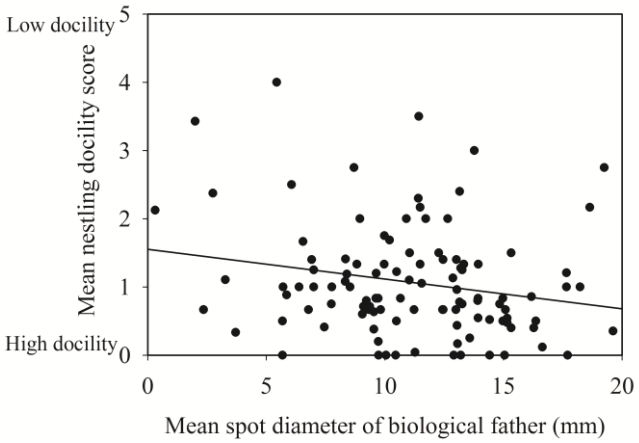

Figure 1.4 Mean docility score of nestling barn owls raised by foster parents in relation to the spot diameter of the biological father. A higher score means the individuals are less docile (see explanation in MATERIAL AND METHODS). We calculated mean sibling values so that a single value per foster parent pair is presented in the figure.

Covariation between behavioral antipredator traits

Most individually tested traits were correlated with each other. Breathing rate was negatively correlated with tonic immobility duration (Spearman's correlation, r_s = -0.48, n = 70 individuals, P = 0.0004) and positively with tonic immobility number of attempts (r_s = 0.34, n = 70, P = 0.018). The tonic immobility duration was negatively correlated with the number of attempts needed to induce tonic immobility (r_s = -0.67, n = 70, P < 0.0001). Finally, less docile nestlings breathed at a higher rate (r_s = 0.35, n = 70, P = 0.014), tended to stay less long on their back (r_s = -0.23, n = 70, P = 0.058) and hissed at a lower level than docile individuals (r_s = -0.63, n = 16 nests, P = 0.009).

Discussion

In the barn owl several antipredator strategies were associated with the size of black eumelanic spots: larger spotted nestlings invested more energy in hissing behavior and feigned death more easily and for longer than smaller spotted conspecifics, which were more agitated when handled and stressed as evidenced by breathing rate. Using cross-fostering experiments we could test how 2 of these color-specific behaviors were inherited from one generation to the next. Interestingly, the

covariance between spot size and tonic immobility was inherited from the biological mother, whereas the covariance between spot size and docility was passed on through the biological father. This indicates the presence of genetic or maternal/paternal effects.

Boldness-shyness

When confronted with a predator, small-spotted barn owl nestlings appeared to be particularly bold by being aggressive and agitated while handled, whereas large-spotted owls are rather shy by staying calm when handled, feigning death and hissing loudly. Shy behavior seems to have as a goal the avoidance of a direct confrontation or even a fight. Given that docile individuals were hissing more intensely than less docile nestlings, we conclude that the hissing behavior reflects a shy and calm behavior. It might confuse or scare away the predator without the direct risk of a physical confrontation. In other species where a physical confrontation would pose a serious risk of injury or death it is also used for instance by burrowing owls (*Athene cunicularia*) mimicking rattle snake calls to deter ground squirrels (Rowe et al. 1986) or skunks (Medill et al. 2011), who can advertise their danger by hissing. As shown in our study, hissing was more pronounced in large than small broods probably because the likelihood that there is an individual with a fearful personality that continues to hiss during long periods of time is higher in large than small broods. Thus, the higher hissing response to a predator of large- compared with small-spotted owlets could be socially mediated. However, we did not find support for this hypothesis given the absence of statistical interaction between brood size and spot diameter. Another possibility that remains to be tested is whether the intense hissing behavior of larger spotted individuals is mediated by social dominance interactions and aggressiveness within the brood. To sum up, more data are required to investigate whether the association between spot diameter and hissing is genetically inherited or socially driven.

Even though the tonic immobility test is widely regarded as a measure of fear in poultry (Jones 1986), differences in duration can also be regarded as different strategies (Erhard et al. 1999). Tonic immobility might increase survival chances after an attack, as demonstrated in ducks (Sargeant and

Eberhardt 1975) and quails (Thompson et al. 1981). It can even be interpreted as a calculating, selfish strategy, where an individual tries to divert attention away from itself to redirect it toward its conspecifics, which are still moving (Miyatake et al. 2009). The negative association we found between tonic immobility and breathing rate supports the idea that in barn owls tonic immobility is a calculating strategy because a higher breathing rate, which is an indicator of stress, goes together with shorter tonic immobility duration and more attempts needed to induce it.

As we have seen, in the barn owl docility is inherited to offspring from the biological father. Because we know males are also selected to have fewer and smaller spots than females (Roulin et al. 2010, Roulin et al. 2011a), selection on boldness may indirectly affect the evolution of spot diameter and vice versa. In other words, antipredator behavior may indirectly influence the evolution of sexual dimorphism in the degree of eumelanin-based coloration. Thus, in males small and few spots may honestly signal the ability to survive in environments where predators are frequent given that boldness increases reproductive success early in life possibly at the cost of reduced lifespan (Smith and Blumstein 2008, Reale et al. 2009). We would thus predict that males displaying smaller black spots produce offspring with low survival prospects. Interestingly, this is exactly what we found in a recent study showing that smaller spotted males produce daughters with a particularly low survival (Figure 1c in Roulin et al. 2010). A similar pattern is found in a population of the common buzzard (*Buteo buteo*), where light males are more aggressive than dark males, whereas in females this pattern is reversed (Boerner and Kruger 2009). This might reflect the different demands made on the 2 sexes in antipredator behavior.

Consistency of behavior

Repeatabilities reported in this study range from 0.15 to 0.5, which indicates that individuals are consistent in their behavior toward potential predators. The repeatability of tonic immobility duration and number of attempts is clearly lower than found in other studies on chickens (rank scores within individuals; Jones 1988) or beetles (r=0.94, Nakayama et al. 2010), but in a review on

pigs, cattle and poultry, repeatability was found to be very variable (Forkman et al. 2007). A meta-analysis of animal personality studies by Bell et al (2009) shows that it is not uncommon to find low but significant repeatability values and our reported values are all still well within the range of repeatabilities reported there. In field studies, many variables are beyond our control that can impact individual behavior, thus it remains complex to study consistency of behavior. This is highlighted by a recent study on rufous-collared sparrows (*Zonotrichia capensis*) where geographic variation in repeatability of the same traits in different populations of the same species was found (van Dongen et al. 2010).

Melanin-based coloration and stress

Associations between melanin pigmentation and aggressiveness (Ducrest et al. 2008), courtship behavior (Yeh et al. 2006), glucocorticoid-dependent stress responses (Kittilsen et al. 2009) and antipredator strategies (Venesky and Anthony 2007) have already been observed in several species. A high response to stress could be a way to explain the agitation and aggressiveness against handlers observed among small-spotted barn owls because a previous study has shown that barn owls with larger spots mount a lower corticosterone response to capture-induced stress than those with smaller spots (Almasi et al. 2010). In rainbow trout, individuals with more spots also showed a lower response to stress, both in hormone levels and locomotor response (Kittilsen et al. 2009). There is evidence suggesting that individuals behaving bold when faced with a predator respond more strongly to handling stress (i.e. breathe faster) and explore their environment more intensely (Fucikova et al. 2009). The link between stress response and exploratory behavior proposes an interesting hypothesis that dispersing behavior is related to spot diameter, which could explain why in our barn owl population immigrant females (who have dispersed) have smaller spots than resident females (Roulin and Altwegg 2007). This proposition is currently studied further in our barn owl population.

Selection pressure on stress response

Prolonged exposure to predators, as experienced in areas with high predator pressure, might facilitate habituation, resulting in a decreased response to this stress source, to avoid the costs associated with chronic stress (McEwen and Wingfield 2003). A reduced response to repeated stressors has been demonstrated in a number of taxa including fish (Brown et al. 2005, but see Bell et al. 2010) and rats (Caldji et al. 2000). In our study, we found a reduced response to stressors in the large-spotted individuals, suggesting that predation pressure might have selected for this trait to coevolve with melanin-based coloration. It would therefore be interesting to test whether predation is a major selective force that could account for the observed pronounced worldwide variation in spot size in the barn owl (Roulin et al. 2009, Roulin and Salamin 2010).

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2. Melanin-based colouration in juvenile kestrels (*Falco tinnunculus*) covaries with anti-predatory personality traits

Valentijn van den Brink[1], Isabelle Henry[1], Kazumasa Wakamatsu[2] and Alexandre Roulin[1]

[1]Department of Ecology and Evolution, University of Lausanne, CH-1015, Lausanne, Switzerland

[2]School of Health Sciences, Fujita Health University, Toyoake Aichi 470-1192, Japan

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Personal contribution: I performed the behavioural experiments and analysed them together with the co-authors. I also wrote the manuscript, in collaboration with the co-authors.

Abstract

Recent studies have shown that melanin-based colouration is associated with the ability to cope with stressful environments, potentially explaining why colouration covaries with anti-predator behaviours, boldness and docility. To investigate whether these relationships are consistent across species, we performed a study in the European kestrel (*Falco tinnunculus*). Similar to our results found previously in the barn owl (*Tyto alba*), nestling kestrels displaying a larger sub-terminal black tail band stayed on their back longer (tonic immobility test) and breathed at a lower rate than individuals with a smaller black band when handled. However, in contrast to barn owls, nestling kestrels with a larger black tail band were more aggressive and more agitated. Our results strengthen the hypothesis that melanin colouration is related to stress response and in turn to the reaction to predators, a very important personality trait (i.e. boldness).

Introduction

Data is accumulating on the possibility that melanin-based colouration is associated with behavioural syndromes. Darker melanic individuals are usually more aggressive and sexually active (Ducrest et al. 2008) and a recent study in tortoises has shown that dark colouration is associated with anti-predator behaviour (Mafli et al. 2011). The possibility that melanin-based colouration could be related to all five categories of behavioural syndromes (aggressiveness, boldness, activity, exploration and sociality; Reale et al. 2007) therefore starts to attract researchers (e.g.: Mateos-Gonzalez and Senar 2012). Given that in many species inter-individual variation in melanin-based colouration is under strong genetic control, covariation between melanin colour and personality traits could arise if genes encoding for colour pleiotropically regulate behaviour (Ducrest et al. 2008). From a physiological point of view, glucocorticoids may explain why behaviour is associated with melanin colouration. In several species glucocorticoid-dependent stress responses are differentially expressed in dark and pale coloured individuals, as shown in Harris's sparrow (*Zonotrichia querula*, Rohwer and Wingfield 1981), the barn owl (*Tyto alba*, Almasi et al. 2010), fish (Kittilsen et al. 2009,) deer mice (*Peromyscus maniculatus*, Hayssen et al. 2002) and dogs (Bennett and Hayssen 2010).

Based on the finding that eumelanism is associated with stress-response, Van den Brink et al. (2012) proposed the hypothesis that dark and light individuals may differ in their response to predation risk. Accordingly, these authors showed that when exposed to the threat of a potential predator, barn owl nestlings with larger eumelanic spots hissed more, were more docile, needed fewer attempts to induce death feigning (tonic immobility test) and breathed at a lower rate than those with smaller spots. Given that glucocorticoid stress-responses appear to be colour-dependent in organisms that are phylogenetically as distinct as fish, mammals and birds, the results found in the barn owl with respect to predation response might also apply to other animals. In a first attempt to answer this question, we performed a study in the Eurasian kestrel (*Falco tinnunculus*). The kestrel was chosen because it can use nest boxes not occupied by barn owls (and hence can be considered a sort "by-catch" of our long-term barn owl research in the same area). The kestrel is a suitable species

to study relationships between behavioural traits and colour, since individuals from the same population also vary in melanic colouration. It differs from the barn owl in that it is diurnal, so although a closely related species, we feel it could provide a first indication if the previously found results can be generalised to other species. Another important difference is that in the kestrel males are darker eumelanic than females, whereas in the barn owl this is the opposite, with females being darker eumelanic than males.

Kestrels are small diurnal raptors, common in most of Europe, which display colour traits that vary between individuals in the form of a large sub-terminal black-eumelanic tail band and pheomelanic reddish-brown colouration of the back and tail feathers. We conducted similar experiments as those performed in the study on nestling barn owls (Van den Brink et al. 2012a)) aimed at measuring the direct stress involved in encounters with potential predators. We began by measuring eumelanin and pheomelanin content of feathers to confirm the type of melanin in the traits studied. Then, we measured how docile nestling kestrels were when handled. Corresponding tests involved holding an individual by the legs and scoring if it then threatens or grabs the observer with its claws and presenting a stick to the nestling to measure the strength with which it grabs the stick. Then, we performed the Tonic Immobility test (TI) to assess how long individuals remain immobile in front of a predator (Gallup 1974). Finally, we counted the number of times an individual breathes per unit time when handled. This provided us with a measure of the level of stress, since also in great tits (*Parus major*) breathing rate is correlated with corticosterone levels in the blood (Carere and van Oers 2004) and exploratory behaviour later in life (Fucikova et al. 2009).

Based on the results obtained in the barn owl (Van den Brink et al. 2012a)) we predict individuals that display a larger sub-terminal black-tail band to be more resistant to stress by being more docile when handled, to stay longer on their back when placed there and to show a lower breathing rate. We also studied pheomelanic colouration, since a recent study by Roulin et al. (2011a) showed that physiological traits can be inversely related to eu- and pheomelanin

colouration. These results suggest that a relationship between phenotypic traits and pheomelanin could perhaps be of opposite sign than the relationship of these phenotypic traits with eumelanin.

Methods

Study species and study area

The kestrel is one of the most common small diurnal raptors of Europe (Glutz von Blotzheim 1987). It lives in open habitat where it feeds mainly on various vole species. Adults are sexually dimorphic, with females about 20 % larger and heavier than males. Females are reddish-brown with black bars on the back and tail. In contrast, adult males show blue-grey feathers on the head, rump, upper tail-coverts and tail. They are reddish with black spots on the back (Village 1990). Individuals of both sexes display a large, sub-terminal black tail band.

We studied a kestrel population breeding in nest boxes in western Switzerland (46”49’ N, 6”50’ E, 500-600m altitude). These boxes are placed on the side of farms and are intended for barn owls (*Tyto alba*), but when unoccupied by barn owls they can be used by kestrels. We studied the kestrels in 2009 and 2010, which were exceptionally poor years for barn owls, leaving many boxes available for kestrels to breed. Between June and August we visited 30 nests with a total of 62 nestlings (30 females and 32 males) in 2009 and 25 nests with 86 nestlings (of unknown sex) in 2010. In 2009 we visited each box two times for our experiments, once when the nestlings were (mean ± SE) 15 ± 0.2 days old and once at 22 ± 0.2 days. In a separate visit, just before fledging at 26.7 ± 0.2 days we only measured colour traits. In 2010 we wanted to focus on certain aspects of our 2009 study and therefore we only visited each nest once, to minimise unnecessary disturbance. As a result, we could therefore only determine approximate age of the nestlings, because we did not visit the nests on the day of hatching. We based our estimates on predicted hatching dates and comparison of wing length and body mass to measurements of nestlings of known age from previous years. We only performed the assessment of behaviour on one visit, shortly before fledging, when nestlings were between approximately 25 and 30 days old.

Body measurements and assessment of plumage traits

At each visit to the nest we recorded the body mass of the nestlings to the nearest 0.1 g and measured the length of their left wing and tail to the nearest mm and tarsus length to the nearest 0.1 mm. We also measured to the nearest 0.1 mm the width of the sub-terminal black band on all twelve tail-feathers during the last (and in 2010 during the only) visit, shortly before fledging. Subsequently, for each individual we calculated the mean width of all sub-terminal black bands to be used in statistical analyses. The width of this band does not covary with the age at which we measured it (Pearson's correlation: $r = 0.03$, $n = 81$, $p = 0.8$), but the length of the tail does covary with age ($r = 0.401$, $n = 82$, $p = 0.0002$) and the width of the tail band also correlates with the length of the tail ($r = 0.28$, $n = 81$, $p = 0.01$). The increased width of the tail band with longer tail length might be related to a possible signalling function, but since we are interested in the amount of eumelanin produced and deposited, we will focus on the absolute width here. To remove variation due to tail length, we used the residual of tail length with age at measuring as covariate.

