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Faculté de biologie et de médecine

**Département d'Ecologie et d'Evolution**

# Heritability and fitness value of glucocorticoid hormones and the interaction between the steroidogenic pathway and the melanocortin system in the Barn owl

**Thèse de doctorat ès sciences de la vie (PhD)**

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

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## Monsieur Paul Béziers

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**Heritability and fitness value of glucocorticoid hormones and the interaction between the steroidogenic pathway and the melanocortin system in the Barn owl**

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Prof. Sophie Martin

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**Summary**

Hormones orchestrate many characteristics of an organism's morphology, physiology and behaviour, and thus, stem as key components in the evolution of many phenotypic traits, including sexually selected colour ornaments. Determining the genetic basis and the mechanisms by which selection processes have shaped endocrine traits are crucial to better understand how hormone levels and hormone-mediated traits have evolved.

In this thesis, I explored different aspects associated with the endocrinological system of the barn owl, including the link between steroid hormones and melanin-based colour traits. In the first part, we investigate the potential of baseline and stress-induced secretion of corticosterone, the main stress hormones in birds, to evolve (heritability) in response to selective processes (chapter 1) and how variation in corticosterone levels, including baseline and stress-response levels, are associated with survival (chapter 2). To evaluate the contribution of genetic variation to the overall variation (heritability) in the secretion of baseline and stress-induced corticosterone levels (chapter 1), I used 7 years of corticosterone measurements taken on nestlings from a free-living population of barn owls, combined with a long-term pedigree (20 years). In parallel, I analysed 13 years of corticosterone and capture-recapture data collected from adult barn owls to estimate the association between circulating corticosterone and survival. The analyses showed that baseline and stress-induced corticosterone secretions are, respectively, negatively and positively associated to survival (chapter 2) and that the variation in baseline and stress-induced corticosterone levels are low ($h^2$ = 0.199) to mildly heritable ($h^2$ = 0.441) but are strongly genetically correlated ($r$ = 0.735) (chapter 1). These results suggest that the stress-induced corticosterone response is susceptible to respond faster to selective processes, and therefore to evolve faster, than baseline corticosterone levels. However, because they are strongly genetically correlated selection acting on one trait may constrain the evolution of the other.

In the second part of the thesis, I explored the proximate mechanisms linking melanin-based traits with the HPA axis (Chapter 3), the signalling function of melanin-based traits in relation to testosterone and the potential role of sex hormones (testosterone, estrogen) in the expression of melanin colour traits in the barn owl (Chapter 4). With this aim, I assessed the relationships between coloration and the expression of genes associated with the melanocortin system and melanogenesis with plasma testosterone levels in barn owls, with the expression levels of genes related to the metabolism and biological action of steroid hormones, including glucocorticoid and mineralocorticoid receptors. I showed that the expression of glucocorticoid and

mineralocorticoids receptors, which mediate the action of corticosterone, are negatively associated with melanin-based traits and positively with the expression the *PCKS2* gene which is involved in the processing of melanocortin hormones and thereby in melanin synthesis (chapter 3). I also showed that the size of eumelanin black spots displayed at the feather tips in barn owls covary with testosterone levels. Interestingly, in breeding adults this relation is of opposite sign in the two sexes (chapter 4), which suggests that testosterone-dependent behaviour and physiological processes are differently associated with melanin-based feather spottiness in males and females. Moreover, I showed that dark-reddish individuals express higher levels of *5α*-reductase in their feathers than light reddish individuals. This enzyme transforms testosterone into the more potent hormone DHT, which in turns binds to androgen receptors. This finding is consistent with the hypothesis that testosterone might be involved in the expression of reddish-brown pheomelanic pigments.

In summary, the present thesis provides new knowledge on the genetic basis of corticosterone levels and its capacity to evolve, given that the collected data showed that corticosterone levels, including baseline and stress-induced response, are partly heritable and that part of this variation between the two traits are genetically correlated. Accordingly, corticosterone levels are associated with survival and hence, are currently under selection. Additionally, this thesis adds new information on the signalling value of melanin-based traits in barn owls and the potential role of sex hormones in the expression and adaptive function of melanin-based traits.

**Résumé**

Les hormones régulent l'expression de caractères morphologiques, physiologiques et comportementaux dont le développement d'ornements et caractères sexuels secondaires. Afin de comprendre comment ces traits ont pu évoluer, il est essentiel de déterminer le rôle des gènes impliqués dans leur expression et les mécanismes par lesquels la sélection a façonné le système endocrinien.

Au cours de cette thèse, nous avons exploré différents aspects liés au système endocrinien, y compris le lien entre les hormones stéroïdiennes et la coloration mélanique chez la Chouette effraie (*Tyto alba*). Dans un premier temps, nous avons étudié le potentiel évolutif (chapitre 1) et la valeur adaptative de la corticostérone (chapitre 2), une hormone qui est importante dans la régulation des fonctions métaboliques chez les oiseaux et permet à un organisme de surmonter des événements stressants, tels que le manque de nourriture, l'attaque d'un prédateur ou des imprévus climatiques. Nous avons pu montrer que le niveau de corticostérone basal et celui induit par un événement stressant sont, respectivement, négativement et positivement associés à la survie annuelle des chouettes adultes (chapitre 2), et que la variation du niveau de corticostérone basal et celui induite par un stress est faiblement ($h^2$ = 0.199) à moyennement héréditaire ($h^2$ = 0.441, chapitre 1). Ces résultats suggèrent que les niveaux de corticostérone sont susceptibles d'évoluer. Cependant, comme le niveau basal et celui induit lors d'un stress sont génétiquement associés (r = 0.735, chapitre 1) et qu'ils sont différemment sélectionnés (chapitre 2), cet antagonisme pourrait empêcher l'évolution des niveaux de corticostérone selon la force et la stabilité des processus de sélection.

Nous avons également exploré les mécanismes physiologiques et génétiques reliant la coloration mélanique à l'axe hypothalamus-hypophyso-surrénalien (HHS) qui régule l'expression de la corticostérone (chapitre 3), la fonction des signaux de coloration mélanique en lien avec la testostérone et nous sommes intéressés au lien entre les hormones sexuelles (testostérone, estrogène) et les colorations mélaniques (chapitre 4). Nous avons mis en évidence que l'expression des récepteurs des stéroïdes liant la corticostérone sont négativement associés à la coloration mélanique, mais en revanche, qu'ils sont positivement associés à l'expression du gène *PCSK2.* Ce gène code pour une enzyme impliquée dans le processus de maturation de différents peptides du système des mélanocortines, dont l'α-MSH et l'ACTH. Ces hormones régulent respectivement la synthèse de la mélanine et la sécrétion de corticostérone (chapitre 3). L'association entre la couleur et l'axe HHS chez la Chouette effraie pourrait ainsi venir du

processus de maturation de ces hormones et de l'expression de cette enzyme. Nous avons également montré que la taille des taches noires ornant le plumage de la Chouette effraie est associée au niveau de testostérone dans le sang. Toutefois, cette relation est opposée entre les sexes (chapitre 4), ce qui laisserait suggérer que la testostérone n'est pas impliquée dans l'expression des taches. Au lieu de ça, l'expression des taches pourrait être contrôlée par des gènes pléiotropes, qui régulent conjointement la synthèse de la mélanine et le niveau de testostérone dans la circulation. En revanche, nous avons trouvé que la 5α-réductase, une enzyme qui métabolise la testostérone en DHT, est exprimée en plus grande quantité dans les plumes des individus roux que blancs. La DHT étant une hormone qui a, une plus forte affinité pour le récepteur des androgènes que la testostérone, cela suggère que la testostérone pourrait être indirectement impliquée dans l'expression de la couleur rousse chez la Chouette effraie.

En conclusion, cette thèse montre que le système endocrinien est susceptible de répondre à des processus de sélection et donc d'évoluer. De plus, les gènes impliqués dans la synthèse de pigments mélaniques pourraient potentiellement influencer la sécrétion des hormones stéroïdiennes, telle la testostérone. Toutefois, il est également possible que la testostérone puisse, par le biais de la DHT, influencer l'expression de pigment roux chez la Chouette effraie.

## General introduction

To be able to survive and reproduce in a continuously changing environment, an organism has to integrate and coordinate a number of behaviours and physiological functions. Hormones, such as steroids (e.g., glucocorticoids, testosterone, estrogen), are chemical messengers that regulate, orchestrate and coordinate the most important developmental, daily and life-history processes. Their capacity to control diverse physiological and behavioural functions makes them key components during early development (Arnold, 2009; Phoenix *et al*, 1959; Wallen, 2009) and between and within cyclic life-history stages of adulthood (Landys *et al*, 2006; Romero, 2002). However, due to their ubiquitous presence in the organism, their pleiotropic actions and, often, their antagonistic effects on a number of behavioural, physiological and morphological traits, hormones are at the origin of many constraints and trade-offs that have influenced the evolution and development of different morphological and life-history traits (Flatt *et al*, 2005; Hau *et al*, 2010; Ricklefs and Wikelski, 2002; Zera *et al*, 2007).

Some hormones, such as glucocorticoids, are thought to be important in regulating the trade-offs between reproduction and survival (Bonier *et al*, 2009a; Breuner *et al*, 2008; Crespi *et al*, 2013) whereas others, such as sex hormones, might be at the origin of the variation in the expression of secondary sexual traits such as colour ornaments (Kimball, 2006b). For instance, testosterone, which is recognised as an important mediator of trade-offs (Hau *et al*, 2010; Ketterson *et al*, 2001; Marler and Moore, 1988), including between ornamental traits and immune functions (Casto *et al*, 2001; Folstad and Karter, 1992), has been found to be associated with the expression of melanin-based colour traits in a number of species being, in some of them, essential to produce male-like traits (Bokony *et al*, 2008; Buchanan *et al*, 2001; Evans *et al*, 2000; Peters, 2007). Thus, understanding and determining the proximate mechanisms contributing to the expression and the association between hormones and other phenotypes are of a great importance in order to better comprehend the adaptive function and evolution of hormones, and related traits such as sexually selected colour traits. However, to understand how the different endocrine patterns might have evolved to mediate such associations and trade-offs, it is crucial to determine how selective processes shape endocrine traits. This, in turn, requires a good understanding of both the genetic component contributing to the phenotypic variation of hormonal titers and their consequences on fitness.

In the following sections, I will introduce the role and regulation of glucocorticoid hormones by the hypothalamic-pituitary-adrenal axis, the mechanisms by which glucocorticoid hormones

induce biological effects and the importance of assessing the contribution of genetic variation to endocrine traits, such as glucocorticoids. I will also describe the role and link of the melanocortin system with the hypothalamic-pituitary-adrenal axis and melanin-based traits, discuss the function of melanin-based traits and the potential effect of sex hormones, including testosterone, in the regulation of such traits.