We also collected three feathers from the back and the tail for reflectance spectra measurements. We measured the reflectance spectra in the 300-700 nm range using an Ocean optics S2000 spectrophotometer (Ocean Optics, Dunedin, FL, USA) with a deuterium halogen 2000 light source (Mikropack, Ostfildern, Germany). The reflectance is expressed as the proportion of light reflected in comparison with a spectralon white standard disk (WS type). For each reflectance spectrum, we calculated the brown chroma as $(R_{700nm} - R_{600nm})/R_{mean}$, where R_{mean} is the mean reflectance in the visible light (300-700 nm). The brown chroma represents the contribution of the red part of the spectrum relative to the complete visible part of the spectrum and we used this as a proxy for pheomelanin colouration.

Determination of melanin content from feathers

In 2006, we plucked wing coverts, upper-tail coverts and tail feathers from three adult kestrels (one male and two females) found dead along roads and stored in the laboratory at -20°C. Our aim was to identify and measure the concentration of melanin pigments in the reddish, black and greyish parts of feathers. We thus pooled feathers from the different body parts presenting the same colour patterns.

K. Wakamatsu identified the concentration in pheomelanin and eumelanin in kestrel feathers (Wakamatsu and Ito 2002, Ito et al. 2011). Microanalytical methods were developed to quantify the amounts of eumelanin and pheomelanin. Those assays were based on the formation of specific degradation products, pyrrole-2,3,5-tricarboxylic acid (PTCA) by alkaline H_2O_2 oxidation of eumelanin and aminohydroxyphenylalanine (AHP) by reductive hydrolysis of pheomelanin with hydriodic acid (HI). Alkaline H_2O_2 oxidation of feathers was performed as follows. 1.0-1.5 mg of feather was taken in 10 ml screw-capped test tubes, to which 375 µl 1 mol/l K_2CO_3 and 20 µl 30% H_2O_2 were added. The mixtures were mixed in the room temperature for 20 hrs. The residual H_2O_2 was decomposed by the addition of 50 µl 10% Na_2SO_3 and the mixtures were then acidified (pH4.0) with 135 µl 6 mol/l HCl. After vortex-mixing, the reaction mixtures were centrifuged at 4,000 g for 1 min, and aliquots (80 µl) of the supernatant fluids were directly injected into the HPLC system. HI hydrolysis was performed by heating 1.0 -1.5 mg of feather, 30 µl 30% H_3PO_2 and 500 µl 57% HI in a screw-capped tube at 130˚C for 20 hrs, after which the mixture was cooled. An aliquot (100 µl) of each hydrolysate was transferred to a test tube and evaporated to dryness using a vacuum pump connected to a dry ice-cooled vacuum trap and two filter flasks containing NaOH pellets. The residue was dissolved in 200 µl 0.1 mol/l HCl. An aliquot (10 µl) of each solution was analysed on the HPLC system. H_2O_2 oxidation products were analysed with the HPLC system consisting of a JASCO 880-PU liquid chromatograph (JASCO Co., Tokyo, Japan), a Shiseido C_{18} column (Shiseido Capcell Pak MG; 4.6 x 250 mm; 5 µm particle size) and a JASCO UV detector. The mobile phase was 0.1 mol/l potassium phosphate buffer (pH 1.9): MeOH, 99: 1 (v/v). Analyses were performed at 35˚C at a flow rate of 0.7 ml/min.

Absorbance of the eluent was monitored at 269 nm. HI reductive hydrolysis products were analysed with an HPLC system consisting of a JASCO 880-PU liquid chromatograph, a JASCO C_{18} column (JASCO Catecholpak; 4.6 x 150 mm; 7 µm particle size) and an EICOM ECD-300 electrochemical detector. The mobile phase used for analysis of AHP was 0.1 mol/l sodium octane sulfonate and 0.1 mol/l Na_2EDTA: MeOH, 98: 2 (v/v). Analyses were performed at 35˚C at a flow rate of 0.7 ml/min. The electrochemical detector was set at +500 mV versus an Ag/AgCl reference electrode.

Tonic Immobility Test

Using the method described by Jones and Faure (1981), Jones (1986) and Van den Brink et al. (2012), a single person (VvdB) put each nestling on its back and restrained it for 10 seconds with a hand on its breast in a large wooden box (40 x 40 cm) fitted with a wire mesh cover to prevent escape. The hand was then removed and the time until the nestling moved to turn itself and stand on its feet again was measured. The same person stayed nearby within sight of the nestling until the end of the test. Some individuals are more readily induced into the state of TI than others and it is recommended to repeat the test several times in a row if an individual does not stay in the first attempt. Therefore, we tested those individuals that stayed less than 15 seconds again for a maximum of three times (Jones and Faure 1981, Jones 1986, Van den Brink et al. 2012). Thus, each individual was tested between one and three times (mean number of attempts: 1.9 ± 0.1 SE), which gave us a first measure of the motivation to stay on its back. Over the one to three trials, we considered the longest duration this individual stayed on its back as a second measure of tonic immobility (mean duration: 53.9 s ± 5.1 SE). If an individual stayed longer than 120 s, we stopped the test and hence 120 s was the maximum duration recorded. Individuals that never stayed on their back were scored a zero value for the TI duration. As an extra measure of personality we also measured “righting time”; the time it took for an individual to right itself again when placed on either side of its body, without restraint (mean duration: 3.2 s ± 0.8). The main difference with the TI test is that this procedure does not involve restraint and only places the individual on its left or right side

(mean values for three repeats on both sides were recorded), which might provoke a different reaction than the TI test.

Breathing rate

The number of times an individual breathes in one minute, as measured by counting the number of breast movements is an indication of response to handling stress (Carere and van Oers 2004, Fucikova et al. 2009). In 2010, we assessed breathing rate in nestlings by counting breast movements in a one-minute period immediately after being taken out of their nest box. Because we recorded breathing rate in more than one nestling per visit, and stress levels may rise after removal from the nest box (Carere and van Oers 2004), for each individual we recorded the time elapsed between when we opened the nest box for the first time and when we started to count breast movements (mean number of breathing movements: 127 ± 2.8 SE), thus we considered it a stable measure of response to handling stress.

Docility

We assessed aggression towards the handler and the degree of agitation during the two visits for all nestlings on a 0-3 scale. Score 0 was assigned to nestlings that did not express any aggressive behaviour, score 1 to those that tried to bite us once or a few times, score 2 for those that frequently scratched, attacked or tried to pinch with their beak, and score 3 was assigned to individuals that were extremely aggressive by grabbing with their bill and claws and when trying to catch the bird to be handled were on their back with claws raised (mean score: 0.9 ± 0.1 SE). Assessments of agitation were given according to a similar index (0-3), the minimal score (0) being assigned to nestlings that did not move or call during manipulation, whereas the maximal score (3) corresponds to nestlings which were struggling, flapping their wings and/or hissing all the time (mean score: 1.7 ± 0.1 SE).

Stick grabbing

Nestling kestrels can adopt a defensive posture where they position themselves on their back and strongly thrust their claws at the predator. To record this aggressive behaviour we presented individuals with a round (2 cm diameter) wooden stick and recorded the intensity with which the stick was grabbed. Score 0 was assigned to individuals that did not grab the stick at all, score 1 to individuals that moved their talons towards grabbing, but did not grab the stick, score 2 to individuals that grabbed the stick, but weakly (i.e. the stick could be easily removed from the grip of the individual again by simply pulling the stick away). Finally, score 3 indicated that individuals had a very strong grip, where the individual held on powerfully to the stick and its talons had to be removed one by one from the stick (mean score: 1.3 ± 0.05 SE).

Lifting by claws

We also measured the tendency to grab and hold onto our hand when we slightly lifted the birds up while holding them by their tarsi. We measured this on a scale of 0 to 3. Zero meant the bird did not grab at all, one was weak grabbing, two strong grabbing and three was recorded as very strong grabbing with flapping of wings in an attempt to sit upright (mean score: 1.2 ± 0.06 SE).

Statistical procedures

All statistical tests were performed with the software program JMP 9.02 (SAS 2010), tests were two-tailed, p-values smaller than 0.05 were considered significant and model fits were compared using ΔAIC. All results are presented as means with standard errors.

Main experiment in 2009

We analysed the data for 2009 and 2010 separately, because in 2010, only one visit per nest was made. To reduce the amount of variables we tested and to avoid using multiple correlated variables in our models (Quinn and Keough 2002), we performed a principal components analysis

(PCA) on the mean individual values for 2009 and another PCA for the mean nest values in 2010. For the data of 2009 the initial PCA contained TI duration, number of TI attempts, righting time, aggression, agitation, stick grabbing and lifting from claws. We retained two variables of this PCA, because they had eigenvalues > 1 (2.3 and 1.6; Quinn and Keough 2002). After inspection of the loading matrix for the different variables of the PCA (Figure 2.1a), we found that the variable "righting time" did not contribute strongly to the overall fit of the PCA as defined by a loading lower than 0.4 on both components (loading on PC1 and PC2 of 0.38 and -0.20 respectively). The variance explained by the first two components also increased from 32.6% to 36.8% for PC1 and from 22.4 to 25.8% for PC2 after removal of this variable. The eigenvalues of these two new PCs were 2.21 and 1.54, respectively. They were used in subsequent analyses with eumelanin- (width of the black tail band) and pheomelanin-based (brown chroma-value) traits. We calculated repeatabilities and standard errors following Becker (1984) and Lessels & Boag (1987) for PC1 and PC2, but found only PC2 repeatable between visits (PC1: $F_{1,89} = 0.64$, $p = 0.98$; PC2: $F_{1,89} = 1.97$, $p = 0.002$, $r = 0.35 \pm 0.09$). We constructed a repeated measures mixed model with individual identity nested in nest of rearing and individual as random variables to account for repeated measures and similar genetic background of siblings. The dependent variable in two separate models was PC1 or PC2. Predictor variables were sex, age rank in nest of rearing (because the dynamics of sibling interactions in the nest could perhaps influence behaviour due to age differences between siblings), age at the experiment (to account for possible influences of developmental stage), residual tail length (tail length corrected for age) and width of black tail band. In an identical set of models we used pheomelanic colouration instead of the width of the black tail band.

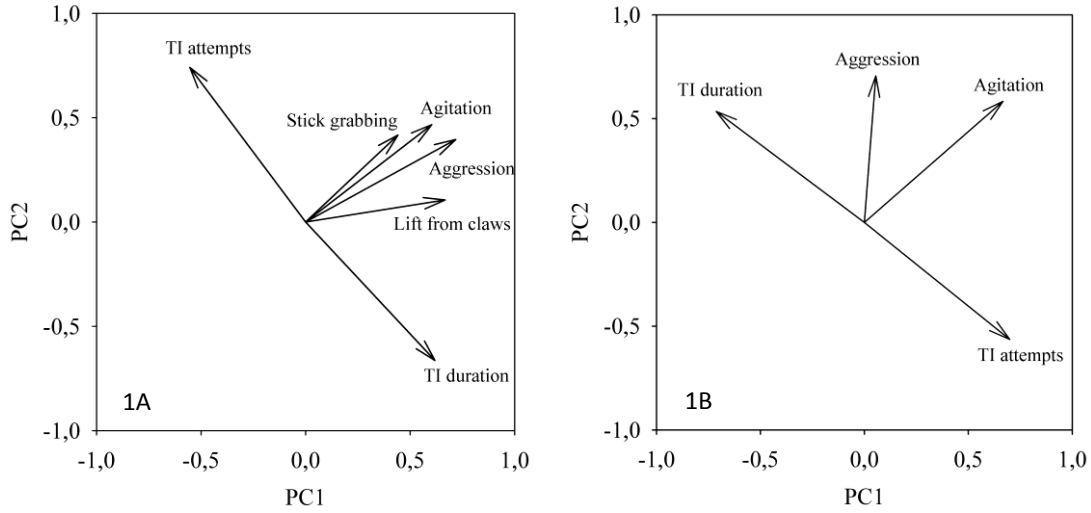

Figure 2.1 Loading plots for A) 2009 and B) 2010 of contribution of different variables to the first two components of a principal component analysis on behavioural traits in nestling kestrels.

Minor experiment in 2010

To confirm part of the results obtained in 2009, we performed a small study in 2010. We did not find an effect of sex on personality traits in 2009 and because the width of the black tail band was not sexually dimorphic (see results) we did not identify nestling sex in 2010. We also did not know the exact age (see above) and to reduce variation that might have been caused by this, we decided to use mean values per nest for the analyses. Furthermore, given the absence of any relationship between personality and pheomelanin-based colouration in 2009, we did not measure this colour trait in 2010. After analysing the 2009 data, we removed several behavioural tests that were least informative from our protocol for 2010. Righting time, stick grabbing and lifting from claws were all redundant with TI or docility measurements. When we re-analysed the 2009 data with only the remaining variables and with mean values per nest over all visits, as in 2010, we find qualitatively similar significant results as for the complete analysis with all variables. We measured three personality traits (TI, aggression and agitation) and breathing rate in 2010 and we calculated mean values per nest. We performed a PCA on these traits and found that the first two PCs explained 34.3% and 29.9% of variation (Figure 2.1B). The loading matrix showed that the breathing rate

(loadings of 0.02 and 0.13) did not contribute to the first two PCs (eigenvalues of 1.74 and 1.45), but it was the only variable that contributed strongly to the variation (loading of 0.97) in the third PC (eigenvalue of 1) and therefore breathing rate was removed from the PCA and analysed separately. This improved the overall fit of the PCA to 42.5% for PC1 and to 36.9% for PC2, with eigenvalues of 1.74 and 1.44, respectively. We used the resulting two PCs in subsequent analyses with the width of the black tail band as predictor. For the analysis of breathing rate we introduced as a covariate the mean time span between the moment when we opened the nest box and recorded breathing rate.

Results

Melanin content

Eumelanin pigments were more abundant than pheomelanin pigments in black (mean ± SE: 41'809 ± 10'592 ng/mg *vs.* 536 ± 271) and grey feathers (62'696 ± 15'664 *vs.* 763 ± 303), while the opposite was found for reddish feathers (3'367 ± 589 *vs.* 7'923 ± 1761). Thus, the ratio eumelanin / pheomelanin was 374 for black feathers, 217 for grey feathers and 0.5 for reddish feathers. These measurements confirm the eumelanin and pheomelanin composition of the studied colour traits.

Main experiment in 2009

The final model showed that the wider the black tail band ($F_{1,78.07} = 5.3$, $p = 0.02$, Figure 2.2) and the older the nestlings when tested ($F_{1,88.6} = 109.8$, $p < 0.0001$), the higher the value for PC1 was. Nestling sex had no significant effect on PC1 ($F_{1,50.3} = 0.16$, $p = 0.69$), nor did age rank ($F_{1,72.11} = 0.05$, $p = 0.83$) and residual tail length ($F_{1,89.5} = 2.4$, $p = 0.13$). Model selection by comparing ΔAIC between full and reduced models showed that the final model with only width of tail band and age during experiment produced the best fit (ΔAIC between final and next closest model was 8 points). The PCA showed a clear clustering of the aggression, agitation, lifting from claws and stick grabbing (Figure 2.1A). This was also supported by similar loadings of these variables onto the PCs. Based on the loading values, this means that individuals with larger tail bands were more aggressive, more

agitated, grabbed the stick more strongly, reacted stronger to lifting from the claws and stayed in TI longer than individuals with smaller tail bands. TI was also achieved in fewer attempts in individuals with a wider black tail band. The models for PC2 and the model for PC1 with the chroma-values were all non-significant (all p-values > 0.21). Correlations between the different behavioural traits are shown in Table 2.1.

Table 2.1. Pairwise correlations for variables used in principal component analysis of behavioural traits for data from 2009 (A) and 2010 (B) in nestling kestrels. N.S. = not significant. *: P <0.05, **: P < 0.01, ***: P < 0.001. Variables in italics (recovery in 2009 and breathing rate in 2010) were removed from final PCA analyses.