*The hypothalamic-pituitary-adrenal axis*

The secretion of glucocorticoid hormones (stress hormones) by an organism, controls a broad range of physiological and behavioural functions (Figure 1) that are orchestrated by a complex set of interactions between the hypothalamus, the pituitary and the adrenal gland (i.e., the hypothalamic-pituitary-adrenal, HPA, axis). Corticosterone, which is the primary glucocorticoid in birds, reptiles, amphibians and several species of rodents, is involved in many daily life processes, including energy storage, metabolic function, foraging behaviour and sleep (reviewed in Dallman *et al*, 2004; Landys *et al*, 2006; McEwen, 2008). Corticosterone plays also a determining role in initiating and coordinating emergency mechanisms in case of unpredictable (e.g., inclement weather conditions, food restriction periods) and life-threatening events (e.g., a predator attack) (Wingfield *et al*, 1998). During such events, the HPA axis triggers an important release of corticosterone in the circulation, which induces the redirection of an organism's resources towards behavioural and physiological functions that are vital to its immediate survival (reviewed by Crespi *et al*, 2013; Landys *et al*, 2006; Sapolsky *et al*, 2000). For instance, elevated corticosterone levels increase heart rate, blood pressure, peripheral blood supply and glycaemia (reviewed inSapolsky *et al*, 2000). The rapid increase of corticosterone in the circulation is initiated by a neuronal signal to the hypothalamus and followed by a complex set of neuroendocrine reactions that triggers the release of corticotropin-releasing hormones (CRH) from the paraventricular nucleus (PVN) of the hypothalamus into the anterior pituitary gland, that in turn, stimulates the release of adrenocorticotropic hormone (ACTH) (Figure 1)(Charmandari *et al*, 2005). The release of ACTH into the circulation subsequently induces the secretion of corticosterone by the adrenal cortex into the adrenal vein within seconds to minutes (Johnson *et al*, 1992; Sapolsky *et al*, 2000). Although, this physiological response aims to help an organism to overcome immediate and short life-threating events (i.e., minutes), long term (i.e., days or week), chronic exposure to high levels of corticosterone can have permanent or temporary deleterious effects, contributing to health problems and, thereby, decreased survival prospects (Bremner, 2007;

Breuner *et al*, 2008; de Kloet *et al*, 1999; Martin, 2009; Sapolsky *et al*, 2000). Therefore, to protect the organism from an over-exposure of corticosterone, the HPA axis is carefully regulated by a negative feedback loop (Figure 1) via glucocorticoid receptors (mineralocorticoid and glucocorticoid receptors) in different brain regions (Charmandari *et al*, 2005).

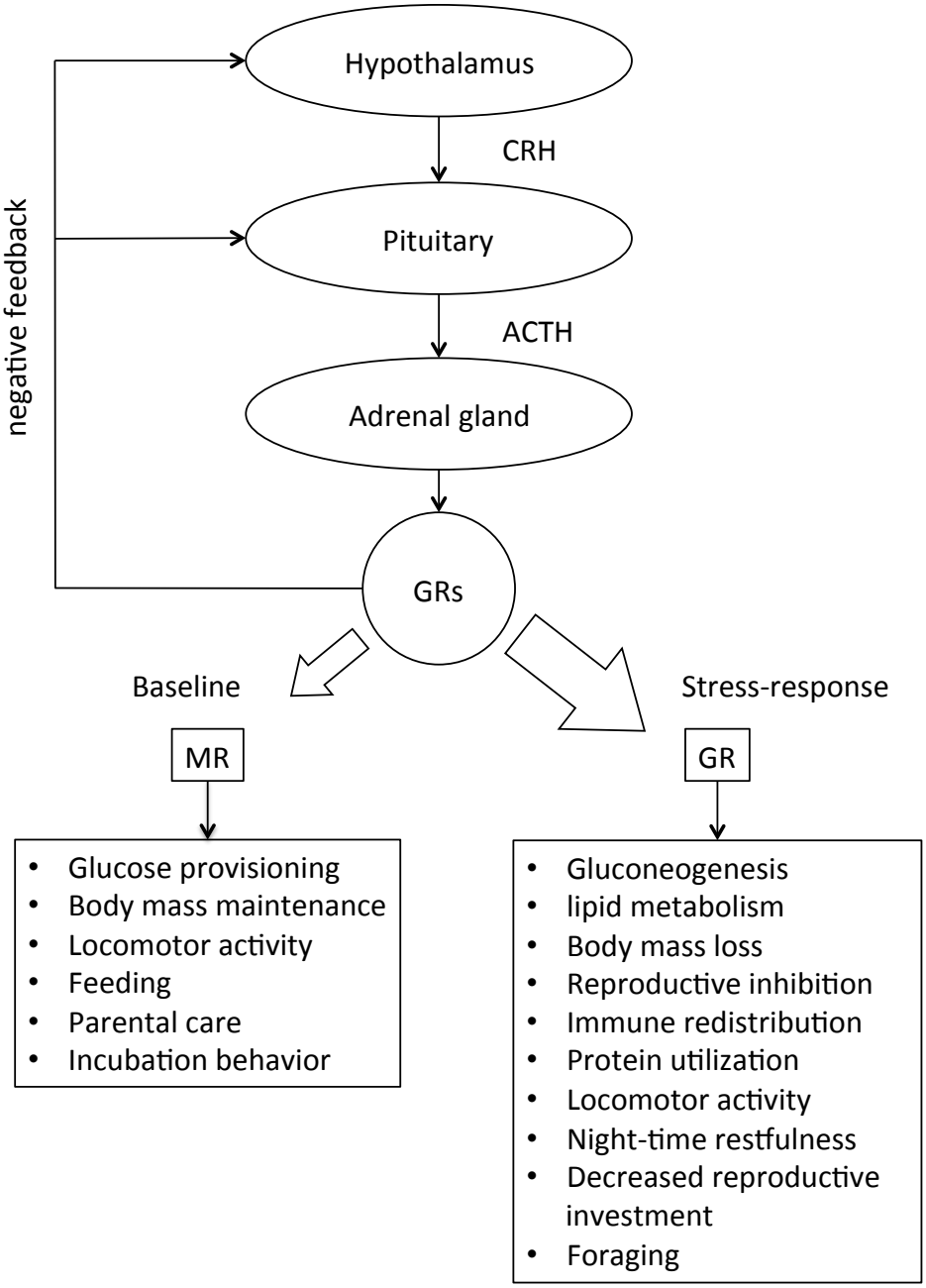


**Figure 1**. Schematic illustration of glucocorticoid synthesis pathway regulated by the hypothalamic-pituitary-adrenal (HPA) axis with the major phenotypic action of glucocorticoids (GSs) in vertebrates. *CRH*, corticotropin-releasing hormone; *ACTH*, adrenocorticotropic hormone; *MR*, mineralocorticoid receptor; *GR*, glucocorticoid

receptor. The negative feedback loop is regulated through both receptors, including the hippocampus. Redrawn from (Hau *et al*, 2016).

*Mechanism and action of glucocorticoid hormones*

The secretion of glucocorticoid hormones, such as corticosterone, is only one component of the HPA axis involved in the physiological action of glucocorticoids. Once released in the circulation, corticosterone travels to target tissues attached to a plasma globulin protein named corticosteroid-binding globulin (CBG). In the circulation, most corticosterone molecules are bound to CBGs, the remaining being bound to albumin or remain unbound (Malisch and Breuner, 2010; Siiteri *et al*, 1982). These unbound molecules are called the "free" part and are believed to be the only ones that enter target tissues where they can have a biological activity (Mendel, 1989). The binding capacity or affinity of CGBs may vary according to environmental or physiological conditions, between sex, population and species or even life-history stages (reviewed in Malisch and Breuner, 2010). The role of CBGs is still debated (for details see Romero and Wingfield, 2016), some authors suggesting that CBG acts as a buffer to restrain the effect of glucocorticoids while allowing a rapid mobilization of glucocorticoids when needed (Breuner and Orchinik, 2002; Rosner, 1990). Others have suggested that CBGs play the role of carrier (Rosner, 1990) with the capacity to induce physiological actions (Nakhla *et al*, 1988; Strelchyonok and Avvakumov, 1991) or to prolong the life-span of glucocorticoids by preventing them from being taken up into the liver where glucocorticoids are metabolized into its inactive form (Shultz and Kitaysky, 2008). The action of glucocorticoids depends also on the activity of the isozyme 11*β*-hydroxysteroid dehydrogenase (11*β*-HSD) in target tissues. The enzyme 11*β*-HSD type 2 is implicated in the conversion of the active glucocorticoids, such as corticosterone into its inactive form, 11-dehydrocorticosterone or inversely, the 11*β*-HSD type 1 catalyses 11-dehydrocorticosterone to corticosterone (Chapman *et al*, 2013; Tomlinson *et al*, 2004).

As like other steroid hormones, corticosterone is a ligand-dependent transcription factor that affects the expression of numerous genes (e.g., ≈2% of human genome is regulated by glucocorticoids) by binding to the promoter region of corticosteroid-responsive genes (de Kloet *et al*, 2005; Le *et al*, 2005; Reddy *et al*, 2009). The action of corticosterone requires the mediation of one of two receptor types, the glucocorticoid (GR, type I) and the mineralocorticoids (MR, type II) receptors (Figure 1). These receptors are mostly located in the cytoplasm, with some being in the nucleus, where they are associated to heat-shock protein (HSP) that prevents them from binding

to DNA (Norris, 2006). The binding of corticosterone to MR or GR induces the migration and binding of the ligand-receptor complex to the corticosterone response element present in the promoter region of corticosteroid-responsive genes. This results in the activation of the transcriptional machinery to transcribe the DNA sequence of the activated genes, and ultimately to protein synthesis. GR and MR can also repress the expression of certain genes by preventing the binding of other transcription factors to their target genes (de Kloet *et al*, 2005; Ray and Prefontaine, 1994).

The biological effects of corticosterone hormone is also tissue specific as it is strongly influenced by the levels of specific enzymes activating or deactivating the hormone (Chapman *et al*, 2013; Joels *et al*, 2008; Tomlinson *et al*, 2004) and the number receptors that binds corticosterone (Iida *et al*, 1985; Vanderbilt *et al*, 1987; Yang *et al*, 1989). The abundance of receptors (MR and GR) and enzymes (e.g., 11*β*-HSD type 1 and 2) is thus a determinant factor, as well as the concentration of circulating corticosterone, given that both are essential in triggering the biological effects and the magnitude of such effects (Breuner and Orchinik, 2001; Csaba, 1986; Lattin and Romero, 2015; Lattin *et al*, 2013). Moreover, as corticosterone receptors are present in most tissues and cells across the body (Iida *et al*, 1985; Vanderbilt *et al*, 1987; Yang *et al*, 1989), many traits are susceptible to be influenced by variations in corticosterone levels.

*Glucocorticoids variation and evolution of the hypothalamic pituitary-adrenal (HPA) axis*

As previously mentioned, hormones, such as glucocorticoids, are powerful molecules that coordinate, orchestrate and regulate different physiological and behavioural functions in response to internal (e.g., plasma levels of free fatty acids, glycaemia, diseases, circadian rhythm, life stage and so on) and external cues (e.g., climatic and environmental changes, food scarcity, predation risk). Glucocorticoids are important transducers between an organism and its environments, allowing it to adopt appropriate physiological and behavioural actions to cope with environmental perturbations. The environment is a source of unpredictable perturbations (e.g., social interaction, diseases, environmental challenges), which results in a variety of interactions between organism's and their environment and has led to the evolution of a diversity of physiological adaptations. These coping mechanisms have translated into great variation in glucocorticoids levels within or among individuals, populations and species. For instance, glucocorticoids vary with social rank (Gesquiere *et al*, 2011), sex (Jones *et al*, 1998; Rivier, 1999; Trainor *et al*, 2010), but also a variety of environmental factors, such as seasonality (Romero, 2002), human disturbance (Abolins-Abols

*et al*, 2016; Almasi *et al*, 2015; Partecke *et al*, 2006) population density (Dantzer *et al*, 2013; Glennemeier and Denver, 2002; Meylan and Clobert, 2004), predation risk (Monclus *et al*, 2005; Scheuerlein *et al*, 2001; Sheriff *et al*, 2009) and unpredictable climatic events (Bize *et al*, 2010; Jenni-Eiermann *et al*, 2008; Romero *et al*, 2000). Glucocorticoid levels have also been shown to be associated to different personality traits (Carere *et al*, 2003; Garland *et al*, 2016; Stowe *et al*, 2010), life-history strategies (Crespi *et al*, 2013) or fitness metrics, including reproductive success (Angelier *et al*, 2009; Blas *et al*, 2007; Crespi *et al*, 2013; Romero and Wikelski, 2001) and survival (Blas *et al*, 2007; Comendant *et al*, 2003; Goutte *et al*, 2010; Rivers *et al*, 2012).

These findings have only recently led evolutionary biologists to try to understand the proximate mechanisms underlying such phenotypic variation, their consequences and the processes promoting the evolution of such adaptations (Bonier and Martin, 2016; Cox *et al*, 2016; Hau and Goymann, 2015). However, many questions remain unanswered. As depicted in the sections above, the emergency life-history stage is the result of a cascade of complex neuroendocrine interactions, between different glands, hormones, receptors and binding proteins. Given all these components of the HPA axis, it remains unclear which ones are the most important in determining an individual's fitness. There are several candidates, including the amount of circulating glucocorticoids, number and distribution of glucocorticoids receptors and CBG, or the capacity to respond to stress (e.g., sensitivity of the HPA axis to respond to a stress or magnitude of the glucocorticoid response) and to induce a feedback loop. We also need further information about the genetic determinism of the response of the HPA axis to environmental stressful factors and given this information, the extent to which the HPA axis can respond to selective process and further evolve.

Investigating these issues is important because hormones, like glucocorticoids, form a tight and dynamic link between an organism and its environment. Therefore, understanding the proximate mechanisms and evolutionary processes driving the HPA axis is crucial to determine whether and how environmental changes, such as human disturbance and habitat degradation, could be threatening certain animal populations or species. This may also help us understand why some species manage to deal with our challenging world while others cannot (Bull and Maron, 2016; Hunter, 2007).

*Testosterone and the hypothalamic-pituitary-gonadal (HPG) axis*

Like glucocorticoid hormones, sexual hormones are regulated by a complex set of neuroendocrine

pathways linking the hypothalamus, the pituitary and adrenal glands (i.e., hypothalamic-pituitary-gonadal (HPG) axis). Secretion of sexual hormones is controlled by the release of gonadotropin-releasing hormone (GnRH) by the hypothalamus. This hormone regulates, via the stimulation of gonadal hormones (e.g., luteinizing hormone and follicle-stimulating hormones (FSH)), sexual development, sex hormone secretion and reproductive functions. The luteinizing hormone and FSH stimulate the secretion of testosterone in males and estrogen in females, and are responsible for the production of gametes in both sexes (Norris, 2006). Sex hormones are principally synthesised from cholesterol in gonads but can also be synthesised in other organs, including adrenals (e.g., dehydroepiandrosterone, androstenedione) and the skin (Norris, 2006; Slominski, 2012; Zouboulis *et al*, 2007). Like other steroids, sex hormones are transcriptional factors (see above for details) that, once bound to their receptors, regulate the expression of many genes and cellular processes (Becker *et al*, 2002; Gambineri and Pasquali, 2000; Gruber *et al*, 2002). Sex hormones play a critical organizational role during early development, as they can permanently affect the structure of the nervous system, behaviour and body development. These organizational effects are crucial because they determine in a permanent way the hormonal response of an organism throughout its life (Arnold, 2009; Phoenix *et al*, 1959; Wallen, 2009). At adulthood, sex hormones promote and coordinate a number of physiological, morphological and behavioural traits essential to reproduction. For instance, testosterone mediates sexual and courtship behaviours, territorial aggression, sperm production and the development of secondary sexual characters (Norris, 2006). Although empirical evidence shows that testosterone increases male reproductive fitness (McGlothlin *et al*, 2010; Reed *et al*, 2006), presenting high testosterone levels may also come with some costs for the lifetime fitness of an individual. For instant, high levels of testosterone can be immunosuppressive, metabolically costly, and can reduce survival and investment in parental care (Dufty, 1989; Folstad and Karter, 1992; Reed *et al*, 2006; Wingfield *et al*, 2001). Therefore, like all hormones, testosterone secretion is controlled by a feedback mechanism that maintains hormone homeostasis. Testosterone can also be influenced by numerous factors, including environmental, social and internal factors (Garamszegi *et al*, 2008; Goymann *et al*, 2004; Lamba *et al*, 1983; Wingfield *et al*, 2001).

Testosterone circulates in the plasma attached to transport proteins, but unlike most vertebrates, birds do not possess sex-hormone-binding proteins (SHBG) (Wingfield *et al*, 1984). Therefore, in birds testosterone is mostly bound to glucocorticoid-binding globulin (CBG, see above) (Charlier *et al*, 2009; Deviche *et al*, 2001). Sex hormones can circulate to target tissues

either under an active (e.g., testosterone, estrogen) or inactive form (e.g., DHEA, androstenedione) (Norris, 2006) and once in a specific tissue, sex hormones can be metabolized into different sex steroid hormones (Figure 6). For instance, testosterone can be metabolized into estrogen by aromatase or into dihydrotestosterone (DHT) by *5α*-reductase, two hormones that have different physiological functions (estrogen) than testosterone or similar functions but in a more active form (DHT) (Ball and Balthazart, 2008; Dessifulgheri *et al*, 1976). Therefore, as like glucocorticoids, the effect of sex hormones may also be regulated in a tissue-specific manner, as it will depend on their circulating concentration, their affinity with carriers (CBG, SHBG), the sensitivity of the target tissue (e.g., number of receptors) or the enzymatic activity of the tissue (e.g., activity of aromatase or *5α*-reductase).

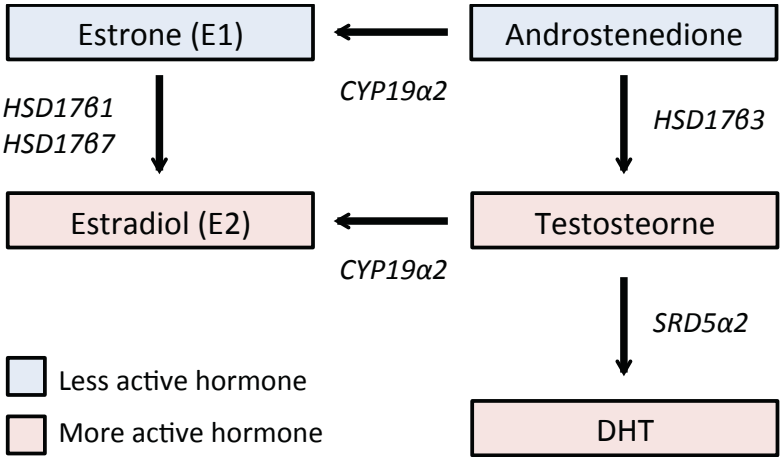


**Figure 6.** Schematic overview of the steroid pathway for sexual hormones. The enzymes that regulate the biosynthesis of androgens and estrogens are represented in italics. DHT, dihydrotestosterone; *CYP19α2*, aromatase; *SRD5α2*, *5α*-reductase; *HSD17β*, 17β-hydroxysteroid dehydrogenases.

*Melanin-based coloration*

Melanin-based colour traits are present in a wide range of taxonomic groups and species (Majerus, 1998) where they have evolved in variety of forms and a diversity of functions. For instance, melanin pigments (see Box 1) can be used to protect tissues from UV radiation or abrasion, to hide from both predators and preys or to communicate information (e.g., warning, quality, behavioural strategy, genetic compatibility, individual identity) (Hill and McGraw, 2006;

Hoekstra, 2006; Majerus, 1998; Roulin, 2004c). Such diversity has fascinated evolutionary biologists and raised their interest on how and why such a diversity of patterns and functions has evolved.

In many species, colourful ornaments are thought to have evolved as honest signals of quality because their expression often relies on the individual's condition (e.g., Hill, 1991; Hill, 2000; Hill and Montgomerie, 1994; Zahavi, 1975). For instance, in species that display carotenoid-based traits these colourful ornaments are considered as a reliable indicator of individual quality because they derived from food and can have antioxidant capacity (Olson and Owens, 1998) and hence reflect resistance to oxidative stress (Amengual *et al*, 2011), nutritional status or foraging ability (Hill, 1992; Kodricbrown, 1989; Walker *et al*, 2014). In contrast, because melanin-based traits are directly synthesised by the animals from amino-acids and hence often under tight genetic control and weakly affected by environmental factors (Badyaev and Hill, 2000), the means by which melanin-based traits can reflect the quality of an individual remains obscure. However, melanin-based traits might be considered as a reliable signal of quality if their expression involves high-costs (e.g., social costs) or if there is a mechanistic connection between the expression of the traits and a phenotypic or genetic quality (Roulin, 2016). Under this hypothesis, genes regulating aspects of the quality of an individual may also affect the expression of melanin-based traits.

**Box 1. The melanogenesis pathway**

Melanins are the most common colour pigments and can been found in bacteria, plants, fungi and animals (Butler and Day, 1998; Cheynier *et al*, 2013; Gonzalez *et al*, 1997; Ito and Wakamatsu, 2003; Langfelder *et al*, 2003; Majerus, 1998; Plonka and Grabacka, 2006). In vertebrates, melanin-based traits are composed of two pigments, the black eumelanic pigment and yellow-to-brown pheomelanic pigment, which are synthesised through two distinct pathways (Figure 2) (Wakamatsu and Ito, 2002). The ratio of eumelanin to pheomelanin pigments deposited in tissues or integuments determines the variation in melanin coloration (Jawor and Breitwisch, 2003). Melanin is synthesised in the melanosomes, which are organelles also involved in transporting and delivering melanin pigments to the keratinocytes (Yu *et al*, 2004). The synthesis of melanin is largely regulated by the melanocortin 1-receptor (MC1R), a seven transmembrane G protein-coupled receptor, which binds the $\alpha$-melanocyte-stimulating hormone ($\alpha$-MSH) or its antagonist agouti signalling protein (ASIP). Binding of $\alpha$-MSH activates the cAMP signalling system, which in turn induces the expression of the microphthalmia-associated transcription factor (MITF) gene. MITF is a protein that regulates the expression of different enzymes involved in melanin synthesis, including tyrosinase, which is the main catalytic agent and a rate-limiting factor for the synthesis of melanin production (Ito *et al*, 2000). Tyrosinase transforms tyrosine to dopaquinone that can generate either eumelanin by an intermediate step involving the tyrosinase related protein (TYRP 1) and the dopachrome tautomerase (DCT) (Kobayashi *et al*, 1995; Lin *et al*, 2013), or pheomelanin, in the presence of the amino acid cysteine (Figure 2). Once melanin pigments are formed, melanosomes migrate along microtubules down to the dendritic part of melanocytes, where they are phagocytised into keratinocytes. The binding of the antagonist ASIP to MC1R on melanocytes switches the balance of eumelanin to pheomelanin contents in melanosomes or, in other cases, prevents any melanin synthesis producing white colourations (Wakamatsu and Ito, 2002).