Table 1A	TI attempts	TI duration	*Recovery*	Stick Grab	Lift from claws	Aggression
TI attempts	-	-	-	-	-	-
TI duration	**-0.70 *** **	-	-	-	-	-
Recovery	***-0.25 ****	***0.20 N.S.***	-	-	-	-
Stick Grab	**0.007 N.S.**	**0.009 N.S.**	***0.11 N.S.***	-	-	-
Lift from claws	**-0.28 ****	**0.19 N.S.**	***0.05 N.S.***	**0.32 ****	-	-
Aggression	**-0.03 N.S.**	**0.26 ***	***0.33 ****	**0.26 N.S.**	**0.33 ****	-
Agitation	**-0.04 N.S.**	**0.07 N.S.**	***-0.05 N.S.***	**0.21 N.S.**	**0.24 N.S.**	**0.58 *****

Table 1B	TI Attempts	TI duration	Aggression	Agitation
TI duration	**-0.78 *** **	-	-	-
Aggression	**-0.24 N.S.**	**-0.24 N.S.**	-	-
Agitation	**0.14 N.S.**	**-0.09 N.S.**	**0.64 *****	-
Breathing	***-0.14 N.S.***	***0.28 N.S.***	***0.07 N.S.***	***0.002 N.S.***

The different number of tests per individual for the TI experiment could have biased our results and therefore we repeated our analyses with only the result of the first attempt to induce TI. The results remain significant (age, $F_{1, 46.69} = 31.8$, $p < 0.0001$; terminal black tail band, $F_{1,48.2} = 7.7$, $p = 0.008$) and therefore we are confident that our method of testing has not influenced the outcome of our analyses.

Minor experiment in 2010

Both PC1 and PC2 did not show a significant relationship with the width of the black tail band (all p-values > 0.54). The placement in opposite corners on the loading plot (figure 2.1B) of the TI duration and the number of attempts to induce TI is confirmed by their strong negative correlation (Pearson's correlation: r = -0.79, p < 0.0001), whereas aggression and agitation are positively correlated (r = 0.64, p = 0.0007, Table 2.1). The larger the black tail band, the fewer breathing movements per minute were made (multiple regression analysis: $F_{1,23}$ = 7.137, p = 0.014; Fig. 2.3). The time after opening of the nest box had no effect on the number of breathing movements ($F_{1,22}$ = 1.639, p = 0.21), hence we removed this variable from the final model.

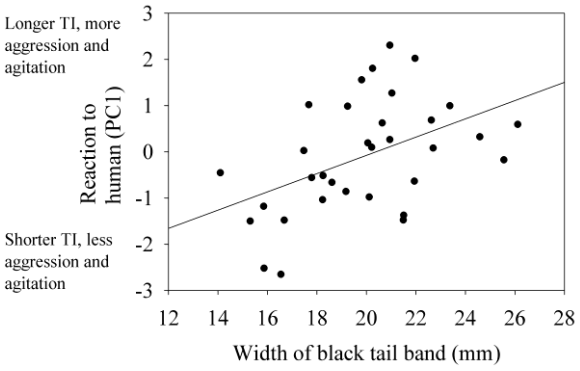

Figure 2.2 Relationship between PC1 of a PCA on the reaction to human intrusion of the nest box and the width of the sub-terminal black tail band in nestling kestrels in 2009. A higher value in PC1 reflects longer Tonic immobility (TI) duration, more aggression and agitation, stronger grabbing reflex and strength of grabbing, along with fewer attempts necessary to induce TI.

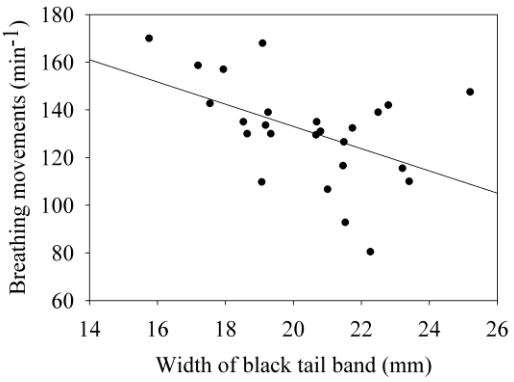

Figure 2.3. Relationship between number of breathing movements and width of the sub-terminal black tail band in nestling kestrels in 2010.

Discussion

We found that in 2009 the wider the black tail band in nestling kestrels was, the more they were aggressive, agitated, grabbed a stick stronger and remained in TI longer. In 2010 we showed that individuals with a wider black tail band breathed slower. These results are very similar to those reported in barn owls (Van den Brink et al. 2012a)), with the exception of aggression (in kestrels

darker individuals were more aggressive while in the barn owl lighter coloured individuals were more aggressive).

Behaviour and feather melanin content

In contrast to eumelanin colouration we did not detect any significant relationship between behaviour and the reddish-brown pheomelanic colouration in both the kestrel and barn owl. The absence of covariation between these phenotypic traits is still unclear and might be specific to these two species or, alternatively, such covariations may occur under specific conditions. Indeed, other unpublished studies performed in the pheomelanic tawny owl (*Strix aluco*) showed that the degree of reddishness correlates with anti-predator behaviour (Van den Brink et al. unpublished data; **chapter 4**, this thesis). Based on the chemical analyses of feather pigments, it could be that the traits we tested here are only influenced by variation in the amount of eumelanin and not of pheomelanin (in contrast, in the tawny owl variation in the degree of reddishness is due to variation in both eumelanin and pheomelanin feather content; Gasparini et al. (2009). Thus, more data need to be accumulated to investigate under which circumstances pheomelanism correlates with anti-predator behaviour. Note however, that in the barn owl we found that only the degree of pheomelanin-based coloration is associated with dispersal distances (Van den Brink et al. submitted). Thus, it is possible that eumelanin and pheomelanin traits are correlated with different personality traits.

Behavioural syndromes

The loadings onto the PCA show clear clusters of behavioural types. Even though the results from 2009 could not be replicated in 2010, the different types of behaviour cluster in the same manner in the two years (Figure 2.3). The relatively low contribution to the total variance of PC1 and PC2 might be a further indication of this fact. The duration of TI, aggression and agitation all show a positive relationship to the sub-terminal black tail band in both years, whereas the number of attempts needed to induce TI is correlated negatively to it. It appears that these traits are different

types of behaviour; confrontational (aggression, grabbing, agitation, refusal to remain in TI) versus risk-avoiding (easy induction and long duration of TI). This apparent separation between the different types of behaviour suggests that they might be part of different behavioural axes as defined by Reale et al. (2007). It is tempting to place the two differently correlated behaviours on opposite ends of the boldness/shyness axis. However, even though TI is commonly used to quantify fearfulness in poultry (Gallup 1977; Jones and Faure 1981; Jones 1986), it can also have adaptive value, since it can increase survival after attack, presumably by causing the predator to loosen its grip (Thompson et al. 1981) or it might serve to divert the predator's attention away from the individual towards conspecifics (Erhard et al. 1999, Miyatake et al. 2009). Therefore, it is not certain if TI is purely a "fear" reaction, adding to the doubt whether fear is a behaviour that can adequately be measured (Boissy 1995, Erhard et al. 1999, Gregory 2008). This is why breathing rate was also measured, because we know this is related to stress (Carere and van Oers 2004).

Differences between results obtained in 2009 and 2010

Our results point out that although we performed a number of similar tests in 2009 and 2010, the number and type of tests performed might cause variation in the results. We believe it is most likely the differences in results between the two years are not due to the difference in analysis method, because re-analysis of the 2009 data in the same way as in 2010 (i.e. with mean nest values) did not change our conclusions. We can however not completely exclude the possibility that the differences are caused by the different methods or reduced number of tests used. A last possibility is that the behaviour is influenced by environmental factors that change from year to year, which would not be surprising given that covariation between melanin-based colouration and other phenotypic attributes varies between environments as demonstrated by opposite relations between reproductive success and melanin based colouration in tawny owls in two different populations (Brommer et al. 2005, Roulin et al. 2008), underlining that generalisations from field studies on animal personality are not easy to make as already mentioned by other authors (e.g. Bell 2005).

Similarity and differences between kestrels and other species

We have several hypotheses to explain the inter-specific difference observed in the link between docility and colouration. Indeed, we found that kestrels displaying larger black tail bands were more aggressive towards human whereas in barn owls individuals showing larger black spots were less aggressive. From an ultimate point of view, it might pay off more to stay calm and quiet for the kind of predators a barn owl faces, whereas for a kestrel the best might be to be more aggressive. The nocturnal barn owl and the diurnal kestrel might well face different predator pressures (diurnal predators are mainly avian, such as buzzards *(Buteo buteo*) and goshawks (*Accipiter nisus)*, nocturnal predators are mainly mustelids or foxes (for recently fledged nestlings). The barn owl can in fact also be a threat to kestrels). There is some evidence that predation might select for boldness or docility (Reale and Festa-Bianchet 2003). From a proximate point of view, this inter-specific difference in the sign of the association between boldness and melanin coloration could be explained by the level of stress. Indeed, the kestrel is diurnal and barn owl nocturnal implying that they may experience difference levels of stress due to differences in human disturbance. The third option might be that differences are species-specific, as demonstrated by the difference in breathing rate (56.4 min^{-1} ± 1.2 breathing movements for barn owls versus 127 min^{-1} ± 2.8 for kestrels) along with adult barn owls being calmer than adult kestrels when handled (pers. obs.). Finally, the differences between the species might be caused by different sex-linked associations between behaviour and colouration. In the barn owl, females display bigger black spots than males, whereas in kestrels females display smaller black tail bands than males (Piault et al. in press). Thus, eumelanism is a typical female-like trait in the barn owl and typical male-like trait in the kestrel. As a consequence, individuals behave as typical males when displaying small black spots in the barn owl and large black bands in the kestrel.

In both the kestrel and barn owl eumelanin colouration is associated with behaviour and breathing rate. There are other examples of relationships between melanin colour and behaviour. A

study on Marsh harriers (*Circus aeruginosis*), which display colouration most likely due to melanins, also showed different anti-predatory responses for males of different colour morphs (Sternalski and Bretagnolle 2010). In this study it was found that greyer males were far less often involved in mobbing a predator than browner males, while the number of alarm calls was similar between the two morphs, suggesting morph-specific roles. Boerner and Kruger (2009) showed that in common buzzards (*Buteo buteo*) there were differences between different (supposedly eumelanic) morphs which were, interestingly enough, reversed between the two sexes. In males lighter coloured individuals were more aggressive towards predators, whereas in females it was the reverse. Towards conspecifics, both sexes responded most strongly to similarly coloured individuals. These two examples show that individual differences in behaviour related to melanin colouration can occur, although the relationships can be sex- or species-specific. Indeed, it has been found that within individuals some phenotypic traits can be positively correlated with eumelanin content but negatively with pheomelanin content (Roulin et al. 2011a). However, until the mechanism responsible for the covariance between behaviour and melanin colour traits has been clearly identified, as well as until the identity of melanin pigments have been determined in each species, it remains difficult to draw general conclusions.

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3. Melanin-based colouration predicts natal dispersal in the barn owl *Tyto alba*

Valentijn van den Brink, Amélie N. Dreiss and Alexandre Roulin

Department of Ecology and Evolution, University of Lausanne, Switzerland

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Personal contribution: Together with the two co-authors I designed and performed the analyses and wrote the manuscript.

Abstract

Searching a suitable breeding site is an important decision in the life of most animals. The decision about where to settle and how far to travel before doing so depends on many factors. Individual differences in dispersal distance could be either the result of different strategies (e.g. specialists versus generalists), which might result in similar reproductive success in different habitats, or of different competitive abilities to acquire a territory close to the natal site. The barn owl (*Tyto alba*) displays a polymorphism in melanic colouration which is associated with many physiological and behavioural traits such as habitat choice, stress response and docility, raising the possibility that the colouration is also related to dispersal. We studied the movement of individual barn owls from the site of rearing to the site where the first breeding attempt takes place (natal dispersal) and from one breeding site to the next (breeding dispersal) using a long-term dataset. Darker reddish individuals moved farther than paler individuals during natal dispersal, but not during breeding dispersal. A cross-fostering experiment where we swapped hatchlings between randomly chosen nests showed that the colour of the biological and foster parents had no influence on dispersal distance. The distance dispersed of parents and same sex offspring is correlated, whereas natal and breeding dispersal were not repeatable within individuals indicating that they are two different processes. Given that the distance travelled in natal dispersal appears to be heritable, the underlying genes might be coupled to those related to colouration. We discuss different hypotheses to explain the potential adaptive function of the link between colouration and natal dispersal.

Introduction

Dispersal is an important aspect of population dynamics. The choice of a territory can determine to a large extent breeding success (Cody 1981), making it an important event in the life of an individual. The movements in the period preceding a breeding attempt are usually divided into two categories: natal dispersal, which can be defined as the movements from the site where an individual was born to the site where it will first breed; and breeding dispersal, which is the distance between the two locations of successive breeding events (Greenwood and Harvey 1982). The distances covered in natal dispersal are usually greater than those in breeding dispersal.

How individuals decide where to settle, and how great a distance they will travel before settling depends on many factors. Individual strategies and environmental factors can explain inter-individual variation in dispersal distances. Intraspecific competition for mates or other resources contribute greatly to dispersal (Johnson and Gaines 1990, Perrin and Mazalov 2000), so that individuals that are outcompeted have to move more. Body condition might also be of importance (Bowler and Benton 2005, Clobert et al. 2009), although it is not clear if individuals in better condition are more or less likely to disperse than individuals in worse condition (Belthoff and Dufty 1998, Meylan et al. 2002, Bonte and de la Pena 2009). Local adaptation sometimes also plays an important role in the decision where and when to settle down (Edelaar et al. 2008). Some traits are better suited for different local environments and this would greatly influence the decisions individuals have to make when searching a breeding location. Local adaptation can also impact the direction or the distance moved to a new nest site. For example, in response to predators or as a predator, individuals with different skin or plumage colouration might be better camouflaged in different habitats (Bortolotti in: Hill & McGraw 2006).

Another possibility that might lead to observed differences in dispersal behaviour is that the searching and moving behaviour is influenced by innate individual differences. Dispersal is a risky undertaking, with the possibility of predation during dispersal (Yoder et al. 2004) or of not being able to find a suitable breeding site in the unknown destination area. It is therefore not surprising that

dispersers can differ in personality, physiology or morphology from non-dispersers (Cote et al. 2010). When individual differences in behaviour are consistent and repeatable, they can be called temperament or animal personalities (Sih et al. 2004a, Reale et al. 2007). Suites of personality traits can also be correlated. For example, explorative and less explorative individuals are often divided into “bold” and “shy” categories, although these are extremes of a continuum (Reale et al. 2007). An example is the Trinidad killifish (*Rivulus hartii*), in which, when corrected for sex, predation risk and body size, individuals categorised as bold in behavioural tests in the laboratory, dispersed farther than shy individuals when returned in natural conditions (Fraser et al. 2001). Bold individuals are also more likely to migrate in roach fish (*Rutilus rutilus*; Chapman et al. 2011) and even in humans individuals with increased activity levels (a trait that is associated with boldness/shyness; Reale et al. 2007) migrate more often (Jokela et al. 2008). Thus boldness and dispersal seem related to each other.

On a European scale barn owls (*Tyto alba*) show a gradient in pheomelanic and eumelanic colouration from south to north (Roulin 2003). This gradient might reflect differences in the specific habitat requirements of the different colour morphs (Antoniazza et al. 2010). The females tend to disperse farther than males (Taylor 1994) and we already demonstrated that white non-pheomelanic males have a higher chance to be recruited in our local Swiss population than their darker reddish pheomelanic siblings (Roulin and Altwegg 2007). Reddish individuals eat more voles and grow longer tails than whiter individuals, who eat more mice (Roulin 2004a, 2006). Furthermore, it appears that whiter individuals breed more often in wooded habitats than reddish conspecifics (Dreiss et al. 2012). We also found a relationship between a eumelanic trait (diameter of black spots located at the tip of ventral feathers) and personality traits in our study population of barn owls (Van den Brink et al. 2012a)). The results already found in associations between colour and habitat choice and between colour and personality traits make this species suitable to investigate a possible relationship between dispersal behaviour and both pheomelanin- and eumelanin-based colouration. Since

dispersal is sometimes also associated with personality traits we can assume that either of the two possible mechanisms plays a role in possible differences in barn owl dispersal distances.