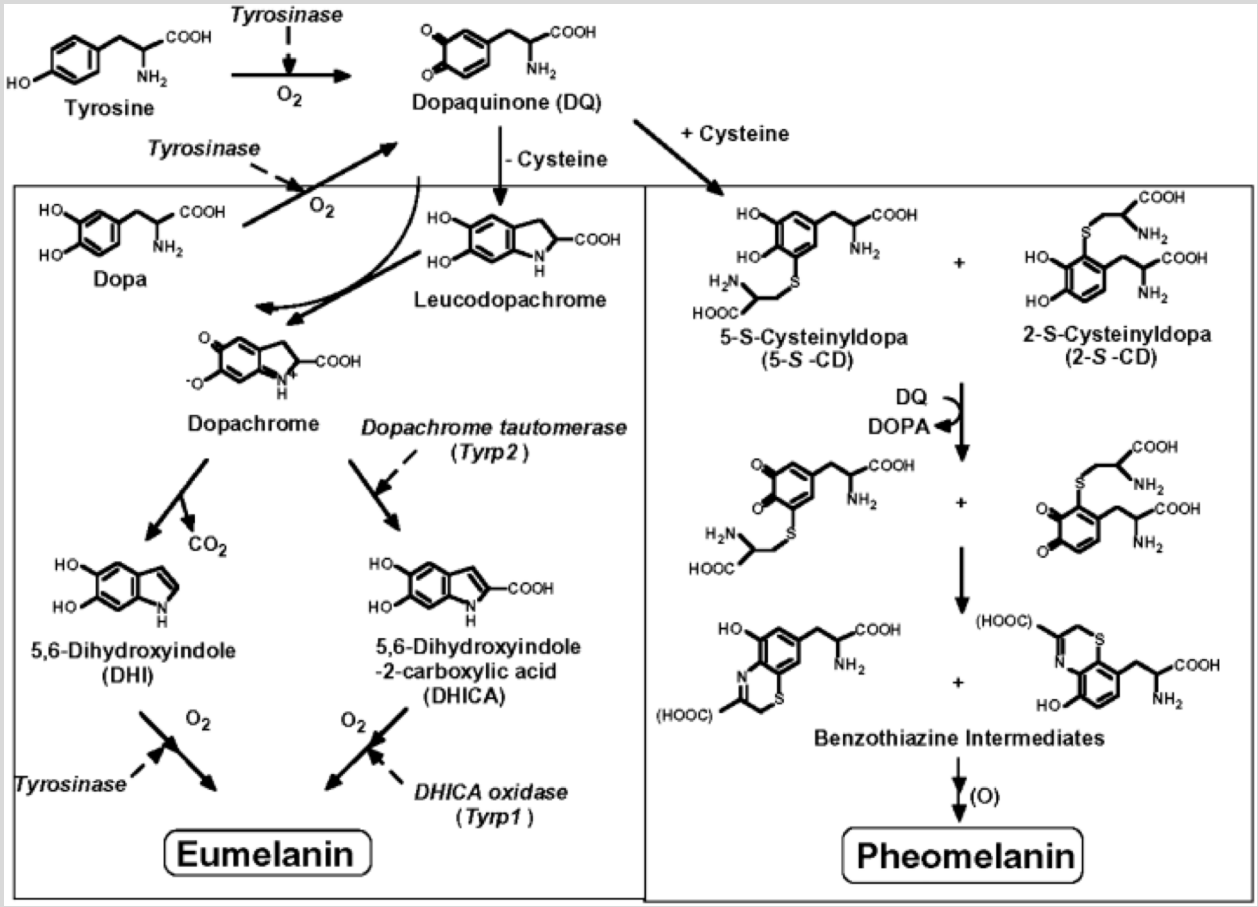


**Figure 2.** Synthetic pathways for eumelanin and pheomelanin. Note that the amino acid cysteine is necessary for the synthesis of pheomelanin and that the enzyme tyrosinase (TYR) in involved at several levels of eumelanin synthesis pathway, but not in pheomelanin after conversion of tyrosine to dopaquinone. The enzyme tyrosinase related protein (TYRP) -1 and TYRP-2 (also known as dopachrome tautomerase (DCT)) are only present in the eumelanogenesis process. Redrawn from (Ito and Wakamatsu, 2011)

*The hypothalamic-pituitary-adrenal axis and melanin-based traits*

In the last decade, many studies have shown that melanin traits can be associated to different physiological, behavioural or life-history strategies (reviewed by Roulin and Ducrest, 2011). An interesting aspect in the evolution of melanin-based traits is that the genes involved in the synthesis of melanin pigments can pleiotropically influence other physiological and life-history traits through the melanocortin system (Figure 4, Ducrest et al. 2008).

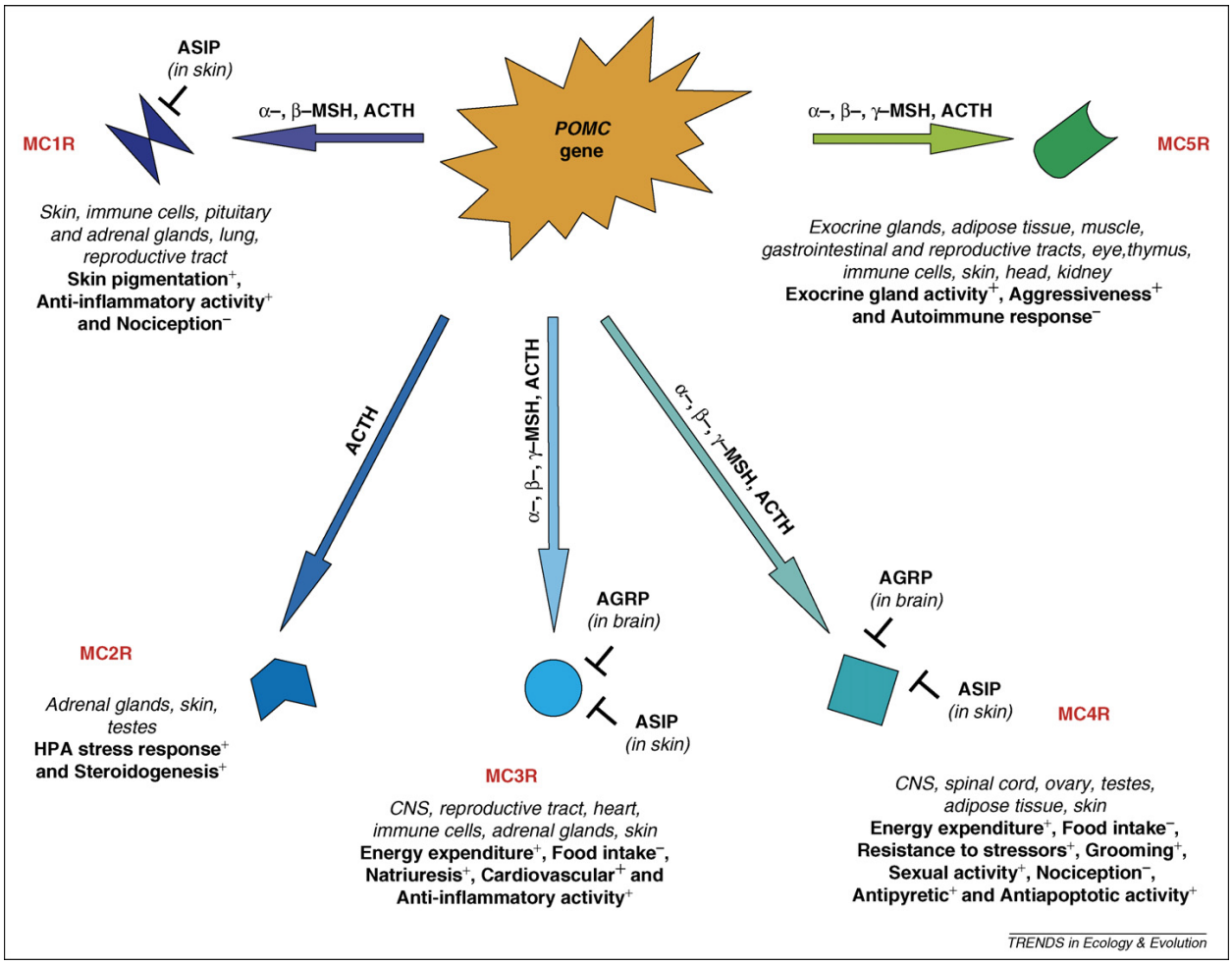


**Figure 4**. Representation of the melanocortin system. The posttranslational cleavage of the proopiomelanocortin (POMC) prohormone produces the melanocortins (i.e., α-, β- and γ-MSH and ACTH), which bind to the different melanocortin receptors (MC1-5R). The location of the five melanocortin receptors (MC1-5R) in vertebrates is given in italics while their function is written in bold. For each function, we report whether binding of the melanocortins to the different MCRs has positive (+) or negative effects (-). For example, binding of melanocortins to MC3R increases energy expenditure but reduces food intake. The agonists and inverse antagonists (agouti-signalling protein, ASIP or agouti-related protein, AGRP) for each MCRs are indicated with the symbol ⊥. Reprinted from (Ducrest *et al*, 2008).

The melanocortin system is composed of five G-protein-coupled melanocortin receptors and their agonist melanocortin hormones (i.e., melanin-stimulating hormones α-, β- and γ-MSH, ACTH) and antagonist the agouti-signalling protein (ASIP). All melanocortins are derived from the cleavage of the proopiomelanocortin (POMC) prohormone by protein convertases (PC1/3, PC2) (Figure 5) (Chretien and Mbikay, 2016; Eipper and Richard, 1980; Pritchard and White, 2007) and bind to one or more of the five melanocortin receptors (MC1-5R). For instance, binding of ACTH to the

receptor MC2R induces the production of corticosterone in the adrenal glands (see above, Figure 4), whereas the binding to MC1R by α-MSH, which is the product of the cleavage of ACTH by convertase protein 2 (PC2) triggers melanin production in melanocytes (see Box 1). Thus, because each of the melanocortins are derived from the same precursor (i.e., POMC) and that they can bind to different melanocortin receptors which have very different functions (Figure 4), the pleiotropic effect of the melanocortin system has been proposed as an explanation for many of the covariations found between melanin-based traits and physiological, behavioural or life-history traits (Roulin, 2016; Roulin and Ducrest, 2011). This effect could be reinforced by the fact that adrenal glands and pigment cells (melanocytes) both originate from the neural crest cells. Their common developmental origin could also be an explanation to why melanin-based traits covary with glucocorticoids in many vertebrate species (Wilkins *et al*, 2014).