We first studied the relationship between the individual colour and the natal dispersal of recruits; individuals born in the study area, that ultimately also breed there. During the study period, cross-fostering experiments were performed, giving us the opportunity to study genetic and environmental influences on dispersal. Secondly, we followed the movements between consecutive breeding attempts of individuals breeding in our study area during their breeding career, the breeding dispersal. We investigated if differences in breeding dispersal distance between individuals were related to their melanic colour and several other traits such as habitat characteristics or life history traits such as breeding success, age at first breeding and wing and tail length. Natal and breeding dispersal take place in different life stages and therefore we might see differences in distance travelled and underlying factors causing the two types of dispersal. Finally, we investigated whether dispersal distance and/or colouration are also related to reproductive success. We will discuss if local adaptation, personality or other factors can explain colour-specific dispersal behaviour.

Methods

Study species and study area

Between 1994 and 2010 we studied a population of barn owls breeding in the Broye plain, a 25 km x 15 km area in western Switzerland (46°49′ N; 06°56′ E). The Veterinary Service of the Canton of Vaud, Switzerland, authorised this study under license number 1146, allowing us to take blood samples for DNA and cross-fostering of nestlings. The Swiss Ornithological station, Sempach Switzerland, authorised the ringing of individuals. This population breeds mostly in nest boxes placed on farms. The barn owl is a medium-sized owl with worldwide occurrence. It hunts mainly voles and mice in open agricultural areas (Glutz von Blotzheim 1987). It displays a distinct colour polymorphism

ranging from spotless white to dark reddish pheomelanic and heavily marked with eumelanic black spots. Males are generally paler pheomelanic and show fewer and smaller black spots than females, although both sexes can display any phenotype (Roulin 1998b). The laying period ranges from February until August and the last fledglings appear as late as November. Clutches consist of two to nine eggs. Hatching occurs asynchronously with 2.5 day-intervals, because the mother starts incubation directly after laying the first egg. This can result in an age difference of up to three weeks in a nest of nine offspring. After fledging at approximately 55 days of age, juveniles remain in the direct vicinity of their nest site for 1-2 months where they still receive regular feedings from their parents (Glutz von Blotzheim 1987, Taylor 1994). When individuals have become independent they will disperse and try to find an own breeding site. As in most bird species (Clarke et al. 1997), females generally disperse farther than males, which might be driven by inbreeding avoidance (Greenwood and Harvey 1982). The average migrating distances vary tremendously between populations, with mean distances ranging from within 10 km for a population in Britain to between 50km and 100 km for German and Dutch populations (Taylor 1994, Kniprath 2010). Dispersal distances appear related to food availability, with increasing distances in years with low vole densities (Taylor 1994). The distances reported here are mostly recoveries of dead birds, which does not rule out the possibility that dispersal distances are inflated due to transport between death and recovery (e.g. on the bumper of a car; Taylor 1994).

Cross-fostering

Between 1994 and 2010 we performed cross-fostering experiments, where nestlings from one nest were raised in another nest. 55 out of the 185 studied individuals were cross-fostered. For more details see (Roulin and Dijkstra 2003, Roulin 2006). Pairs of nests were matched by hatching date and part or all of the nestlings were swapped between them. We did this by immediately transporting nestlings by car from the nest of origin to the nest of rearing. During this procedure they were placed in a cloth bag, which was kept close to the body of one of the researchers to insure the

nestlings did not get cold during the time it took to drive between nest sites. The transport was always completed within one hour. This allowed us to study if the dispersal behaviour was heritable by comparing dispersal distances between cross-fostered nestlings with those of their biological and foster parents. If local adaptation plays a large role in natal dispersal, we can expect cross-fostered individuals to not be optimally adapted to the habitat where they were raised. This might then lead them to disperse farther than non-cross fostered individuals.

Assessment of colouration

At the age of fledging at about 55 days old, [initials of researcher, removed for review process] measured colour traits (pheomelanic colouration, number of eumelanic spots and eumelanic spot diameter) and biometric measurements for all nestlings. Adult individuals were measured each year during the breeding season with females being distinguished from males by the presence of a brood patch. For our analyses we used the measurements of an individual made in the same year as the year under investigation. The methods of measuring and the exact measurements taken are described in more detail in (Roulin 1999b). Colour traits are heritable, both spot diameter (h^2= 0.82; Roulin et al. 2010) and pheomelanic colouration (h^2 = 0.81; Roulin and Dijkstra 2003). For the adults we determined the age of individuals either by their moult pattern (for method see Taylor (1994); *n* = 192 individuals), or directly if we had ringed the individual as a nestling (*n* = 177).

Individual traits

To demonstrate that a relationship between dispersal distance and colour is not inflated by other variables, we included a number of covariates in our statistical models. We included age rank in the nest, since the pronounced hatching asynchrony in the barn owl might influence the outcome of competition among siblings for nesting sites. The second covariate was the number of fledglings in the nest of rearing, because the distance dispersed might increase with increasing number of siblings fledged, as this increases the chance of inbreeding and competition over nest sites if no dispersal

takes place (Szulkin and Sheldon 2008). Morphology can also influence migration behaviour (Paradis et al. 1998, Dawideit et al. 2009) and therefore we included the adult wing length and tail length in two separate models for natal dispersal and breeding dispersal. Two more traits were included for breeding dispersal only: whether a change of partner between two consecutive breeding attempts occurred and the number of partners in the breeding career, controlled for the total number of breeding attempts. Change of partner may induce dispersal and a high number of partners might also influence dispersal tendency.

Measuring dispersal distance

We used the linear distance in km from the nest site of rearing to the breeding nest site for natal dispersal, or from the breeding site in one year to the site in the next year for breeding dispersal. Here we assumed that natal dispersal can occur in any direction, because other studies found no clear directionality in dispersal, other than determined by geographical features (Taylor 1994, Marti 1999, Matics 2003). We performed a log + 1 transformation on the dispersal distances to improve normality. A second measure available to us was the number of nest boxes that were closer to the nest box of rearing than the nest box where the individual finally attempted to breed. This method does not qualitatively change the results (not shown), and therefore we will focus on the linear distance as a measure of dispersal. For the breeding dispersal we calculated the distances between consecutive breeding locations in different years. If more than one attempt took place in the same year (about 10% of the pairs attempt to breed more than once), the distance between the first attempt of that year and the last attempt of the previous year was used. The average number of attempts during the recorded breeding career for males was 3.5 ± 0.15 SE (range: 1 to 14) and for females 3.3 ± 0.12 SE (range: 1 to 12).

Our measure of dispersal in a limited area has the potential bias that we will miss dispersal events from those individuals that disperse beyond the borders of our study area. The tail of the dispersal kernel might contain individuals that differ in their reproductive output (this could be

different in positive or negative direction). The ring recovery data is too fragmented, however for us to make inferences about events outside our study area.

Breeder density and dispersal distances

When analysing dispersal, simply calculating the distance dispersed is not an appropriate measure, because the number and the location of available nest sites can strongly influence the dispersal distance (Van Noordwijk 1984). Individuals in the centre of a study area are therefore more likely to pass more possible breeding sites before reaching the edge of the study area. An alternative method is to calculate the number of potential breeding sites an individual crosses before it reaches the site where it finally breeds (Mateos-Gonzalez and Senar 2012), but this data is unavailable in our study population, because we do not know the territory sizes and limits. Therefore, we decided to use a number of covariates in our models to take this problem into account and demonstrate that our results are robust. We included the distance from the nest box of rearing to the centre of our study area and the number of nest boxes surrounding the breeding site as a proxy for the number of suitable territories nearby. We included the number of nest boxes present in a 2 km radius around the rearing nest site where the focal individual was raised or bred as an adult and hence from where it dispersed. The mean home range of breeding male owls in our population was shown to be 335 ha, which would give a radius of slightly over 1 km (Arlettaz et al. 2010), but because there is large variation in home range sizes we decided to use a 2 km radius for our analyses (when using a narrower, 1 km radius our results do not change qualitatively). For both natal dispersal and breeding dispersal analyses we only considered individuals recaptured during a breeding attempt inside of our study area.

Natal dispersal of parents and their offspring and individual dispersal tendency

Our long-term dataset provided us with the opportunity to compare natal dispersal distances for 53 individuals that were recruited into our study population with the natal dispersal distances of

their offspring that were also recruited locally. We compared dispersal distances of both parents with those of their offspring of both sexes. We also added cross-fostering status of the offspring to the model (i.e. either raised by biological or foster parents). Another trait tested was the individual tendency to disperse by comparing first natal dispersal distance with breeding dispersal distance for the same individual and second breeding dispersal within the same individual. If dispersal is part of a behavioural type (Sih et al. 2004b), an individual that is more likely to disperse farther in natal dispersal might be also more likely to disperse later in life. We calculated the Pearson's correlation between the individual log + 1 transformed natal and breeding dispersal distances of 75 individuals for which both distances were available. For individual breeding dispersal we calculated repeatability from a linear mixed model, following methods described in Nakagawa and Schielzeth (2010).

Habitat characteristics

We recorded a number of other characteristics of the breeding sites. Since reddish individuals are known to breed more often in open areas than paler individuals who breed more frequently near forests (Dreiss et al. 2012), we also measured the amount of forested area in a 1.5 km radius around the nest box of rearing (see Frey et al. (2011) for a detailed description of methods). If local adaptation plays a role in the natal dispersal movements, we can expect reddish individuals that were raised in a forested area to move farther than if raised in a site where forests are less abundant, perhaps to reach an area similar to the nest site of rearing.

Statistical methods

All statistical tests were performed with the program JMP 9.02 (SAS 2010). Dispersal distances were log+1 transformed, since this improved normality strongly (Shapiro-Wilk's test for goodness-of-fit on transformed values: P = 0.49). All tests are two-tailed, non-significant effects were removed sequentially, non-significant interactions first and p-values <0.05 are considered significant.

Natal dispersal

We studied the natal dispersal distances for 185 individuals (116 males and 69 females) out of 3128 fledglings (5.9%); the other fledglings either did not breed in our study area or did not survive until breeding. With the information of these individuals we then constructed general linear mixed models with the pheomelanic plumage colouration score of the individuals as response variable. In initial models, also eumelanic traits were explored, but number of spots and spot diameter did not explain any variation in distances dispersed and thus we did not study these traits further. The dependent variable was the log + 1 transformed distance between the nest of rearing and the nest where the first breeding attempt took place. Identity of the biological mother was included as a random factor to account for non-independence of nestlings born from the same parents (30 out of 97 mothers produced more than one recruit into the population). This factor explains the most (54%) variation in the final model. If we replace the biological mother with one of the other parents, however, the results (not shown) do not change qualitatively. We also included nest site identity as random effect, to account for offspring originating from the same nest site, but possibly different parents. The other variables included in the model, including interactions with colour are shown in table 1.

Breeding dispersal

We only considered adult individuals that had attempted breeding more than once in the study area, which allowed us to measure the distance between two consecutive breeding sites. Our sample consisted of those recruits used for the natal dispersal analyses that bred more than once (n = 75) and the individuals that we ringed as adults or that were ringed outside the study area by amateur ornithologists and later dispersed to our study area to breed there (n = 295). This resulted in a total of 369 individuals breeding more than once out of 976 ringed individuals that attempted to breed at least once in our study area. We used the log + 1 transformed distance between boxes in consecutive years as the response variable, thus the distance for an individual that bred in the same

location twice was zero here. We included the identity of the individual and the year of birth as two random variables (explaining 14% and 2% of variation respectively) to account for more than one breeding attempt of each individual (mean number of attempts: 3.4 ± 0.1 *SE*; range: 2-14 attempts). We include a change of partners between years, as unpublished data shows that after a divorce, dispersal is likely to occur. We do not limit ourselves to divorce (where both partners are confirmed to be still alive the next year), but analyse all cases where an individual had a new partner between years. For this model the variables and interactions included in the full model are presented in table 2. As with natal dispersal, we did not find any effect of eumelanic traits in initial models and thus we focused on pheomelanic traits.

Dispersal distance and reproductive success

To assess the relationship between dispersal distance and reproductive success in the year following the dispersal event, we constructed models for the recruits, as well as for the breeding birds. Laying date, clutch size, the number of fledglings and whether or not any fledglings were produced were used as response variables in separate models. Predictors were the distance dispersed, sex, colouration (pheomelanic or spot diameter) and the interactions between these traits. Identity of the mother and year were included as random variable in the model for the recruits. The density of nest boxes around the site of breeding was included to account for possible competition for breeding sites. The age at the first breeding attempt and the distance from the box of rearing to the centre of the study area were also included.

Comparison of natal dispersal distance between parents and offspring.

We modelled the log-transformed offspring natal dispersal distance on the same-sex parent natal dispersal distance with a mixed model with restricted maximum likelihood. Six adults were biological parents of more than one recruit and therefore we included parent identity as a random factor in our model. These recruits were always raised in the same year and the same nest (the nest of origin),

therefore no other random variables were included. We included sex and whether or not individual was crossfostered as fixed effects. We also tested dispersal of offspring and their opposite sex parent with a similar model.

Results

Natal dispersal

We studied 185 individuals originating from 155 nests. The mean distance dispersed was 8.7 ± 0.7 km for males and 11.2 ± 0.9 km for females (mean for both sexes: 9.6 ± 0.6 km). The full model and statistics are presented in table 3.1. When controlled for the distance from the centre of the study area where an individual was raised, which positively affects the dispersal distance ($F_{1,75.15}$ = 12.1, P = 0.0009), the final mode l showed that darker reddish pheomelanic individuals disperse farther than those that are paler ($F_{1,98.8}$ = 4.95, P = 0.03). . Interestingly, individuals with shorter wings tended to move longer distances ($F_{1,87}$ = 3.67, P = 0.04). The interaction between the colour and cross-fostering status was also significant ($F_{1,\ 102.8}$ = 10.7, P = 0.001). Upon closer inspection, only in non-cross-fostered individuals (n = 130; $F_{1,66.4}$= 22.3, P < 0.0001, figure 3.1a) is there a significant relationship between natal dispersal and colour. In cross-fostered individuals (n = 55; $F_{130.1}$= 0.46, P = 0.50, figure 3.1b) no differences with colour are detectable. We investigated if the dispersal distance was also related to the pheomelanic colouration of the parents, because colour is strongly heritable. We found no relationship between the colouration of the biological or foster parents and the natal dispersal distance of the recruits (all P-values > 0.29).

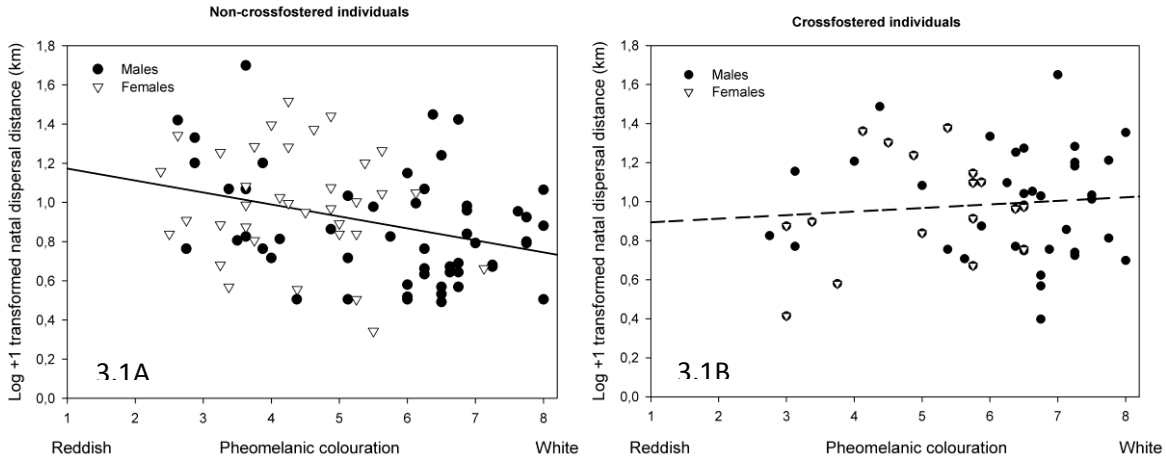

Figure 3.1. Log + 1 transformed natal dispersal distance versus individual pheomelanic colour for non-cross-fostered (1A) and cross-fostered (1B) fledgling barn owls. Males are indicated by closed circles, females by open triangles. Regression line in figure 1B is drawn for illustrative purposes only.

Table 3.1 Model explaining variation in natal dispersal in relation to individual pheomelanic colour of fledgling barn owls. Significant terms and terms involved in significant interactions are written in bold and kept in the final model. Presented are parameter estimates, degrees of freedom, F-ratios and P-values for fixed effect terms included. Terms removed from the model are in order of removal, the last removed on the top.