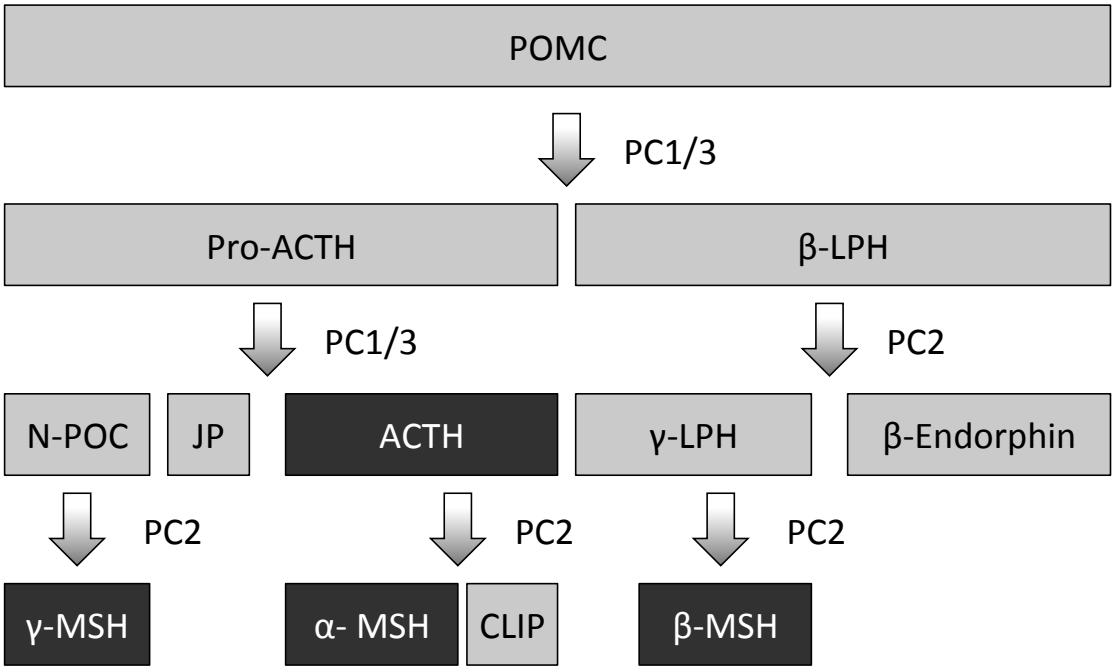


**Figure 5**. Simplified diagram of the posttranslational processing of proopiomelanocortin hormone (POMC). The agonists of melanocortin receptors (MC1-5R) are represented in black. ACTH, adrenocorticotropic hormone; CLIP, corticotropin-like intermediate lobe peptide; JP, junctional peptide; LPH, lipotropic hormone; N-POC, *N*-pro-opiocortin; MSH, melanocyte stimulating hormone; PC, prohormone convertase protein.

Given that corticosterone regulates energy allocation and therefore key condition-dependent traits, corticosterone has been suggested to influence the evolution of ornamental

traits that reflect corticosterone levels or corticosterone-mediated traits (Buchanan, 2000; Husak and Moore, 2008). Although, several studies have shown that corticosterone can negatively affect the expression of certain traits, including male bird song (Buchanan *et al*, 2004; Spencer *et al*, 2003), anuran amphibians vocalization (Leary *et al*, 2006) or melanin colour ornaments (Roulin *et al*, 2008), the mechanisms by which mineralocorticoids (MR) and glucocorticoids (GR) receptors, and thereby corticosterone, affect the expression of melanin-based traits are still unclear. Therefore, to better understand the role of corticosterone in the expression of such traits and their function in an evolutionary context, physiological and molecular studies in parallel with experimental experience are necessary to determine the mechanisms linking such traits.

*Sexual hormones and melanogenesis*

Among factors controlling melanin coloration, steroid hormones like testosterone and estrogen, have been found to regulate melanin-based traits in a number of species (reviewed by Kimball, 2006b; McGraw, 2006). Although several studies have investigated the effect of sex hormones, such as testosterone or estrogen, on melanin coloration (reviewed by Kimball 2006), the proximate mechanism by which those hormones affect melanogenesis remains unclear, given that many of those studies have shown contrasting results. Sexual hormones, such as estrogen have been found to increase melanisation in one or both sexes, for instance by increasing the activity of tyrosinase (see Box 1), whereas in others cases it has been shown to inhibit melanin deposition or the protein ASIP involved in the down-regulation of the synthesis of melanin pigments (Hirobe *et al*, 2010; Jee *et al*, 1994; Oribe *et al*, 2012). Similar evidence shows that estrogen can affect melanogenesis via estrogen receptors, MITF and DCT (Jian *et al*, 2011; Schwahn *et al*, 2005) or estrogen specific G protein-coupled receptors (GPER) (Sun *et al*, 2017). Androgens such as testosterone have also been shown to play a role in melanization via the activity of tyrosinase enzyme (Tadokoro *et al*, 1997; Tadokoro *et al*, 2003). The effect of testosterone could be mediated by the presence of androgen receptors in melanocytes and the enzyme activity of *5α*-reductase in their nuclei (Kim *et al*, 2008), an enzyme that metabolizes testosterone into the more potent androgen dihydrotestosterone (DHT). Therefore, binding of testosterone or DHT to the androgen receptors in melanocytes may influence the expression of genes related to melanogenesis. Although there is clear evidence showing that estrogen and testosterone affect melanin synthesis, the action of these hormones and their receptors on melanogenic genes has not yet been fully explained. Identifying the proximate mechanisms driving the expression of

melanin-based traits is essential to the understanding of the evolution of such traits. For instance, in species displaying hormonally-mediated colour traits, highly ornamented individuals may be of higher quality compared to less ornamented conspecifics because they are able to invest resources into the expression of ornaments while supporting the physiological costs associated with the hormones.

*Aims of thesis*

The aim of this thesis is to determine the degree of heritable variation in the secretion by the hypothalamic-pituitary-adrenal (HPA) axis of corticosterone hormones and their potential association with barn owl survival. Corticosterone is the main glucocorticoid hormone in birds, it is involved in the physiological response to stress and is considered as an important mediator of trade-offs between different life-history traits, including reproduction and survival in different bird species (e.g., chapter 2). Determining the heritability and impact of corticosterone variation on survival is essential to understand the importance of variation in circulating corticosterone levels in selective processes and to assess whether organisms are capable of adapting to continuous and new environmental challenges. Barn owls have the particularity to express sex-specific melanin-based colour traits with an ample level of variation. Variation in coloration covaries with a number of morphological, physiological and behavioural traits, including corticosterone. Although these relations have been proposed to originate from the pleiotropic effect of the genes belonging to the melanocortin system, few studies have firmly investigated the proximate mechanisms linking these traits. To this aim, I specifically explored the role and the potential mechanisms linking steroid hormones to the expression of melanin-based traits in the barn owl, but also, whether individuals with different colour traits regulate hormones (corticosterone, testosterone) differently which could explain why they behave or regulate their physiology differently.

**Picture 1.** On the left, a male adult barn owl (*Tyto alba*) displaying a typical white and immaculate male-like plumage. On the right, a female adult barn owl with a typical reddish-brown and spotted female-like plumage. Picture taken by Isabelle Henry.

*Model species*

The barn owl (*Tyto alba*) is a particularly interesting species for studying all the different questions exposed above for several reasons. First, this species is ubiquitous and therefore has adapted to many different climates and environments. In my study area in Switzerland, the barn owl is a synanthropic species (i.e., a species who lives close to humans and which can benefit from their association and interactions) and therefore has been able to adapt to human presence and human-made disturbances. Secondly, the barn owl shows a pronounced variation in two sexually dimorphic traits, pheomelanin and eumelanin coloration (Picture 1). Although members of the two sexes can exhibit any plumage trait value, males are on average lighter reddish (a pheomelanin-based trait) and display fewer and smaller black eumelanic spots than females. The expression of these three plumage traits is under genetic control and these traits are more strongly heritable in males ($h^2$ for reddish coloration is 0.87 ± 0.12, 0.72 ± 0.14 for number of spots and 0.81 ± 0.03 for spot diameter) than in females ($h^2$ for reddish coloration is 0.79 ± 0.13, 0.50 ± 0.04 for number of spots and 0.58 ± 0.13 for spot diameter) and are barely sensitive to environmental conditions (Roulin and Jensen, 2015). The genes involved in the expression of spot size are located on the Z sex chromosome but also on autosomal chromosomes (Roulin *et al*,

2010b). Spot size is positively selected in females but negatively selected in males (Figure 7), suggesting that the observed incomplete sexual dimorphism may be due to sexual antagonistic selection and the genes involved in the expression of this trait experience intralocus conflict (Roulin, 2004d; Roulin and Altwegg, 2007; Roulin *et al*, 2010b). A key unresolved issue is the proximate mechanisms explaining why spot size is differentially selected in males and females. The variation in spot size could be related to variation in the production of sex hormones having sex-specific physiological effects (either male-specific hormones such as testosterone and dihydrotestosterone (DHT), or female-specific hormones such as progesterone and estrogen) or to their receptors, whose expression levels finally determine the activity of sexual hormones. This hypothesis has received indirect empirical supports because in barn owls, individuals of either sex, which are displaying a male-specific plumage trait, adopt male-specific behaviour by being more aggressive, less cooperative and by dispersing shorter distances (Roulin *et al*, 2012; van den Brink *et al*, 2012a). Moreover, the expression of these traits are associated with a number of morphological, behavioural and

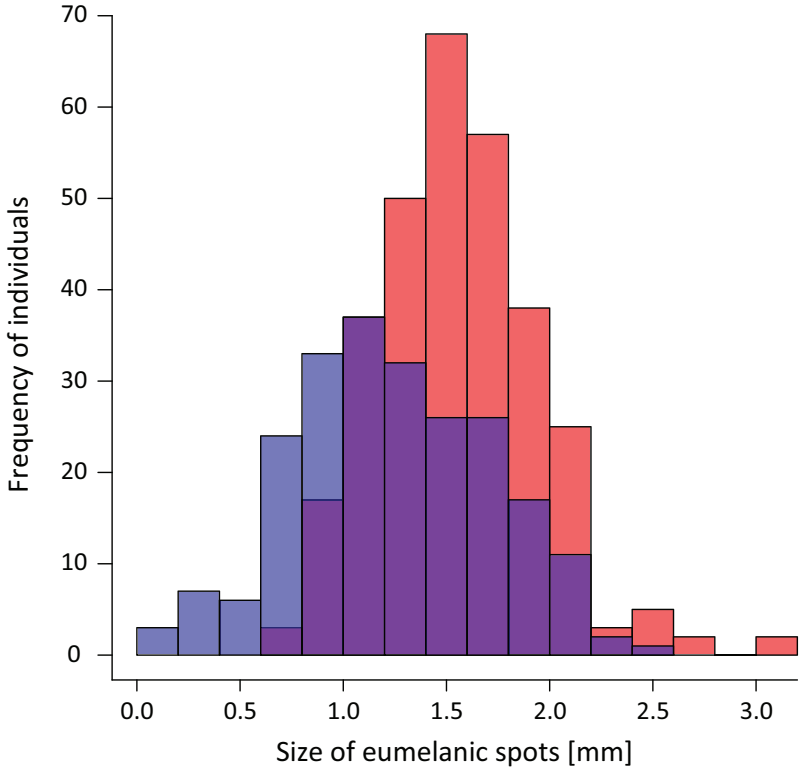


**Figure 7.** Distribution of the mean size of eumelanic spots displayed on the tip of barn owl feathers in our Swiss population. The blue bars represent the distribution of male spot size and the red bars represent the distribution of spot size in females barn owls.

physiological functions and life history strategies (Dreiss *et al*, 2012; Roulin, 2009b; Roulin and Dijkstra, 2003; Roulin *et al*, 2001), including resistance to stress (Almasi *et al*, 2010; Dreiss *et al*, 2010) and are used in female mate choice (Roulin, 1999; Roulin, 2004d; Roulin and Altwegg, 2007; Roulin and Ducrest, 2011).

*Outline of thesis*

The genetic component of a trait is a key factor in the evolution of this trait as it determines its evolutionary responsiveness to natural selection. In **chapter 1**, I investigated the relative contribution of genetic variation to the overall variation in corticosterone secretion (i.e., the main stress hormone in birds) by estimating the heritability ($h^2$) of baseline and stress-induced corticosterone response levels as well as their level of genetic correlation within a wild population of barn owls.