Variable	Estimate	DF (1,x)	*F-ratio*	*P-value*
Log-distance to centre of area	**0.33**	**75.2**	**12.05**	**0.0009**
Cross fostered*Colour	**-0.056**	**102.8**	**10.71**	**0.0014**
Colour	**-0.039**	**98.8**	**4.95**	**0.028**
Cross fostered	**-0.030**	**99.3**	**0.93**	**0.34**
Wing length as adult	**-0.008**	**103.9**	**4.14**	**0.045**
Age at first breeding	0.02	64.1	0.62	0.43
Nr of sites within 2km	-0.006	83.7	0.72	0.40
Forest cover	-0.00005	43.7	0.72	0.40
Sex	-0.015	81.3	0.48	0.49
Age rank in nest of rearing	0.005	56.2	0.06	0.81
Wing length as adult*Colour	-0.003	86.5	1.52	0.22
Age at first breeding*Colour	-0.019	83.2	1.59	0.21
Log-distance to centre of area*Colour	-0.057	76.9	0.41	0.52
Sex*Colour	-0.015	78.4	0.35	0.56
Forest cover*Colour	<0.00001	60.8	0.20	0.65
Age rank in nest of rearing*Colour	0.006	77.7	0.19	0.67
Nr of sites within 2km*Colour	-0.001	87.0	0.04	0.85

Comparison of natal dispersal distances of parents and offspring

We found that natal dispersal distances of parents and same-sex recruits were similar (31 individuals:

estimate= 0.43 ± 0.18, $F_{1, 9.1} = 5.9$, $P = 0.03$, figure 3.2). Sex and cross-fostering had no effect ($P = 0.19$

and P = 0.22, respectively). When testing opposite sex parents and offspring, we found no significant effects (all P-values > 0.23).

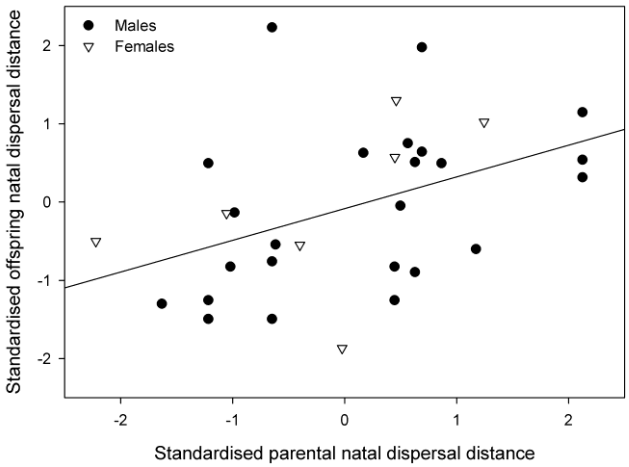

Figure 3.2 Relation between natal dispersal distance of parents and their same-sex offspring in the barn owl. Males are indicated by closed circles, females by open triangles.

Breeding dispersal

Adult females (n = 189) dispersed farther than males (n = 184) between years (3.5 ± 0.2 km vs. 1.8 ± 0.1 km, $F_{1,276.3}$= 49.0, P < 0.0001), as did individuals that had a different partner compared to the year before ($F_{1,857.2}$= 143.7, P < 0.0001; average: 3.1 ± 0.17 km). The greater the number of nest boxes surrounding the nest box of the first year, the farther individuals dispersed ($F_{1,475.8}$ = 8.2, P = 0.004) and the older individuals were, the more site-faithful they became ($F_{1,386.7}$ = 6.7, P = 0.01). The details of the model are displayed in table 3.2. The interaction between sex and change of partner was also significant ($F_{1,856.6}$ = 54.3, P < 0.0001) and upon closer inspection of the results we found that females dispersed almost twice as far as males in individuals that had changed partner ($F_{1,220}$ = 92.2, P < 0.0001). If we look at the two sexes separately, both in females ($F_{1,429}$ = 142.16, P <0.0001) and in males ($F_{1,426}$ = 18.14, P <0.0001) individuals that changed partners between years dispersed farther than individuals that did not (figure 3.3). No colour trait influenced the distance dispersed from year to year (P-values all > 0.48). The differences between individuals in distance dispersed were relatively small and to ensure we did not miss any possible relations because our statistical power was too low,

we also built models with dispersal as a binomial response. The other variables included were the same as for the distance-models. The results obtained from these models were qualitatively the same as those for the distance-models (not shown).

Table 3.2 Model explaining variation in breeding dispersal of adult barn owls. Significant terms and terms involved in significant interactions are written in bold and were kept in the final model. Presented are parameter estimates, degrees of freedom, F-ratios and P-values for fixed effect terms included. Terms removed from the model are in order of removal, the last removed on the top.

Variable	Estimate	DF (1,x)	F Ratio	P-value
Change of partner	**-0.108**	**857.2**	**143.697**	**<.0001**
Sex	**-0.072**	**276.3**	**49.007**	**<.0001**
Sex*Change of partner	**-0.066**	**856.6**	**54.293**	**<.0001**
Nr of sites within 2km	**0.011**	**475.8**	**8.216**	**0.004**
Age	**-0.012**	**386.7**	**6.688**	**0.010**
Forest Cover	0.000	363.1	2.991	0.085
Log-distance to center of area	-0.063	295.2	1.879	0.172
Colour	0.007	272.1	1.033	0.310
Age at first breeding	0.009	205.7	0.906	0.342
Nr of partners	0.006	216.8	0.471	0.493
Nr of breeding attempts	-0.004	188.8	0.203	0.653
Sex*Colour	-0.008	28.6	1.171	0.280
Age*Colour	0.002	573.8	0.610	0.435
Nr of partners*Colour	-0.005	255.3	0.769	0.381
Log-distance to center of area*Colour	-0.023	268.5	0.777	0.379
nr of sites within 2km*Colour	-0.002	500.5	0.845	0.358
Age at first breeding*Colour	-0.005	430.2	0.434	0.511
Forest cover*Colour	-0.00002	521.3	0.220	0.640
Change of partner*Colour	-0.002	769.8	0.086	0.769
Nr of breeding attempts*Colour	0.002	356.5	0.091	0.763

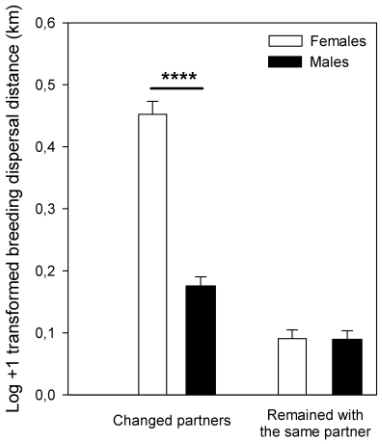

Figure 3.3 Log +1 transformed breeding dispersal distances for male and female barn owls that changed partners or that remained with the same partner between consecutive years. The distances are shown on a log scale. Stars indicate significant differences between categories (<0.0001).

Within-individual repeatability in dispersal behaviour

We found no relationship between natal dispersal and the mean distance dispersed in all breeding dispersal events (r = 0.13, P = 0.26) for the 75 recruits of which we know both the natal and at least one breeding dispersal distance. Breeding dispersal is repeatable between years for the same individual when adult however (adjusted repeatability: 0.26, CI 0.19-0.33; following recommendations in Nakagawa & Schielzeth (2010)). This repeatability might seem low, but migration tendency is among the personality traits with lowest repeatability and these results fall within the reported range for the personality traits recorded (Bell et al. 2009).

Dispersal distance and reproductive success

We did not find any relationship between dispersal distance and colouration with any of the reproductive parameters, neither for the recruits in the first attempt after natal dispersal, nor for the breeding birds in consecutive breeding attempts. We do see that for the recruits, a higher density of nest boxes surrounding the nest box where the first breeding attempt took place means that individuals start breeding later in the season ($F_{1,156.7}$ = 4.7, P = 0.03) and tends to result in a lower number of fledglings produced ($F_{1,152.2}$ = 3.4, P = 0.07).

Discussion

In the barn owl, darker pheomelanic individuals moved greater distances between the site where they were raised and the site where they bred for the first time than paler individuals. In contrast, the degree of eumelanin-based coloration was not associated with breeding dispersal behaviour. Our results were robust because the relationship between natal dispersal and pheomelanin-based coloration remained significant even after controlling for a number of covariates. There was also no relationship between reproductive parameters and the distance dispersed in the preceding year, which might indicate that the differences we find in dispersal distance are caused by an innate tendency to disperse, which in turn might be linked to colouration. However, the

reproductive parameters measured are for individuals that are fit enough to attempt breeding, since we could not measure dispersal distances of those individuals that do not manage to breed or breed outside of our study area. Inter-individual variation in pheomelanin-based coloration is strongly heritable (h^2 = 0.81; Roulin and Dijkstra 2003) and dispersal distances are partially heritable (i.e. parents and offspring showed similar dispersal distances). Therefore, the relationship between natal dispersal and coloration may be, at least in part, genetically inherited.

We found an association between dispersal distance and coloration only for natal dispersal but not for breeding dispersal. We did not find a relation between the natal dispersal distance and the breeding dispersal distance indicating that natal and breeding dispersal are two separate processes with different underlying motivations (Greenwood and Harvey 1982). Generally, breeding individuals become more philopatric, as we also showed here, making the distances travelled between consecutive nesting sites shorter and more similar for all individuals and thereby reducing our power to detect differences. Males are holding the nest sites (Taylor 1994, Roulin 1998a), making a female that leaves her partner more likely to also leave the nest and increasing the distance needed to find a suitable new partner and nest site. The strong effects of sex and of a change of partner on dispersal distance in the breeding dispersal seem to point out that there are other factors than coloration contributing strongly to breeding dispersal. One of those might be competition, since the number of nest boxes (which can be a proxy for possible competition for nest sites in the area) surrounding the site of breeding negatively influences the number of fledglings produced and increases the laying date for the recruits.

We can only make inferences about those individuals that remain inside our study area and attempt to breed there and it could be that one colour is more likely than the other to disperse much farther, thus biasing our results. This is a central issue in dispersal studies and cannot be easily remedied (Koenig et al. 1996). Given the strongly skewed dispersal kernels usually found in barn owls (Taylor 1994, Marti 1999, Kniprath 2010) it is nevertheless likely that most individuals remained and

tried to breed inside the area. However, fitness results might be different for those few individuals in the tail of the kernel (Doligez and Pärt 2008). Since we do not know where they went and what became of them we cannot speculate on how the results would have changed if we could have included all individual breeding attempts.

Potential mechanisms explaining colour-specific dispersal

In the following we discuss five potential mechanisms that can explain the relationship between natal dispersal and pheomelanin coloration. A first possibility is that darker reddish individuals might be more sensitive to inbreeding than lighter coloured conspecifics leading them to disperse larger distances to reduce the risk of inbreeding. Dispersal is often mentioned as mechanism to avoid inbreeding and thus it might also play a role in our population. Such a relation could be formed through linkage disequilibrium, where a trait related to sensitivity for inbreeding is also related to colour. Unfortunately, no data are yet available to further test this hypothesis. Second, whiter birds, that disperse shorter distances in our study, might be favoured if they would be locally adapted and in this case the results we find between colour and dispersal could be specific to our population. Assuming that the adaptation is to different habitats and assuming that parents breed in their optimal habitat, we would expect cross-fostered nestlings to disperse farther. We do not, however, find a relationship between colour and dispersal distance for cross-fostered individuals. This is slightly surprising since we could at least expect them to follow a pattern similar to non-cross fostered individuals if local adaptation played a role. We also do not find a difference in distance dispersed between cross-fostered and non-cross-fostered individuals. We do not know what causes this difference but because we see different patterns, but no different distances, we believe local adaptation (alone) is unlikely to explain our results. Third, as proposed in Roulin & Altwegg (2007), whiter birds could have a mating advantage and in this case our results could be applicable to other populations as well. If so, both males and females would enjoy such a mating advantage. Fourth, individuals may disperse greater distances if a dark coloration is in linkage disequilibrium with

another phenotypic trait that is important for dispersal. An example of such linkage disequilibrium was found in our study population where darker reddish males were mated with longer-tailed females and hence produced dark offspring with long tails (Roulin 2006). However, we only found that wing length, but not tail length, tended to be associated with natal dispersal distances but in an unexpected direction: longer-winged birds tended to disperse shorter distances which is surprising given that longer wings might equal better flying abilities and thus easier dispersal (Dawideit et al. 2009, but see Paradis et al. 1998). Perhaps it has to do with manoeuvring ability in different habitats (e.g. forested versus more open), which would point towards an effect of local adaptation, but currently we cannot say if this is a statistical artefact or a genuine difference. There is thus no evidence that darker birds disperse more because of genetically correlated traits that facilitate dispersal. This leads us to the fifth hypothesis advocating that individual differences in the distance dispersed could be due to colour-specific personality. Dispersal is often linked to boldness and exploration (Budaev 1997, Dingemanse et al. 2003). It might be particularly beneficial for reddish birds to be explorative, which would lead to the observed difference. The phenotype of reddish birds could allow them to explore and settle in unknown areas more easily than white birds. As a consequence they could invade already existing populations of whiter barn owls quicker or allow settlement in areas previously free of barn owls. Another option is that selection is being exerted on another (personality) trait that is genetically correlated to exploration or dispersal behaviour, but that there is no direct selection on reddish birds to be more explorative than white birds.

Our findings have brought up many new and exciting questions about the mechanisms that could explain the causes for the observed relationship between colouration and dispersal. We believe that it would be interesting to continue measuring personality traits such as boldness and exploratory behaviour in individual barn owls so that when more data becomes available we can construct pedigrees and calculate heritability estimates to investigate if the personality traits and colouration measured as juveniles will match dispersal behaviour later in life.

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4. Pheomelanin-based colouration 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

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

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

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

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Personal contribution: I helped design and perform the experiments, analysed part of the data and wrote the paper.

Abstract

Nest defence is an activity where individuals take risks and hence jeopardize future reproduction. To test whether individuals vary in nest defence behaviour, we studied the tawny owl (*Strix aluco*), an emblematic species as it attacks humans who approach their nest. A potential phenotypic correlate of nest defence behaviour is melanin-based colour polymorphism, since dark and light melanic individuals display alternative life history strategies. During daylight hours our presence induced females to be active more often when they were dark than light reddish. This suggests that darker females are more willing to defend their progeny against humans. To test this hypothesis, we performed playback experiments. As expected, when we broadcasted territorial calls of a male tawny owl, darker females defended their nest more intensely. A similar playback experiment performed at night the following year showed the stronger nest defence by dark than light reddish birds is not restricted to females. Furthermore, they responded more vigorously to a stuffed dark than light reddish tawny owl placed beside their nest. Indeed, darker males responded stronger when we broadcasted calls of a male tawny owl in their territory. The bolder behaviour of dark than light reddish individuals is adaptive since dark birds suffer lower nest predation. The fact that dark compared to reddish stuffed owls elicited a stronger nest defence reaction from breeding birds strongly suggests that coloration itself is used in social interactions.

Introduction

Life history theory proposes that individuals have to optimally allocate resources among energy-demanding and time-consuming life history traits (Stearns 1992). Some of the most important decisions are to decide whether to invest in reproduction vs. maintenance, in offspring number vs. quality, or 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). 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, and where thus different strategies quickly develop if information about the competitors intentions is available. It is also possible for different behavioural types to evolve by 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(Gross 1996). Examples are colour-specific mating strategies in male ruffs *(Philomachus pugnax;* van Rhijn 1973, Lank et al. 1995) or reproductive strategies in the white throated sparrows (*Zonotrichia albicollis;* e.g. Tuttle 2003).

Anti-predator behaviour is critical to organisms because nest depredation is one of the major causes of reproductive failure in many bird species (Martin 1995). Nest defence behaviour is therefore likely to be linked with key life-history trade-offs between offspring quality vs. number or between current offspring vs. future reproduction as predicted by 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 because of the relative value of current reproduction compared to future reproduction. Parents that invest more energy in anti-predatory behaviour

ensure the production of a large number of high quality offspring. In this case, the cost incurred by this defence behaviour (i.e. higher adult mortality caused by predators, Montgomerie and Weatherhead 1988) and physiological costs due to aggressive behaviour, such as the cost of increased testosterone levels (Marler and Moore 1988) should be lower than the benefits generated by rearing high-quality offspring. The cost/benefit ratio may differ between individuals (Montgomerie and 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).