Because glucocorticoid hormones, such as corticosterone, are crucial in the maintenance of day-to-day energy homeostasis and in the physiological response of stress during life threatening events, they are thought to be physiological cues enabling to predict fitness components, including reproductive success and survival. In this **2nd chapter**, I used 13 years of capture-recapture data with 11 years of corticosterone measurements taken on breeding barn owls to evaluate whether baseline and stress-induced response levels predict their survival.

In the last decade, many studies in several species have shown an association between glucocorticoid levels and melanin-based traits. This association has been proposed to stem from the pleiotropic effects of the melanocortin system. However, to our knowledge few studies have explored the proximate mechanisms linking the melanocortin system to glucocorticoids and the HPA axis. In **chapter 3**, I explored the proximate mechanisms linking the melanocortin system to the HPA axis by measuring the expression of mineralocorticoid (MR) and glucocorticoid receptors (GR) in tissues where melanin is synthesised with genes involved in the melanocortin system and melanogenesis.

In many species, dark melanin-based traits individuals are more aggressive and present higher levels of testosterone levels. This association is thought to come from the fact that testosterone can induce the deposition of melanin pigments in integuments or that genes implicated in the

expression of melanin pigments also affect the secretion of testosterone. In **chapter 4,** I first investigated the relation between testosterone and the expression of melanin-based traits in adult and nestling barn owls. Then, I explored the hypothesis that sex hormones may control the expression of melanin-based colour traits in the barn owl, by measuring in the feather-tissues, where melanin pigments are synthesised, the expression levels of genes associated to the metabolism and action of testosterone and estrogen.

In parallel to my thesis work, I also investigated the different reproductive strategies in the barn owl. As in many species, barn owls may adopt different reproductive strategies to maximize their reproductive fitness, for instance some individuals may prefer to reproduce once a year by reproducing less often to produce high quality offspring. Whereas other individuals may prefer to produce more than one annual clutch per year but at the expense of offspring quality. In this **5th chapter,** I investigated the determinants of double brooding and fitness consequences based on 24 years of data collected in a Swiss population of barn owls.

During my thesis, I also had the opportunity to collaborate with a number of colleagues and studies that resulted in different publications in different topics. Thereafter, you will find a list of the publications for which I collaborated during my thesis.

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# *Chapter 1*

## Heritability of baseline and stress-induced corticosterone levels in the barn owl

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**Abstract**

The hypothalamic-pituitary-adrenal (HPA) axis is responsible for the regulation of glucocorticoids, hormones that are essential in the mediation of energy allocation and physiological stress. The potential for corticosterone levels to evolve depends not only on the strength of selection exerted on it but also on the extent to which the regulation of corticosterone is heritable. Because little is known about the heritability of corticosterone levels in wild populations, we used a long-term data set to estimate the heritability of baseline and stress-induced corticosterone levels in the barn owl. Although baseline and stress-induced corticosterone levels are strongly genetically correlated ($r$ = 0.735; BCI 95%: 0.600-0.857), the heritability estimate of stress-induced corticosterone levels ($h^2$ = 0.441; BCI 95%: 0.243 - 0.640) is 2.2 times higher than that of baseline corticosterone levels ($h^2$ = 0.199; BCI 95%: 0.101 - 0.297). These findings suggest that the regulation of stress-induced corticosterone levels has the potential to evolve faster than baseline levels when selection acts with the same intensity on both traits and that selection on either baseline or stress-induced level is likely to induce indirect selection on the other given their strong genetic correlation.

**Introduction**

Little is known about the potential of the endocrine system to respond to selection compared to morphological or life history traits (Charmantier and Garant, 2005; Kempenaers *et al*, 2008; Pavitt *et al*, 2014; Zera *et al*, 2007). Yet, hormones such as glucocorticoids play an important role in regulating and coordinating physiological and behavioural functions to respond adaptively to internal demands and external challenges (Breuner *et al*, 2008; Romero, 2004; Wingfield *et al*, 1998). Hormones typically affect the expression of multiple traits, which may induce constraints and trade-offs in the evolution of life history traits (Flatt *et al*, 2005; Zera *et al*, 2007). Despite the acknowledged importance of hormones in selective processes, little is known about the contribution of genetics to variation in glucocorticoids levels and the potential of the HPA axis to respond to selection in natural conditions.

The hypothalamic-pituitary-adrenal axis (HPA) regulates the secretion of glucocorticoids (cortisol in most mammals and fish and corticosterone in birds) in the blood stream from where it is distributed in tissues and organs where it regulates physiological processes. At baseline levels, corticosterone plays an important role in daily life processes, including energy intake (Dallman *et al*, 1993; King, 1988), water/salt balance (Bramanti *et al*, 1997), glucose supply in cells (Kuo *et al*, 2015) and supports the allocation of metabolic demands between life history traits and between life history stages (Crespi *et al*, 2013; Landys *et al*, 2006; Sapolsky *et al*, 2000). In many taxa baseline corticosterone levels vary between different life history stages (Lattin and Romero, 2015; Romero, 2002) in a predictable manner. For instance, the increase of baseline corticosterone levels during reproduction is supposed to allow parents to reallocate resources from body maintenance to reproductive activities (Beletsky *et al*, 1989; Bonier *et al*, 2009a; Bonier *et al*, 2009b; Comendant *et al*, 2003; Love *et al*, 2004; Love *et al*, 2014). Many studies show an association of baseline corticosterone to fitness and survival (Bonier *et al*, 2009b; Comendant *et al*, 2003) but the nature of this relationship varies, with some beeing positevly, negatively or non-significalty associated to fitness components (reviewed in Bonier *et al*, 2009a). However, above a certain threshold high baseline corticosterone levels can suppress immune functions (Sapolsky *et al*, 2000; Stier *et al*, 2009) or reduce parental investment (Almasi *et al*, 2008; Thierry *et al*, 2013). Further increase in corticosterone levels can be triggered by unpredictable stressful events leading to the so-called “stress-induced corticosterone levels” to allow animals to adjust their physiology and behaviour to, for instance, inclement weather conditions, predator attacks or food shortage (Landys *et al*, 2006; Rodrigues *et al*, 2009; Sapolsky *et al*, 2000). Similarly to baseline

corticosterone levels, the adrenocortical stress response may also be under selection as the capacity to mount an important corticosterone stress response has been shown to predict survival and reproductive success in different species (Angelier *et al*, 2009; Blas *et al*, 2007; for a review see Breuner *et al*, 2008; Crespi *et al*, 2013; Romero and Wikelski, 2001). In certain species and populations, some individuals have even been able to adapt their HPA axis to cope with specific and stressful environments (Abolins-Abols *et al*, 2016; Atwell *et al*, 2012; Müller *et al*, 2007). For instance, nestling European Blackbirds (*Turdus merula*) native from urban areas show a lower corticosterone stress-response than their rural conspecifics (Partecke *et al*, 2006), an adaption that could be essential to survive in urban environments.

The rate at which the HPA axis will evolve under selection has been investigated in captive animals by applying intense artificial selection. These studies showed that the heritability of stress-induced corticosterone levels varies between $h^2$ = 0.15 and 0.41 (Brown and Nestor, 1973; Evans *et al*, 2006; Odeh *et al*, 2003; Pottinger and Carrick, 1999; Satterlee and Johnson, 1988), values that are comparable to the $h^2$ of other physiological traits (Coviello *et al*, 2010; Mills *et al*, 2012; Pavitt *et al*, 2014; Travison *et al*, 2014) and tend to be lower than $h^2$ for morphological traits (Mousseau and Roff, 1987). Unfortunately, little is known about the situation prevailing in natural populations where the environment is more variable, population structure is less homogeneous than in laboratory conditions, and where the strength of artificial selection will not bias heritability estimates (Charmantier and Garant, 2005; Huey and Rosenzweig, 2009; Sgro and Hoffmann, 2004). To the best of our knowledge, only one field study performed in American barn swallows (*Hirundo rustica*) has investigated the heritability of baseline ($h^2$ = 0.152) and stress-response corticosterone levels ($h^2$ = 0.343) during one single year (Jenkins *et al*, 2014). The estimate for stress-induced corticosterone response was similar to the values obtained by artificial selection studies (Brown and Nestor, 1973; Evans *et al*, 2006; Odeh *et al*, 2003; Pottinger and Carrick, 1999; Satterlee and Johnson, 1988).

Like many other hormones, corticosterone induces distinct physiological and behavioural processes depending on its concentration in the blood. Although the response to baseline and stress-induced levels are mediated by different protein receptors and may function as two complementary hormonal systems (Landys *et al*, 2006; Romero, 2004), baseline and stress-induced corticosterone levels share parts of their physiological pathway. Both are regulated by the secretion of corticotropin-releasing hormone (CRH), which activates the HPA axis by stimulating the pituitary secretion of adrenocorticotropic hormone (ACTH) into the bloodstream from where it

flows to the adrenal glands and induces the secretion of corticosterone. The secretion of CRH is influenced by many factors, such as the circadian rhythm (Buckley and Schatzberg, 2005), physical activity (Chennaoui *et al*, 2002; Kawashima *et al*, 2004), blood corticosterone levels and stressful events (Carsia and Harvey, 2000; Harvey and Hall, 1990). There are evidences that baseline and stress-induced levels may evolve indirectly, in response to selection on behavioural traits (Albert *et al*, 2008; Carere *et al*, 2003; Garland *et al*, 2016; Stowe *et al*, 2010). For instance, Albert *et al* (2008) observed lower levels of both baseline and stress-induced corticosterone levels in rats selected for tameness compared to those selected for aggressiveness. We are only aware of one study that has looked at the genetic correlation between these traits in a wild population and this study did not find any evidence that same genes contributed to the variation in baseline and stress-induced response (Jenkins *et al*, 2014). Assuming that baseline and stress-induced corticosterone levels are genetically correlated, selection exerted on one component will indirectly affect the evolution of the other component.

Over a period of seven years, we measured baseline and stress-induced corticosterone levels in 927 nestling barn owls to estimate heritability and genetic correlation between the two components of the HPA axis. We performed animal models using a pedigree established with 21 years of data and including 7705 individuals.

### *Methods*

*Data collection*

From 1992 to 2012, we monitored a population of barn owls in western Switzerland (46°49'N, 06°56'E) breeding in nest boxes fixed to barns. This nocturnal species lives in open landscapes and preys upon small mammals. In our study site, the 2 to 12 eggs are laid from mid-February to the beginning of August. Eggs hatch asynchronously as they are laid every 2 to 3 days and incubated directly after the first egg is laid, which generates a pronounced size hierarchy among the progeny. The first flight occurs at *ca*. 55 days of age. We ringed all nestlings and most breeding adults. To disentangle the genetic effect from possible environmental factors, we cross-fostered eggs or nestlings between pairs of nest that had similar laying dates. If clutch size between pairs of nests was of similar size (± 1 egg), we swapped all eggs, whereas if there was a difference of more than one egg, we cross-fostered the same number of eggs between pairs of randomly chosen nests. In this case, we made sure to keep intact the within brood age hierarchy by swapping eggs at the

same stage of incubation. Of the 925 nestlings we sampled for corticosterone, 563 had been cross-fostered and hence raised by foster parents.