Melanin-based colouration 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 both melanin pigments (grey-black eumelanin and reddish-brown pheomelanin) is frequently reported to covary with morphological, physiological, behavioural or life history traits (Roulin 2004b). In many vertebrates, eumelanic individuals were frequently found to be more resistant to stress, more sexually active and more aggressive (Ducrest et al. 2008). In the common buzzard (*Buteo buteo*) for instance, melanin-based colouration covaries with aggressiveness (against both predators and conspecific competitors) and fitness components (Boerner and Kruger 2009). Such covariations are likely to be the result of pleiotropic effects of key regulators of the melanogenesis (Ducrest et al. 2008). For instance, melanocortins which are products of the proopiomelanocortin gene (*POMC*) can indeed induce eumelanogenesis by binding to the MC1 receptor and promote aggressiveness by binding to the MC5 receptor (Morgan et al. 2004). Melanin-based colouration can thus be associated with some behaviours (*e.g.* boldness, aggressiveness) 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 colouration 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 (Galeotti and Sacchi 2003, Roulin et al. 2003, Brommer et al. 2005, Roulin et al. 2008, Gasparini et al. 2009, Piault et al. 2009, Karell et al. 2011b, Roulin et al. 2011b). 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 (Roulin et al. 2003). In contrast, reddish individuals seem less flexible, adopting a constant reproductive investment (Piault et al. 2009, Roulin et al. 2011b) and produce fewer but higher quality offspring (Roulin et al. 2004, Roulin et al. 2008). Given their parental investment in high-quality offspring, reddish individuals may be more aggressive and bolder during nest defence to increase their offspring survival. 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 human who approach their nest too closely. We thus predict that dark reddish individuals display bolder behaviour than light 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 1987), females are also responsible for nest defence (Wallin 1987), whereas, in territorial disputes, males and females were found to be equally active (Sunde and Bolstad 2004). Since tawny owls remain territorial during the breeding season (Glutz von Blotzheim 1987), we performed playback experiments in two consecutive breeding seasons, during daylight and at night. In one year

we also presented a mounted model of stuffed owls (either dark or light reddish) during these playback experiments to record if response differed according to the colour of intruder (i.e. stuffed bird) or defender. Several studies already reported associations between anti-predator behaviour and melanin-based coloration in marsh harriers (*Circus aeruginosus;* Sternalski and Bretagnolle 2010), common buzzards (*Buteo buteo;* Boerner and Kruger 2009), barn owls (*Tyto alba;* Van den Brink et al. 2012) and European kestrels (*Falco tinnunculus;* Van den Brink et al. 2012b). Therefore, we predict that if reddish colouration 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 1987). 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 species exhibits sexual dimorphism; females are larger than males (Glutz von Blotzheim 1987) and defend the nest against predators more than males (Wallin 1987). The reddish colouration is independent of age and sex and is highly heritable (h^2 = 0.72-0.80; Brommer et al. 2005, Gasparini et al. 2009, Karell et al. 2011b). The variance in colouration is explained by a mix of pheomelanin (68%) and eumelanin (21%), whereas no carotenoids have been recovered in the feathers (Gasparini et al. 2009).

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 randomly 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. 2011b).

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) providing a good estimation of overall colouration (Roulin et al. 2005). A grey colouration is characterized by an absence of pheomelanin pigments. Between- and within-year visual assessments of individual colouration were found to be significantly repeatable for both females (280 individuals, r= 0.86 ± 0.023 SE, $F_{279,613} = 11.37$, P < 0.0001) and males (226 individuals, r= 0.88 ± 0.025 SE, $F_{225,354} = 12.59$ P< 0.0001) following methods of Becker (1984) and Lessels and Boag (1987).

Brood size manipulation

Based on the criterion that clutches were laid on similar dates (r = 0.923, 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 effort (Roulin et al. 2011b). 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 colouration was not associated with clutch size and brood size before or after the manipulation (Pearson's correlations; all 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 not always both partners were captured.

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

Between 2005 and 2010 we examined whether colouration of the parents is 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 1987) 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. To test whether the amount of male responses we had could be informative too, we nevertheless constructed a model with the same parameters as for the females, but found no significant effect of colour on presence ($p = 0.39$)), as expected if nest defence is the main responsibility of females (Wallin 1987). We determined sex either visually based on the sexual dimorphism in size (Baudvin and Dessolin 1992) or the type of alarm calls (Galeotti and 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)*. 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, t49 = 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 colouration 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. Passerine alarm is 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 disturbed by passerines. We saw the breeding female in 24% of the playback experiments and 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%). Passerines are known to actively mob raptors (Hogstad 1995) and in particular owls as passerines are a possible prey to them (McPherson and Brown 1981). We therefore decided to use the passerine alarm calls as a proxy for the presence of an adult tawny owl since they appear better able to notice movements of camouflaged tawny owls than we were.

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. We obtained data of 31 nests that were visited twice between April 19 and May 23. 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). 8 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. Starting with three minutes of silence as an acclimation period, we then repeated a playback sequence of eight minutes (i.e. three minutes of calling followed by five minutes of silence) 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 also that colouration of both parents was unknown at the time of the experiment. In addition, we presented next to the loudspeakers a taxidermically mounted owl of either dark or light reddish morph; also here we chose to reduce variation caused by the specific mounted owl used and thus only presented two differently coloured owls. The mount was placed when setting up the speakers and was covered under a sheet until the recording started. 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. The playback experiment took place at night (mean time 23:15h ± 12 min, range: 21h16 – 01h18)), 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. The recorder (Marantz PMD 661) was placed 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.

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. Observers were unaware of the bird coloration at the time of recordings.

Statistics

All analyses were performed using JMP 9.0.2 (SAS 2010) and SAS v9.1 (SAS 2008). 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, clutch identity and year as three 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 (enlarged or reduced treatment), time of the day (mean: 14h05 ± 35min, range: 6h-21h), date (i.e. number of days after the 1st of January; range: 34-174) 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 colouration 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 an ANCOVA mixed model with 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 colouration, sex, brood size manipulation, hatching date and two way interactions.

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, $0.33 < |r| < 0.77$, $P < 0.027$), we performed a principal components analysis (PCA). Where necessary, variables were log-transformed to improve normality. 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 and 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 model 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.

There were only three males that responded in both replicates and 16 females, 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, χ^2 = 0.08, P = 0.78). We tested repeatability by first performing an Analysis 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, Lessels and 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 colouration and predation ($F_{1,9}$ = 0.48, P = 0.5).

Playback experiment during daylight hours in 2010

The probability that passerines alarmed was associated with female plumage colouration in interaction with the brood size manipulation experiment (logistic regression analysis: female colouration: χ1 = 3.02, P = 0.08; brood size manipulation: χ1 = 3.25, P = 0.07; interaction: χ1 = 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 4.1).

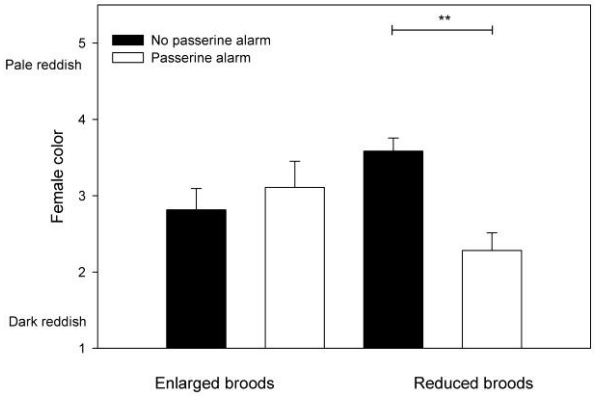

Figure 4.1 Relation between passerine alarm and colour of female tawny owls depending on brood size manipulation. Data from the playback experiment carried out in 2010. Significant differences are indicated by stars.

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 4.2). In this model, the reaction of individuals rearing a reduced brood was borderline significantly more intense than that of females with an enlarged brood ($F_{1,24.59}$ = 3.6, P = 0.07).

Both PC1 and PC2 are not repeatable within individuals (analysis of variance, with individual as factor, all p-values > 0.34). 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, minimum distance, distance at first response and number of flights were not significantly repeatable (all 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 4.3). Males showed 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).

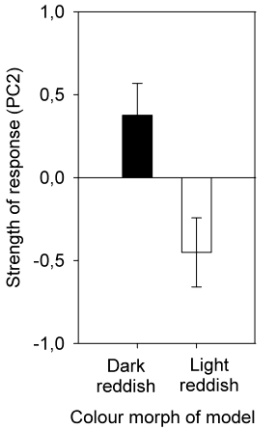

Figure 4.2 Strength of the response of female individuals (measured as latency to respond and frequency of alarm calls in principal component 2 of a principal components analysis) to a dark or light reddish mounted tawny owl.

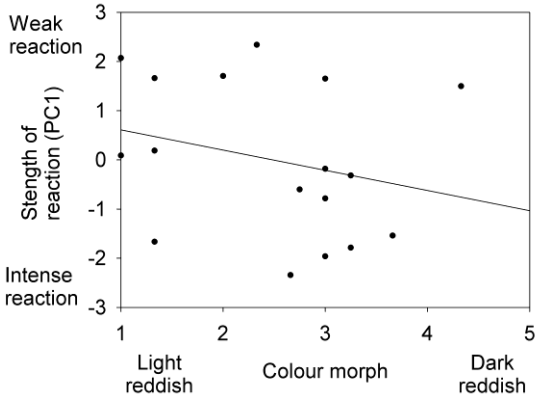

Figure 4.3 strength of reaction by male individuals in relation to their colour morph, measured as principal component 1 (PC1; call frequency, latency to respond, number of lights and distance to the intruder) in a general linear model analysis. A negative value for PC1 indicates a stronger reaction.

Discussion

Our results have shown that differently coloured tawny owls differ in their response to intruders. The long-term dataset has 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 suggests that our presence around nests and broadcasted male calls induce owls to

move from their hide particularly when the female is dark rather than light reddish and mainly when brood size is small. This demonstrates that parents invest more in defending few, presumably high quality offspring, rather than many, lower quality offspring. 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 than a light reddish intruder. This is consistent with the finding that light reddish owls tend to suffer more predation than dark reddish conspecifics.

Colour, reproductive success and survival

Colour is not selectively neutral in tawny owls as results from a Finnish population show 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 both in Italy and Switzerland (Galeotti and Sacchi 2003, Gasparini et al. 2009), although not at the cost of reproduction. In Finland, occurrence of parasites is similar, 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 (Roulin et al. 2003). Furthermore, lighter reddish individuals lose less weight after an immune challenge (Gasparini et al. 2009) 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, which could mean that partners select for complementary rather than similar 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 and 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 and Saurola 2004) 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 and Kruger 2009). Colour might then signal such an agonistic behaviour, like the black badges in the siskin (*Carduelis spinus*; Senar and Camerino 1998), the house sparrow (*Passer domesticus;* Moller 1987) or Gouldian finch (*Erythruria gouldiae;* Pryke and Griffith 2009).

Colouration 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 colouration 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 colouration and nest defence intensity in great tits (*Parus major;* Quesada and Senar 2007), male American Robins (*Turdus migratorius;* (Row and 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 form fixed routines, in line with the active/proactive or bold/shy behavioural types (Koolhaas et al. 1999), normally measured in traits such as aggression, risk-taking, fear, exploration and reaction to environmental changes (Sih et al. 2004b). The Pace of Life Syndrome (Reale et al. 2010b) 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. 2010b).

Melanocortin system as potential proximate explanation

For selection to act on the colour as signal of boldness, a genetic basis for this behaviour needs to be present. A candidate is the melanocortin system. In the melanocortin system, pheomelanic red colouration 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 colouration (Gasparini et al. 2009) and thus an increase in reddish colouration 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. 2011b). 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 colouration and aggression, here measured in nest defence. Future work needs to demonstrate if the melanocortin system is causing the relation between colouration 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.

5. MC1R polymorphism is associated with pheomelanin colouration and natal dispersal distance in barn owls.

Susana Pinto, Valentijn van den Brink, Anne-Lyse Ducrest and Alexandre Roulin

Department of Ecology and Evolution, University of Lausanne, CH-1015 Lausanne, Switzerland

Keywords: MC1R, melanocortin system, polymorphism, barn owl, dispersal, melanin

Unpublished manuscript

Personal contribution: I performed the statistical analyses and wrote the manuscript.

Introduction

The MC1R gene is part of the melanocortin system and is responsible for melanic colouration in vertebrates (Gantz and Fong 2003, Cone 2005). The melanocortin system in birds is very similar to that in other vertebrates (Mundy 2005).

Mutations leading to colour polymorphism are common and most are in some way related to the MC1R (Robbins et al. 1993, Gantz and Fong 2003). The MC1R is part of the melanocortin system, which is involved in many physiological and behavioural traits (Ducrest et al 2008).

In barn owls (*Tyto alba*) as in many other birds (Boswell and Takeuchi 2005, McGraw 2006b), melanic colouration is caused by binding of the agonist melanocortin melanocyte stimulating hormone (MSH) to the melanocortin 1 receptor (MC1R). If the antagonist Agouti stimulating protein (ASIP) binds to MC1R, production switches from eumelanin to pheomelanin production. MC1R shows polymorphism in the barn owl (unpublished data).

A specific mutation (*V126I*) is related to pheomelanic colouration (unpublished data). We found in another paper a strong association between pheomelanic colouration and natal dispersal distance (Van den Brink et al. in press) and therefore we decided to test whether the genotypes for MC1R could also explain the observed differences in natal dispersal distance we found in barn owls. If a direct association between the genotype and a behavioural trait can be demonstrated, this could give us some clues on the underlying mechanisms of previously shown associations between melanin based colouration and behavioural traits. We obtained genotypes for 85 out of the 185 individuals we studied. For these individuals the MC1R allele was sequenced and the genotype determined. We then used the genotypes in analyses of natal dispersal distance, controlling for possible covariates.

We will discuss our results and compare them to the similar analyses performed with colouration traits. We will also examine possible explanations and implications of our results.

Methods

We used the same 185 individuals for which we had studied natal dispersal in an earlier paper (Van den Brink et al in press). For these 185 recruits into the local breeding population, individual differences in natal dispersal distances and pheomelanic colouration were known. We then sequenced the MC1R gene for 85 individuals we had also obtained blood samples from. Subsequent statistical analyses were performed on these 85 individuals only.

Allelic discrimination V126I of MC1R

MC1R isolation

The polymorphic sequence of the MC1R gene of *Tyto alba* has been identified by developing two primers in conserved MC1R regions of vertebrates (unpublished data). Then a library was made and used to find the start and the end of the sequence.

Pre-amplification PCR

MC1R is a difficult sequence to amplify, which sometimes yields low amplification results. Thus, before starting the allelic discrimination (AD) assay, the gene needs to be pre-amplified to ensure enough replicates are present. We started by evaluating the sequence concentration. Then, for each sample, we diluted an aliquot at 2.5ng/µL and 2µL of the resulting product was used for each PCR. The standard Taq (5U/µL) (Qiagen) with their Qsolution was used. The final PCR volume was 20µL. The primers used are the following: 1R_-34fw: 5'-GGGACCCCGGGGTTGAGGCG-3' and 1R_568rev: 5'-GGCAGAGGAGGATGGCGTTGTTGCG-3'. Their final concentration in the PCR volume was 200µM. They amplify the first half of the gene (602 bp). Each plate contained three controls corresponding to the three genotypes (M011405 (II), M011732 (VI), M011851 (VV)) plus at least two NTCs (no template control). The PCR cycling is unusual, with a longer step at 95°C to separate the strands and a longer and more hot annealing: 3min at 94°C / 35x cycles 40sec at 94°C, 40sec at 68°C, 1min at 72°C / 10min at 72°C. Finally, a 1% gel compares the relative DNA concentrations of each

sample that will be run in the same plate during the PCR assay. Concentrations are first adjusted and then diluted by 10^{-2}.

qPCR assay

The qPCR assay will only be described in summary here, as a more detailed description of methods is beyond the scope of this study. Briefly, a short section of MC1R containing the mutation is amplified using another pair of primers in presence of two kinds of fluorescent probes that distinguish two alleles (they differ in only one nucleotide; V126I_wt_probe: 5'-TGCAGCTCCGTCGTCTCCTC-3' and V126I_mut_probe: 5'-TGCAGCTCCATCGTGTCCTC-3'). The fluorescent molecules were FAM and ATTO550 for the wild type and mutant alleles respectively. The mutant probe was less efficient at binding *MC1R* and was consequently used at a higher concentration in the assays (100nM and 250nM for wild type and mutant probes respectively). We used the qPCR MasterMix Plus Low ROX (Eurogentec) with the recommended cycling and an annealing temperature of 57°C in a final volume of 26µL. The qPCR machine used is the ABI 7500 (Applied Biosystems). The fluorescence of each probe is recorded at the end of each cycle. When the run is completed, they are plotted against each other. Outliers were removed, which resulted in 3 clusters where the heterozygotes lay in between homozygotes mutant and wild type. The whole procedure was performed in duplicate for each sample. Samples for which only one genotype assignment could be made or which gave contradictory results were excluded.