*Assessment of baseline and stress-induced corticosterone levels*

We collected blood samples from 431 male and 494 female nestling barn owls (molecular sex identification following (Py *et al*, 2006) between the age of 4 and 63 days (mean ± SD: 31.5 ± 10.8 days) between 2004 and 2012 (Table 1). If we had blood samples from different ages of one individual, we considered only the blood sample of the first encounter for all further analyses. Following Romero and Reed (2005), all samples taken within 3 minutes (1'58'' ± 39'' average sampling time) after opening the nest box were considered to reflect baseline corticosterone levels. Once the first blood sample was taken, the individuals were weighed to the nearest g and their wing and tarsus measured to the nearest mm. Nestlings were then individually kept in cloth bags until a second blood sample was taken within 19' to 33' (24' ± 2'46'') to measure stress-induced corticosterone levels. The blood samples were collected with heparinised capillaries and immediately centrifuged and the plasma stored in liquid nitrogen separately from the red-blood cells. Once back from the field within less than 24 hours, the samples were moved at -20°C until analysis. Plasma corticosterone levels were determined using an enzyme immunoassay (see for details Müller *et al*, 2006). Briefly, ten microliters of plasma was added to 190µl water, and from this solution we extracted corticosterone with 4ml dichloromethane, which was re-dissolved in phosphate buffer and measured in triplicate in the enzyme-immunoassay. The dilution of the corticosterone antibody (Chemicon; cross-reactivity: 11-dehydrocorticosterone 0.35%, progesterone 0.004%, 18-OH-DOC 0.01% cortisol 0.12%, 28-OH-B 0.02% and aldosterone 0.06%) was 1:8000. We used HRP (1:400 000) linked to corticosterone as enzyme label and ABTS as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicate on each plate. If the corticosterone concentration was below the detection threshold of 1ng/ml, the analysis was repeated with 15µl or 20µl plasma. Plasma pools from chicken with a low and high corticosterone concentration were included as internal controls on each plate. Intra-assay variation ranges from 3 % to 20 % and inter-assay variation from 7 % to 25 %, depending on the concentration of the internal control and the year of analysis.

**Table 1.** Number of baseline and stress-induced corticosterone samples taken per year and sex.

| | 2004 | 2005 | 2006 | 2009 | 2010 | 2011 | 2012 |
|---|---|---|---|---|---|---|---|
| *Baseline samples* | | | | | | | |
| Male | 19 | 94 | 38 | 23 | 71 | 74 | 112 |
| Female | 33 | 93 | 41 | 36 | 61 | 109 | 121 |
| | | | | | | | |
| *Stress-induced samples* | | | | | | | |
| Male | | 66 | 30 | 21 | 69 | 72 | 108 |
| Female | | 68 | 34 | 33 | 61 | 107 | 115 |

*Animal models*

We used an "animal model" statistical approach to estimate the heritability of baseline and stress-induced corticosterone levels in nestling barn owls (Wilson *et al*, 2010). The animal model uses a linear mixed model to estimate the genetic additive variance of a trait of interest from a population's pedigree. The pedigree is used to control for relatedness when estimating the breeding value of an individual (i.e., the additive genetic effect on phenotype). In our population, the full pedigree consisted of 7705 individuals (of which 1202 are of unknown origin and hence defined as "founders") captured between 1994 and 2015. It was built using paternities based on field behavioural observations (i.e., the adults observed feeding the nestlings were assigned as parents) and molecular analyses to determine paternity in 1403 offspring of which only 27 (2%) were sired by another male than the one that was feeding them (Ducret *et al*, 2016). Excluding founders, 2.89 % of the individuals have an unknown mother and 10.35 % of the individuals an unknown father (1.2 % belong to the same brood with both parents unknown).

The animal models were fitted using the *MCMCglmm* function implemented in the homonymous R package (Hadfield 2010). Baseline and stress-induced corticosterone levels were considered together in the same multivariate analyses, which allowed us to estimate the heritability of each trait as well as their genetic correlation. In the model, the inverse matrix of relatedness among individuals estimated from the pedigree, year and brood identity were included as random effects. The variance estimated from the pedigree represents the additive genetic variance (Va), the variance associated to brood identity represents the rearing environmental variance (Ve) and the variance associated to year represents the yearly variance (Vy). From these variance components and the residual variance (Vr), we calculated narrow-sense heritability ($h^2$) as Va/Vp (Vp = total phenotypic variance). We modelled the variance terms of the

random effects separately for baseline and stress-induced corticosterone levels (option *idh* in *MCMCglmm*), except for the genetic additive variance.

Based on DIC (deviance information criterion) we compared three separate multivariate models with **i.** no genetic variance associated to the traits, **ii.** a genetic variance term per trait but no genetic covariation between the traits, or **iii.** a genetic variance term per trait and a covariation different from zero (option *us* in *MCMCglmm*). The resulting best model was re-ran including different covariates (sampling time (i.e., duration between the moment when nestlings were first disturbed and blood sampling), sampling date (i.e., Julian date), sampling time of the day (i.e., hour of capture), sex, nestling age, rank in the brood and condition (i.e., residuals from the relation between mass and wing length of nestlings)) either in interaction with the two traits, which allows for estimating different regression lines for baseline (baseline levels were log-transformed to improve the normality of the data) and stress-induced levels or without interactions in separate models. This model was used to assess if these factors, known to have an effect on corticosterone levels (Almasi *et al*, 2015; Almasi *et al*, 2010; Romero and Reed, 2005; Roulin *et al*, 2010a), influence the additive genetic (co)variance estimates, for instance, by being non-randomly distributed with respect individual relatedness (e.g., related individuals are more likely to be measured under similar conditions (Wilson, 2008)). Finally, we re-ran the best model by considering only the 255 males and 308 females that were cross-fostered and hence raised by foster parents. This approach avoids the potential covariation between genotype and rearing environment. All models were fitted with inverse *Wishart* priors as described by (Hadfield, 2010) and ran for 10'300'000 iterations with a burning phase of 300'000 iterations and a sampling interval of 2'000 iterations. The mean effective sample size for all model parameters was 4'730 and the lowest effective sample size was 3'769. To assess the models, we visually inspected the convergence of the chains and checked the congruence of the posterior distributions of the different parameters between runs.

**Table 2.** Comparison of animal models considering different underlying scenarios of additive genetic variation and covariation in baseline and stress-induced corticosterone levels in nestling barn owls. In model 1, we estimated the additive genetic variances for baseline and stress-induced corticosterone levels and their covariation. In model 2, we estimated the additive genetic variances for baseline and stress-induced corticosterone levels but considered that genetic covariance equals zero. Finally, in model 3, we considered no additive genetic variance for

corticosterone levels. In each model, we added the identity of the brood and year as random factors. Each model was run twice to estimate the variability in the DIC estimates for the same model parametrization.

| | DIC run 1 | DIC run 2 | Delta DIC* | Delta DIC** |
|---|---|---|---|---|
| Model 1 | 8716.666 | 8716.291 | 0.375 | 0 |
| Model 2 | 8934.732 | 8934.264 | 0.468 | 218.0658 |
| Model 3 | 8958.182 | 8958.173 | 0.009 | 241.5158 |

** Delta DIC within models*
***Delta DIC between models*

**Results**

The model considering a different genetic additive variance term per trait and a genetic covariation between baseline and stress-induced corticosterone levels different from zero was found to fit the data better than the models considering no genetic covariation (second best model) or no genetic additive variance for both traits (Table 2). The heritability estimate ($h^2$) of the best model for baseline corticosterone levels was lower ($h^2$ = 0.199; Table 3) than the heritability estimate for stress-induced levels ($h^2$ = 0.441). The genetic correlation between baseline and stress-induced corticosterone levels was relatively high ($r$ = 0.735; Table 3). The rearing environment (brood ID) explained a small part of the variance in baseline corticosterone level (Table 4), whereas the part of variance explained by year was much higher albeit a larger 95% BCI. In contrast, both the year and the rearing environment explained a low level of the variance in stress-induced response levels (Figure 1). A great part of the variance for baseline and stress-induced levels remained unexplained (Figure 1). The model including the covariates date, hour, sampling time, age and sex of nestlings gave rather similar estimates as the simplest model, although the estimates of additive genetic variance for baseline and stress-induced response levels as well as their level of genetic correlation had lower estimates than the model without covariates (Table 3). Similarly, the model with only the cross-fostered individuals gave similar estimates of $h^2$ and genetic correlation (Table 3).

**Table 3**. Estimates of heritability ($h^2$) for baseline and stress-induced corticosterone levels and the genetic correlation between these two traits in nestling barn owls. The estimates are based on the model 1 (see table 1).

| Model | Baseline corticosterone $h^2$ estimate (95% BCI) | Stress-induced corticosterone $h^2$ estimate (95% BCI) | Genetic correlation estimate (95% BCI) |
|---|---|---|---|
| Model without covariables | 0.199 (0.101-0.297) | 0.441 (0.243-0.640) | 0.735 (0.600-0.857) |
| Model with cross-fostered nestlings without covariables | 0.234 (0.109-0.363) | 0.489 (0.299-0.675) | 0.713 (0.556-0.859) |
| Model with covariables in interaction with trait | 0.161 (0.062-0.260) | 0.354 (0.125-0.583) | 0.575 (0.380-0.765) |

**Discussion**

We used seven years of corticosterone sampling in a wild population of barn owl nestlings to estimate the heritability of baseline and stress-induced corticosterone concentration. Our models showed that the heritability estimate for baseline levels in barn owl nestlings is lower than that of stress-induced corticosterone response, suggesting that the latter may faster respond to selection than the former (Falconer and Mackay, 1960; Hansen *et al*, 2011). We also detected a high genetic correlation between baseline and stress-induced response levels, indicating that the evolution of basal and stress-induced corticosterone levels are not independent from each other. When accounting in the animal model for the effect of covariates known to be associated with corticosterone levels (in supplementary table S1), we obtained lower estimates of additive genetic variance and covariance (Table 3). Although the estimated values are within the same range as those obtained from the animal model without covariates, the fact that they had lower estimates may indicate that some of the covariates considered are somehow associated to the degree of relatedness between individuals (Wilson, 2008). Such association could result from having measured related individuals under similar conditions (for instance, same study area, nest, time of the day and date) or owing to the existence of unknown genetic correlations between corticosterone levels and some of the variables considered (for instance, genetic basis for condition may have pleiotropic links with baseline and/or stress-induced levels).

**Table 4.** Posterior estimation (with 95% Bayesian credible interval (BCI)) of variance components for baseline and stress-induced corticosterone levels based on model 1 (see table 1). Baseline estimates are based on log-transformed baseline corticosterone levels.

| | Baseline corticosterone | Stress-induced corticosterone |
|---|---|---|
| Additive genetic variance ($V_A$) | 0.176 (0.113 - 0.251) | 256.7 (132.71 - 397.57) |
| Year ($V_Y$) | 0.209 (0.082 - 0.725) | 16.13 (0.12 - 111.19) |
| Rearing environment ($V_E$) | 0.081 (0.052 - 0.12) | 42.54 (0.13 - 118.68) |
| Residual variance ($V_R$) | 0.329 (0.282 - 0.387) | 242.19 (164.4 - 305.11) |

Our analyses gave similar $h^2$ estimates for stress-induced corticosterone (0.343) levels and for baseline corticosterone (0.152) levels as a previous cross-fostering experiment made in the wild on 107 nestling barn swallows (Jenkins *et al*, 2014). Our estimates were also in the range of other corticosterone stress-response heritability estimates (range of $h^2$ is 0.14 - 0.41) obtained in domesticated animals from artificial selection experiments (Brown and Nestor, 1973; Evans *et al*,

2006; Odeh *et al*, 2003; Pottinger and Carrick, 1999; Satterlee and Johnson, 1988) or with other hormones (Coviello *et al*, 2010; Iserbyt *et al*, 2015; Pavitt *et al*, 2014; Travison *et al*, 2014) but much lower than other traits in barn owls (e.g., $h^2$ for reddish coloration is of 0.79 ± 0.13 in females and 0.87 ± 0.12 in males) (Roulin and Jensen, 2015). Although our heritability estimate of baseline levels was lower than that of stress-induced response, our results are consistent with the results reported in barn swallows suggesting that stress-induced corticosterone levels may evolve faster than baseline levels. However, it is important to take into account to what extent baseline and stress-induced corticosterone levels are comparable within the context of our study or previous studies using similar fieldwork procedures. For instance, variation in baseline levels is likely to enclose different sources of internal and environmental variation, which may reduce heritability estimate compared to stress-induced levels (e.g., measuring nestlings of different ages or at different hours or days). Whereas stress-induced levels are often measured as the response to a single type of stress (adapted after Wingfield *et al*, 1994) and therefore do not include variation that might result from a differential response to the distinct stressful factors that may exist in natural conditions. A better understanding of these different natural stressful factors and whether they induce different response effects in corticosterone levels is required to make more robust generalizations of heritability differences between baseline and stress-induced corticosterone levels.