Statistical analyses

All statistical analyses were carried out with JMP 9.02 (SAS 2010). All results are reported with estimates, and P-values. P-values < 0.05 were considered significant.

We first needed to establish that the different variants of the MC1R gene that are present in barn owls also occur in our samples. We found that one allele (II) only occurred twice in our sample, and therefore we excluded this from further analyses. The other two genotypes, VV and VI occurred

61 and 24 times respectively. We then performed a Student's t-test on genotype and pheomelanic colouration to confirm the association between colour morph and *MC1R* genotype. Finally we tested if the genotypes were associated with natal dispersal distances. This was done by using the natal dispersal distances as reported in Van den Brink et al (in press) and correlating them against the reported genotypes of each individual. We used the same modelling approach, where we included several potential covariates in the model. Identity of the biological mother and nest of rearing were added as random variables to account for non-independence of individuals from the same nest or mother, although there were only 9 mothers with 2 recruits. The other variables in the model were log-transformed distance to the centre of the study area, cross-fostering, wing length as adult, age at first breeding, the number of sites available within 2km of the site of rearing, age rank in the nest of rearing, sex, forest cover around the nest of rearing and two-way interactions. Detailed descriptions of variables and rationale for inclusion in the models are given in Van den Brink et al. (in press; table 3.1, **chapter 3**, this thesis). We compared the fit of the full and reduced models using AICs.

Results

The two genotypes are strongly associated with the pheomelanic colouration (Student's t-test, $t_{83} = 9.75$, $P < 0.0001$). VI individuals are darker reddish and VV individuals are more pale reddish. There appears almost no overlap between both genotypes in their colouration (figure 5.1). The model for dispersal showed that natal dispersal distances were strongly correlated with genotype. The genotype is the only significant effect in the final model (Estimate = 0.12, $F_{1,42.03} = 7.77$, $P = 0.008$; figure 5.2), which had a much better AIC than the next model, where the log-transformed distance to the centre of the study area was also included. When genotype and colour are in the same model for dispersal distance, the model with colour is rejected as one of the first (results not shown, P-value for colouration $P = 0.52$). Both genotype and colour traits (see Van den Brink et al. in press; **chapter** 3) give the same results for the dispersal distance.

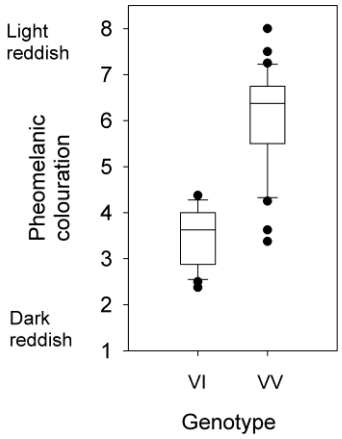

Figure 5.1 Pheomelanic colouration of barn owls of two different genotypes for the MC1R gene.

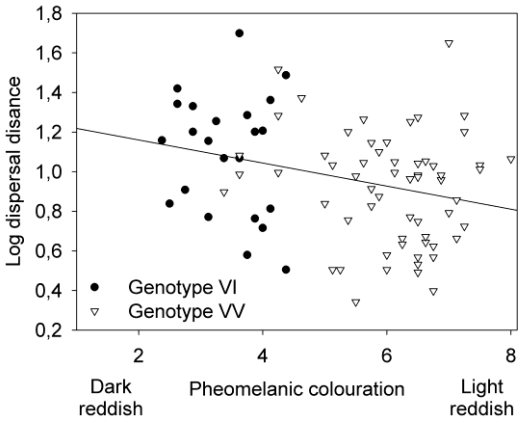

Figure 5.2 Log-transformed natal dispersal distance in km of fledgling barn owls of different genotypes in relation to their pheomelanic colouration.

Discussion

We showed that the polymorphism in the MC1R gene is strongly correlated to pheomelanic colouration. This same polymorphism also strongly correlates with the natal dispersal distances.

There are several possible explanations for this. MC1R could be the causal factor in this behaviour. In that case, the colouration and dispersal are selected for together. It is not very likely that this is the case, as MC1R is mostly involved in colouration and few other functions of the gene are reported (see Ducrest et al. (2008) for more details). There could be correlated selection acting on colour and MC1R. Local adaptation, competitive advantages of one morph over the other could cause dark reddish individuals to disperse farther. In this case the MC1R variants would not be selected for directly, but simply through their strong correlation with colouration. Selection would occur on colour (mate choice) or a trait associated with pheomelanic colouration. A last option is that a downstream effect of the MC1R gene somewhere in the cascade leading to colour production affects behaviour as well. Some components involved in pheomelanin production (such as glutathione) are involved in other behavioural processes as well.

We cannot exclude either of the last two options at this time. Our results do provide further support for the melanocortin system to be involved in (some of) the associations between colouration and behavioural traits we have found in previous work.

The results with colour and genotype differ slightly from each other, in that if colour morph is included in the model, some other variables have a significant effect on dispersal distance. The larger sample size (175 versus 85) might have contributed to this, but it can be that genotype is less affected by other variables than colour, despite the very strong association it has with colour morph.

It would seem worthwhile to sequence more individuals and perhaps also test these against more behavioural traits, which could provide us with more information of possible linkage to other genes that influence behaviour more directly than the *MC1R* gene. In a similar effort, we could sequence the other MCR genes and investigate possible polymorphisms and their effects on behavioural differences.

6. General discussion

The study of behavioural differences in relation to melanic colouration

The melanocortin system consists of 5 receptors and numerous compounds involved in colouration, physiology and behavioural traits. With so many possible places where influence on behaviour or other traits can occur, asserting a mechanism becomes quite complex quickly. A link between colour and behaviour could be due to the binding of one of the melanocortins to MC1R, causing the colour, and at the same time to one of the other receptors, related to several different types of behaviour (such as aggression in MC5R; see also figure 1.2 in Box 1). It could also be indirect, through binding of this melanocortin to MC2R and thus influencing the stress response, which, in turn will influence the behaviour displayed when in a stressful situation (such as contact with predators). Another option is that there is a link through sexual dimorphism, which makes males behave differently than females. In the kestrel and the barn owl, it would appear that female-like individuals and male-like individuals behave differently, independent of their sex.

The study of a link between animal personality and colouration (as an example of a possibly related trait) can become very complex. Nevertheless, in my thesis I have found several interesting leads to start a more detailed examination of the exact mechanisms involved in the relationship between melanin-based colouration and animal personality traits.

Summary of main results

In my thesis I have examined the relationship between melanin-based colouration and individual behavioural traits (animal personality) in barn owls, tawny owls and kestrels. We have shown that the strongly heritable melanic colouration (Gasparini et al. 2009, Roulin et al. 2010) is associated with various individual differences in behaviour, ranging from anti-predator behaviour in adult tawny owls, anti-predator behaviour in nestling barn owls and kestrels, to dispersal behaviour in barn owl fledglings. With these results, I provide a first indication that the melanocortin system

can be involved in associations between colouration and individual differences in behavioural, physiological or life history traits.

In **chapter 1** I showed that individual barn owl nestlings differ in their anti-predator behaviour, and that this behaviour is associated with melanin-based colouration (Van den Brink et al. 2012a)). Nestlings with a larger diameter of black (eumelanin) spots are more docile when handled, hiss more (an anti-predator trait), breathe fewer times per minute when stressed and remained in TI longer and needed fewer TI tests to remain immobile than nestlings with smaller spots. I concluded that melanic colouration was related to stress response. Furthermore, differential predator pressures in the phylogeographical history of barn owls could be responsible for shaping an association between melanin colouration and anti-predator behaviour.

The relationship between melanin-based colouration and behaviour observed in the barn owl is conserved in a closely related species, the Eurasian kestrel (Van den Brink et al. 2012b). When I performed similar behavioural tests as in the barn owl nestlings (**chapter 2**), the results were very similar, since individuals displaying a wider black tail band (a melanic trait analogous to the spot diameter in the barn owl) were breathing slower and remained in TI longer. The exception was that these individuals were less docile. Responses are therefore not always related in a similar manner across species. I proposed several possible explanations for these differences, including differences in predator pressure, stress response and different selection pressures on male- and female traits.

A different behaviour, which is commonly considered to be a life history trait is nevertheless linked to personality because more bold individuals are known to disperse farther (Dingemanse et al. 2003, Cote et al. 2010). **Chapter 3** presents a study on barn owls where for individuals that remain in the study area, natal dispersal distances correlate with pheomelanic (reddish brown) colouration, and that this behaviour is repeatable between parents and their offspring (Van den Brink et al. in press). More reddish individuals disperse farther than their more white counterparts. There are several possible explanations, among which are personality or local adaptation. Cross-fostering

experiments showed that more reddish cross fostered individuals did not disperse farther than non-cross fostered reddish individuals, making local adaptation a less likely explanation while at the same time demonstrating that a genetic link between melanin coloration and behaviour exists.

The generality of a link between melanin-based colouration and personality traits was further elaborated in the **fourth chapter** of this thesis where I studied tawny owls to demonstrate that more melanic individuals respond more strongly to intruders and potential predators near their nest site (Da Silva, Van den Brink et al. unpublished manuscript). I used recorded male territorial calls near nest sites with nestlings present during both day and night and found that more reddish females responded more strongly to these calls during the day and more reddish males during the night. A long term analysis of the reaction of females to nest visits showed that dark reddish females responded more often to our presence with movements or calls than pale reddish females. During the night females responded more strongly to the presence of a dark-reddish mounted stuffed owl than that of a pale reddish owl. Light reddish males suffer more from nest predation than dark reddish males, suggesting a relation between the more intense nest defence I found of dark reddish individuals and their higher reproductive success. The pheomelanic colouration of tawny owls might then signal different behavioural strategies in nest defence, a component of parental care, to conspecifics.

We know from previous studies that the melanocortin system is involved in melanic colouration (Cone 2005, Ducrest et al. 2008, McGraw 2008). My colleagues and I have provided a tentative first glimpse at the underlying mechanisms, demonstrating that the genetic polymorphism in the *MC1R* gene is related to both dispersal behaviour and melanin-based colouration (Figure 5.1 and 5.2, **chapter 5**). This gene has three alleles in barn owls; one is very rare and only occurs in the south of Europe, the other two are strongly correlated with pheomelanic (reddish) coloration in our study population. Other mutations of the MC1R gene involved in colouration are present in other bird species (Mundy 2005).

In my thesis, I have clearly shown that differences between different types of personality traits are associated with melanic colouration in three different species. The first indications for a genetic basis of this association are also present. In this discussion I will present alternative explanations and possible proximate and ultimate mechanisms for the found relationships.

Field versus laboratory (trade-off between control and reality)

With relatively small samples I was able to demonstrate behavioural differences between individuals in their natural environments. The differences observed between years indicate that environmental or habitat-specific differences can influence the behaviour substantially, though. We also observed some limitations of field experiments, with open-field test, mirror image and several other tests not producing any response in the birds. In these cases perhaps the acclimation time or too much stress might have contributed to a lack of response in the individuals tested.

When studying animal behaviour, there is a trade-off between having the control of a laboratory setting and the more natural behaviour of individual animals in their own habitat. The influence of the environment on the behaviour of an animal which is beyond the researcher's control can make measuring personality traits in the field a daunting task. A laboratory is a controlled environment where conditions can be standardized so that the experiment performed or the observations made, represent the same stimulus for all individuals. This advantage can be counteracted by the fact that a laboratory is also an artificial environment, which, although many variables are controlled, introduces much other unwanted variance. The individual animal under study has to adjust to its new surroundings, the capture and transport introduce stress and the natural environment in which the normal behaviour, including the behaviour under study has developed, is not present in the lab (Archard and Braithwaite 2010). It is questionable how far results obtained in a laboratory setting can be extrapolated to natural populations (Calisi and Bentley 2009). A recent study shows that the induced stress can strongly influence results of personality tests and

that longer periods of acclimation and multiple tests are needed to accurately obtain personality traits (Biro 2012). The behaviours that have developed in a natural setting, with all the variables beyond our control are often remarkably stable and repeatable, perhaps because they have developed in a specific environment (Bell et al. 2009). Even with the limitations outlined above, the natural environment is where natural behaviour can be observed and thus where the most useful results can be obtained. I am therefore confident that the observations in the natural environment contribute greatly to the understanding of the natural world. They can help generate questions and can be used to test theories that are developed in more controlled experiments in the field or the laboratory.

Could we explain all the observed associations between colour and behaviour with life history traits?

Life history theory assumes that all individuals in a population try to achieve optimal fitness (Stearns 1992). What I have studied is individual variation. This variation is not variance around the mean as often thought in the past (Sih et al. 2004b), but it likely represents individual, as opposed to population, optimums. In behavioural ecology the general idea was that there exists one, population optimum, with spread around this optimum, shaped like a normal distribution (Wilson 1998, Schuett et al. 2010).

Since individual differences from studies on animal personality often occur in suites of correlated traits and have fitness consequences (Dingemanse 2005, Smith and Blumstein 2008) they can influence life history decisions of individuals. I found repeatable individual differences and even though these are in typical life-history traits, such as nest defence and dispersal, these traits have previously been found related to personality traits.

There is also theoretical support for personality differences associated with life history traits. Wolf et al. (2007) showed that life history trade-offs can promote the development of personality traits and thus that they are not mutually exclusive. Their models demonstrated that trade-offs in

reproduction where individuals have to value current versus future reproduction can lead to differences in boldness. Those individuals that have longer expected lifetime and thus more reproductive opportunities, would show more risk aversive behaviour, whereas individuals with fewer reproductive opportunities could develop more risky strategies. Another example is given in a review by Biro and Stamps (2008) who show that personality traits can occur if they are related to individual differences in life history productivity (growth/fecundity).

Perhaps the separation between life history and personality is more a definition problem, since it is clear that consistent individual differences in life history traits can occur. What is more important is to find proximate and ultimate causes for such consistencies.

Proximate versus ultimate explanations

To explain our results in an evolutionary framework, we need to establish two different things. Not only do we need more knowledge about *how* the link between colouration and behaviour is formed and maintained, but also *why* it is maintained. The *how* is the proximate reason, the mechanism. The *why* is the ultimate reason, the evolutionary process that selection ultimately acts upon.

Before we can find the evolutionary reasons for the maintenance or development of certain traits we first need to know the mechanism behind the personality/colouration link. When we know the mechanism, we can make some predictions about occurrence in other species and its generality. It will also provide clues where to look for evolutionary explanations. At this point, these explanations are still speculative, because we do not yet have concrete proof for which mechanism is responsible for the colouration/behaviour link.

Hints from domestication

In order for a wild animal to be successfully domesticated, it must lose its fear for humans, but also it needs to become less aggressive, which holds true especially for larger animals such as early dogs and aurochs. This process requires several generations of training with resulting phenotypic but most definitely also genetic changes. An unintentional by-product is often an increase in the number of different colour phenotypes (Cieslak et al. 2011).

When studying the domestication literature one can find some, often older studies that show a relationship between behaviour and other traits (for instance colouration), often without explicitly asking what was at the basis of this finding, although sometimes the melanocortin system is suggested as a possible cause. In several dogs the golden coat colour appears more often to display aggressive behaviour than black or multi-coloured dogs. Suggested explanations include linkage disequilibrium between traits responsible for aggression and the neurochemical pathways involved in colouration (Amat et al. 2009, Kim et al. 2010). With current knowledge, we can begin to study whether the behaviours are part of an underlying system. Is there flexibility within an individual, or are multi-correlated traits, driving behavioural types? We could also argue that these apparently unadaptive traits can be part of a trade-off to rewards obtained from being consistent in another trait, which constrains flexibility in the observed trait (reviewed in Schuett et al. 2010).

A long term domestication study took place in Russia (Trut 1999) and it demonstrated that selection for docility "pulls" other traits along. In 1959 in Siberia, Dmitry Belyaev started a program to investigate how the domestication process of dogs might have happened. He selected 130 silver foxes (*Vulpes vulpes*) from fox fur farms for docility and started breeding them. Every generation, only the most docile individuals would be allowed to breed again. The foxes displayed physiological changes in hormonal levels, and after 10 generations, coat colour began to change. They were becoming tamer with time, until now, 60 years later, they behave almost like dogs. Interestingly, there were more changes associated with this change in docility. Melanic colouration also changed. Also several well-known colour patterns from a range of domesticated animals (such as the white

star on the forehead seen in cows, horses, dogs) appeared. (Belyaev et al. 1981). This white star is caused in ontogeny, where melanoblasts are lacking from certain spots in the developing embryo. Many physiological changes, mostly related to hormones and stress response of corticosteroids and indicating strong neurological influences.

Domestication, or more importantly, the increase in docility appears related to colour polymorphism. Experiments on rats (Trut et al. 1997) and observations on fallow deer (*Dama dama*) produced very similar results to those in foxes (Hemmer 1990). Given that docility is a very widespread trait in which individuals differ, we can begin to wonder if docility, colour and other physiological traits could be connected to each other through the same mechanism. Dopamine, a precursor of noradrenalin and adrenalin is involved in the stress response. Dopamine is also a precursor of MSH, a part of melanogenesis (Wakamatsu and Ito 2002; figure 1.1, box 1) and it thus seems likely the melanocortin system can be involved in the relation between docility and colouration

Search for an underlying proximate system

If there is one system that can explain the variation in all the different traits we tested, rather than needing explanations for each separate behavioural type, Ockham's razor predicts that the more simple explanation is most likely to be true. Thus, if we have indications that there is one system that could explain most or all associations, we might be on the right track.

Docility changes related to other physiological traits

Differences in behavioural and physiological traits are sometimes attributed to the Pace Of Life syndrome (POLS; Reale et al. 2010b). A number of interesting correlations have been found, with personality and metabolic traits, that all appear related to focusing on high-risk current reproduction

or low-risk, future reproduction strategies. Several examples from different vertebrates show results that follow the predictions of this hypothesis.

Research in mice shows that fast reacting (aggression) mice have lower serum anti-oxidative capacity than slow reacting mice (Costantini et al. 2008). A study in humans found that glutathione, a compound involved in the resistance to oxidative stress activity has lower levels in shy individuals compared to bold individuals (Matsuzawa et al. 2005). The antioxidant glutathione is necessary for the formation of pheomelanin and in barn owls, also resistance to oxidative stress is associated with differences in pheomelanic colouration (Roulin et al. 2011a). We could then predict that individuals with lower glutathione levels (i.e. pale reddish coloured) will be more shy than those with higher levels. Our results (Da Silva, Van den Brink et al. unpublished manuscript) seem to confirm this. It seems then that many relationships reported between different types of behaviour and other traits are in some way or another also associated with the melanocortin system. Therefore the melanocortin system is a good candidate for a proximate mechanism explaining the association between personality traits and colour polymorphism.

The melanocortin system

There are many genes involved in colouration, but most are in some way related to the melanocortin system, either downstream, or in a component of melanogenesis (Hubbard et al. 2010). Many of these mutations are involved in domestication, which also requires changes in docility and is a common cause for colouration changes (wolves, cichlids, cattle; Cieslak et al. 2011). A widely occurring mechanism for coat (or skin or plumage) colour polymorphism are mutations in *MC1R* ranging from rabbit, sheep, dogs, cats to the woolly mammoth (Rompler et al. 2006, Cieslak et al. 2011), which tends to result in a loss of function of MC1R. Another major mechanism could be mutations in *ASIP*, which competes with MSH to bind to MC1R and can thereby influence the relative

proportions of eumelanin or pheomelanin (Cieslak et al. 2011). Changes in melanin-based colouration are therefore most likely related to different alleles involved in the melanocortin system.

More evidence is gathering that the melanocortin system is involved in many behavioural traits in vertebrates. In salmonid fish, for instance, many analogous findings to those in barn owls have been found associated with melanin-based colour. Stress response (Kittilsen et al. 2009), parasite load (Kittilsen et al. 2012), heart size (Ø. Øverli personal communication, Roulin et al. 2001a).

In humans individuals with red hair appear to sense pain in different ways than others (Liem et al. 2005, Andresen et al. 2011). The red hair colour is due to a mutation in the *MC1R* gene and also the sensation of pain has its roots in the melanocortin system. All these examples share one thing; melanin-based colour is related to important behavioural or physiological differences between individuals. The known effects of the melanocortin system on these physiological or behavioural changes matches with the colouration changes related to MC1R. The melanocortin system combines colour, behaviour and physiology, is located in in the Central Nervous System (CNS), and it is highly conserved in vertebrates (Chunco et al. 2007).The melanocortin system therefore seems the most obvious candidate to start searching for proximate mechanisms that can explain the observed behavioural differences related to this colouration type.

Ultimate explanations

If the mechanism behind the link between colouration and personality traits is known, the ultimate evolutionary explanations can also be examined more closely. In the following I will discuss a few of the most likely ultimate factors. These are not mutually exclusive. What they have in common, is that the behavioural traits do not need to be selected for directly, but could be co-selected for through linkage disequilibrium.

Local adaptation

Microhabitat differences can cause change even on a local scale. Local adaptation could therefore play an important role in individual behavioural differences we observe. We have seen that local adaptation appears to influence diet (Roulin 2006). Predation pressure, one of the hypotheses we proposed as a possible reason for differences in anti-predator behaviour in **chapter** 1 can also shape differences in behaviour for local populations (Bell 2005, Dingemanse et al. 2009). Predator pressure can also influence boldness (Huntingford 1976, Bell 2005) and might have pushed these traits along with colour as a signal for boldness in mate choice.

Environmental conditions, such as temperature, snow cover in winter or precipitation might also play a role in the observed gradient of colouration change from white in the south of Europe to dark reddish in the north of Europe (Antoniazza et al. 2010) and in other directions in other parts of the world (Roulin et al. 2009).

Energy balance/ metabolism

The environment (temperature is most likely) can select individuals on their metabolism and/or on their energy efficiency. We know that the melanocortin system is involved in both these traits (Cone 2005). Studies on barn owls showed that females with larger spots lose less body mass after a day without food (Roulin 2009) and in nestlings the same pattern was found (Dreiss et al. 2010). Other studies show more efficient energy maintenance in darker individuals. In mice (Huszar et al. 1997, Hoekstra 2006) individuals with *agouti* mutations also show increased obesity. In colder climates, individuals need to be able to sustain themselves and produce enough heat to thermoregulate despite the adverse temperatures. In environments where food is scarce, it might pay off to be more energy efficient. Based on a higher metabolism for lighter coloured (low-melanistic) individuals, darker individuals could be better adapted to such environments.

Sexual selected colour traits predicting behaviour

Sexual selection could also help personality traits develop and persist, based on selection for consistency in certain traits. A recent review showed that males are often more consistent in their behaviour than females (Schuett et al. 2010), supposedly because it pays off in competition over females to be predictable in behaviour (Johnstone 2001). In a recent study on barn owls, selection appears to make both males and females display more male-like and female-like traits for colouration traits (Dreiss and Roulin 2010). In our results (**chapter 1**) barn owls with more male-like traits (smaller spot diameter) are less docile, and are thus displaying more male-like behaviour. Females disperse farther, and individuals displaying more female-like colouration (reddish colour) disperse farther, even when controlled for sex (**chapter 3**).

In unpublished work, we also found that male barn owls are less docile than females, and display higher repeatability in this behaviour than females throughout their life (Van den Brink and Roulin, in prep.). In initial analyses, individuals displaying female-like traits (larger spot diameter, more reddish colouration and fewer spots) appear less repeatable in their docile behaviour than individuals displaying male-like traits. This provides further support for the hypothesis that the genes responsible for the link between personality and melanin-based colouration are sex-linked). For males it might pay off to be bolder than females for numerous reasons, such as mate and territory competition. It could be that the genes responsible for the behavioural link to the colouration are sex-linked genes. Many of the traits studied in this thesis (dispersal, nest defence, docility) have something to do with boldness, risk-taking behaviour or aggression). If these behaviours are somehow influenced by genes involved in sex determination, this would help us to search in a more directed way for the ultimate reason for the mechanism behind the link between colouration and personality.

Maintenance of colour polymorphism

So how does animal personality help explain the maintenance of colour polymorphism in these three raptor species? In evolutionary biology the main goal is to understand the molecular mechanisms that lead to phenotypic variation, whether it is in behaviour, morphological or physiological traits. Selection acts not on the phenotype, but on the underlying genotype. Genetics does not follow Mendelian inheritance often. In fact, most of the time, many genes, and the environment contribute together to the phenotype of an individual. Colour itself can be the trait where selection acts upon and indeed, many studies show marked differences in survival related to cryptic colours for prey or success in mate choice. There are only few studies however, reporting a genetic correlation between colour traits and behavioural differences.

Phenotypic correlations are not necessarily genotypic correlations

When we know more about the functional mechanisms, we can gain insight into evolutionary pathways responsible for the development and maintenance of different phenotypes (Hubbard et al. 2010). Phenotypic correlations by themselves do not show shape or even presence of trade-offs (Dochtermann and Roff 2010). However, the genetic correlations are what selection acts on (Lande and Arnold 1983). Correlation of a phenotypic trait with a genetic variant is not enough to prove causality, but several methods can prove that certain mutations can lead to difference in phenotype (e.g. knockout zebrafish, lizards with differences in functional assays. See for examples Hubbard et al. 2010). In field studies, many of these laboratory techniques are not practical and sometimes impossible to use. There are however some methods that can assess the strengths of both the genetic and environmental effects. The utilisation of such methods might help determine the extent of the genetic influence on traits, but it can also uncover hidden associations, when for instance phenotypic and genetic correlations are in opposite directions (Dingemanse et al. 2012). Phenotypic traits are commonly influenced by both genotypic and environmental influences; G x E interactions.

The phenotypic relationships between colouration and behaviour I have found in this thesis do not yet prove that there is a genetic basis for these correlations. In this thesis we only show phenotypic relationships, and no genetic correlations. The associations of various behaviours with a strongly heritable trait (melanin-based colouration in three raptor species, h^2 = 0.7-0.9 (Gasparini et al. 2009, Roulin et al. 2010) suggest that there is at least an underlying genetic basis. Nevertheless, the results do not reject the hypothesis that an underlying system is involved in the relation between colouration and personality upon which selection could act.

Prospects for future research

In order to get closer to determining what the underlying mechanisms are, there are several options for future research that could contribute. When we know more about the proximate mechanisms, we can make inferences and search for evolutionary explanations. We need to make the step from only phenotypic correlations to the underlying genes. This can be achieved in several indirect and more direct ways. The indirect method is through the so-called animal model, where phenotypic and environmental variation can be partitioned to assess the genetic correlations present. The more direct methods are measuring gene expression and QTL-mapping and Genome Wide Association Studies (GWAS). The latter uses next generation sequencing techniques to identify regions of the genome, or specific genes responsible for certain traits.

POMC gene expression

We have developed ways to measure expression levels of genes involved in melanogenesis (Ducrest et al. in prep.). We will be able to sequence the *POMC* gene, and we already have evidence that polymorphism in the *POMC* gene exists. These methods will become available very soon and they will enable us to study personality traits in relation to differences in expression of the genes, or in relation to polymorphism in the *POMC* gene.

G-matrix and the animal model

It is likely that at least part of the ambiguity in phenotypic associations between behaviour and melanin-based ornamentation, can be explained by constraints stemming from an underlying pleiotropy between these traits. Quantitative genetics, specifically the multivariate animal model, allows us for the partitioning of variance and co-variance between multiple traits into variance and covariance due to additive genetic and environmental effects. Up to now these methods have not been employed widely, mostly because not many study systems meet the requirements but see (Dingemanse et al. 2012, Taylor et al. 2012). A common problem in quantitative genetic analysis is a low sample size, which can prevent the estimation of small parameter estimates. The data present for our barn owl study population (more than 15 years of breeding data and pedigree information) allows such analyses to be made. The open nature of the system, with so few offspring recruited into the breeding population, does limit the number of generations present in the pedigree information (pedigree quality), thereby limiting the statistical power to detect small effects, but it should provide us with enough information to tease apart genotypic and environmental effects. Also "hidden" correlations can be uncovered with this method (Dochtermann and Roff 2010). In this case in the phenotype alone we would not be able to observe any meaningful correlations (Roff 1996, 1997, Hadfield et al. 2007).

Next generation sequencing for QTL mapping and GWAS

Recent advances in molecular genetics technology and computing power have made it possible to obtain fine scale genetic data and to investigate genetic basis for phenotypic traits. A genome wide association study (GWAS) based on QTL mapping could identify QTLs involved in colouration and phenotypic traits, such as personality. It might even be able to identify the gene responsible itself.

With GWAS techniques (Baird et al. 2008) markers associated with melanin-based plumage traits could be discovered, as a first step to identify the functional loci involved in melanin colouration, and potentially also behaviour. The technique has not been used widely in natural populations, partly because of the significant effort required to obtain informative markers and pedigrees and the financial costs of sequencing large numbers of individuals. Next generation sequencing techniques can help overcome this problem. The sequencing approach uses restriction enzymes to cut up the genome into short DNA fragments that are distributed across the genome, sequence tens of millions of these fragments on the Illumina next-generation sequencing (NGS) platform, align the fragments to discover genetic variation (facilitated by the fact that specimens were individually barcoded), and then conduct genetic analyses on the resulting thousands of restriction cutting sites. These can then be tested for association with the traits of interest.

Generalisation and conclusions

In my thesis I showed a clear association between behaviour and melanin-based colouration. The results also strongly suggest that there is a genetic basis for this relationship, specially the correlation between parent and offspring behaviour in barn owl natal dispersal (Van den Brink et al. in press) and the correlation between dispersal and variation in the *MC1R* gene. At this point we have tentative suggestions that the underlying melanocortin system can be involved in maintaining differences in behaviour, but we have no clear evidence for the evolutionary processes that might help to maintain it.

Three different species show associations of related personality traits with melanin-based colouration traits. These relationships are all consistent with the predictions and observations made by Ducrest et al. (2008) and Roulin and Ducrest (2011) and strongly suggest that the underlying mechanism is general for these species. Given the conserved nature of the melanocortin system in vertebrates (Boswell and Takeuchi 2005, Mundy 2005) it should be possible to extrapolate results to other taxa. Associations with other traits, influenced by one of the other MCRs, remain to be tested.

In kestrels and barn owls, not all results are the same, notably in aggression. The reasons for this are unknown and warrant closer examination. We suspect that different evolutionary histories of the two species might have influenced the behavioural differences.

This thesis provides correlative results and also gives a first glimpse at the underlying mechanism. We found correlations between melanin-based colouration and personality traits that are consistent in different raptor species. Further support comes from other studies, such as those on salmonids (Kittilsen et al. 2009, Kittilsen et al. 2012) and there are even some indications that results can be extrapolated to humans (Rushton and Templer 2012). The consistency and apparent generality across species is remarkable. It is not very likely that so many correlations in the same direction all occur by chance. These results make me confident that the hypothesis about the melanocortin system as underlying mechanism is correct.

With the results from this thesis, we can proceed with a more directed search for evolutionary explanations. The next logical step is to confirm the genetic basis is in the melanocortin system. This requires polymorphism in genes that correspond to behavioural and colouration differences in individuals. The combined decrease in cost and increase in computing power will bring next generation sequencing within reach, allowing for a search for Quantitative trait loci (QTL) or even the genes responsible for the association between colouration and behaviour. The evolutionary mechanisms responsible for the maintenance of colour polymorphism and its association with personality traits remain unknown. We have speculated about several possibilities, but we cannot provide clear evidence yet. How does this system maintain variation, how exactly is it inherited? These questions remain open, but we could now begin to experimentally test competing hypotheses and begin to search for the genetic basis.

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