Traits that are related to fitness components, such as the HPA axis, are thought to have low additive genetic variance as alleles from genes conferring a higher fitness are expected to be rapidly fixed by natural selection (Charmantier and Garant, 2005; Kruuk *et al*, 2000; Roff, 1997). Selection exerted on baseline corticosterone levels was perhaps so strong in the past that it depleted its genetic variation to a larger extent than stress-induced levels. For instance, the need to finely tune baseline corticosterone in response to environmental variation may have selected for reduced genetic variation. This interpretation is consistent with the finding that the random factor “year” explained 30% of the variation for baseline corticosterone (Figure 1). This could be explained by a suite of environmental factors that vary between years. On the other hand, year explained a lower percentage of the variance for stress-induced corticosterone levels (8.5%). This difference is not surprising because baseline corticosterone levels regulate many daily life processes and supports the metabolic needs of an individual between and during different life history stages, whereas the adrenocortical stress response is sensitive to specific stressful events that are not year-specific. The moderate heritability estimate for stress-induced corticosterone

response suggests that an appreciable part of the variance in hormonal response to stress is genetically determined. This considerable amount of genetic variance may enable for evolutionary process to mould the activity of the HPA axis to stress in order to adapt to specific environments (Abolins-Abols *et al*, 2016; Atwell *et al*, 2012; Müller *et al*, 2007; Partecke *et al*, 2006).

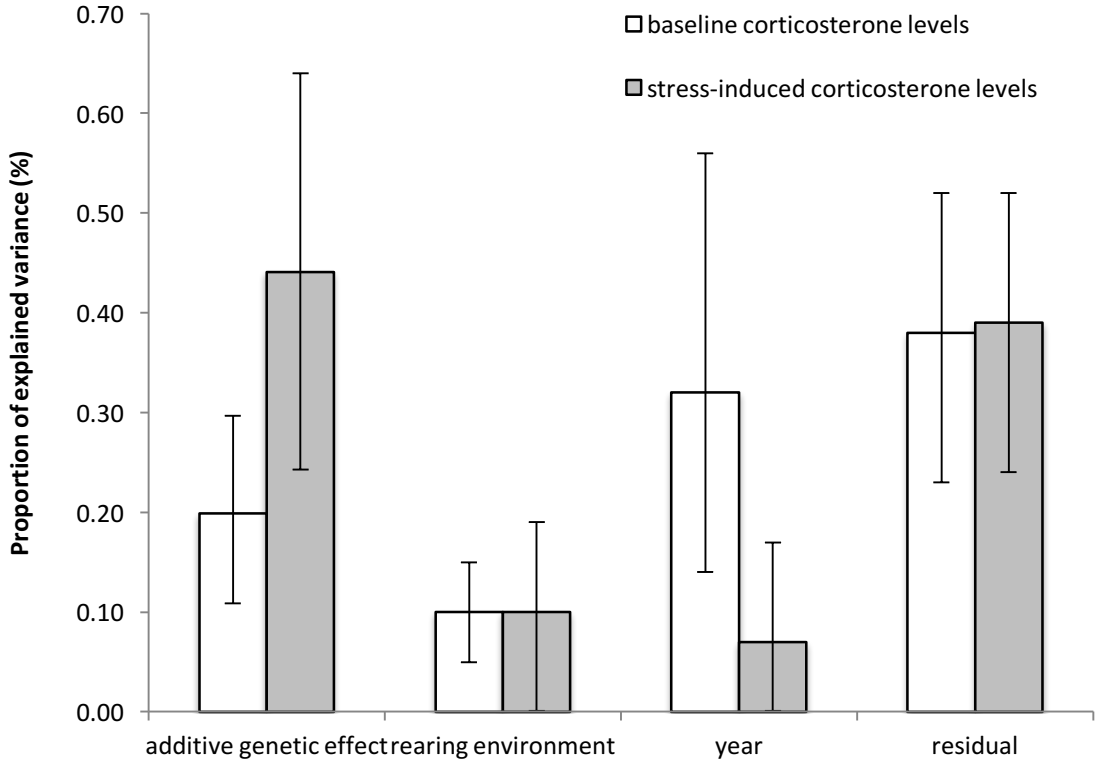


**Figure 1.** Partition of total phenotypic variance into additive genetic, common rearing environmental, year and residual variance estimated from the simplest model in nestling barn owls. The empty bars represent baseline corticosterone whereas the grey bars represent stress-induced corticosterone levels. The error bars represent the 95% Bayesian credible intervals.

The rearing environment explained only 10% of the variance in baseline and stress-induced corticosterone levels (Figure 1). This may indicate that environmental conditions experienced in a given year in different nests are more similar than environmental conditions experienced in different years. For instance, outbreaks of pathogens may occur in some specific years and affect all nests, and fluctuations in food supply may be more pronounced between years than between territories. Other factors including maternal (Almasi *et al*, 2010; DuRant *et al*, 2010; Harris and Seckl, 2011; Haussmann *et al*, 2012; Hayward and Wingfield, 2004; Henriksen *et al*, 2011; Kapoor *et al*, 2006; Muneoka *et al*, 1997; Uno *et al*, 1994; Weaver *et al*, 2004) and epigenetic effects (Franklin *et al*, 2010; Lee *et al*, 2010; Nestler, 2016), as well as parasite loading or hatching

asynchrony that can lead to competitive and developmental hierarchies (Evans, 1996; Nilsson and Svensson, 1996), can also alter the development or functioning of the endocrine system (Love *et al*, 2003; Schwabl, 1999) and thus might potentially contribute to variation in corticosterone secretion between siblings, broods and years.

To the best of our knowledge, we report the first evidence for a genetic correlation between baseline and stress-induced corticosterone levels. From a proximate point of view, this strong correlation ($r$ = 0.735) may be explained by the common synthetic pathway between baseline and stress-induced corticosterone, even if the baseline and stress response corticosterone actions are mediated by different receptors (Romero, 2004). From an ultimate point of view, baseline corticosterone levels have been proposed to play a permissive action, permitting and priming the mechanisms by which an organism will respond to stress (Ingle, 1952; Sapolsky *et al*, 2000). This is consistent with previous studies showing that individuals selected for personality traits can induce correlated changes in baseline corticosterone levels (Albert 2008) and more often in stress-induced corticosterone levels (Baugh *et al*, 2012; Carere *et al*, 2003; Pottinger and Carrick, 2001; Stowe *et al*, 2010). Genetic correlation between baseline and stress-induced corticosterone levels could also potentially induce maladaptive evolution, if selection on one of the traits induces changes on the other traits that are harmful for the survival of an individual (Gratten *et al*, 2008).

To conclude, this study shed some light on the quantitative genetics of corticosterone levels, a rather underestimated topic despite its relevance to understand the evolution of the stress response in vertebrates. It also stresses the need to conduct similar studies on other species in order to better understand the evolution of glucocorticoid stress response and the HPA axis. Future studies should also consider that the evolution of the HPA axis may, however, be more complex as the action of corticosterone not only depends on plasma corticosterone levels (Breuner *et al*, 2013), but also on the tissue-dependent density of receptors to which corticosterone binds (Seckl and Meaney, 2004), the type of receptors (Landys *et al*, 2006; Romero, 2004), the properties and concentration of binding proteins and enzymes involved in the secretion, transportation or degradation of corticosterone. Despite our study support that baseline and stress-induced levels of corticosterone are strongly genetically linked, selection may still be able to act independently on the signalling pathway of baseline activity and stress-induced response through all the factors depicted above. Further studies may therefore investigate in more details the dynamics between baseline and stress-induced corticosterone levels and may also consider the level of genetic variation at other components involved in baseline and stress

response action in order to better understand how phenotypic variation of the HPA axis may respond to selection process.

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**Supplementary statistical data**

**Table S1.** Posterior mean estimates from the bivariate animal model including different co-variables for log transformed baseline- and stress-induced corticosterone levels in nestling barn owls. Shown are the 95% credible intervals for the estimates and their *P* values. Significant variables are written in italics.

| | Estimate | Lower 95% CI | Upper 95% CI | *P* |
|---|---|---|---|---|
| Intercept | -0.258 | -1.018 | 0.483 | 0.505 |
| *Sampling time* | *0.004* | *0.003* | *0.006* | *< 0.0002* |
| Sampling date | -0.001 | -0.003 | 0.001 | 0.28 |
| *Sampling hour* | *0.048* | *0.028* | *0.071* | *< 0.0002* |
| Nestling sex | 0.023 | -0.073 | 0.114 | 0.648 |
| *Nestling age* | *0.024* | *0.017* | *0.032* | *< 0.0002* |
| Nestling rank raised | 0.019 | -0.012 | 0.052 | 0.231 |
| *Nestling condition* | *-0.005* | *-0.007* | *-0.003* | *< 0.0002* |
| Baseline vs. Stress-induced * | -2.057 | -27.460 | 23.700 | 0.869 |
| Sampling time *x* Baseline vs. Stress-induced* | 0.000 | -0.014 | 0.014 | 0.988 |
| Nestling rank *x* Baseline vs. Stress-induced * | 0.173 | -0.764 | 1.079 | 0.701 |
| Sampling date *x* Baseline vs. Stress-induced * | 0.015 | -0.041 | 0.069 | 0.586 |
| *Sampling hour x Baseline vs. Stress-induced ** | *1.418* | *0.814* | *2.021* | *< 0.0002* |
| Nestling sex *x* Baseline vs. Stress-induced * | -1.895 | -4.414 | 0.948 | 0.168 |
| *Nestling age x Baseline vs. Stress-induced ** | *0.867* | *0.651* | *1.077* | *< 0.0002* |
| *Nestling condition x Baseline vs. Stress-induced ** | *-0.212* | *-0.270* | *-0.156* | *< 0.0002* |

* difference between baseline and stress-induced corticosterone levels

# *Chapter 2*

## Baseline and stress-induced corticosterone levels differentially predict adult barn owl survival

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**Abstract**

Glucocorticoids hormones, such as corticosterone, regulate many important physiological functions. At baseline levels, corticosterone is crucial in regulating daily life metabolism and energy expenditure, whereas the acute corticosterone response promotes short-term physiological and behavioral responses to unpredictable environmental challenges. Given their different physiological effects, baseline and acute stress corticosterone response levels may be differentially related to survival. We used 13 years of capture-recapture data combined with 11 years of corticosterone measurements in breeding barn owls to investigate how survival varies in relation to baseline and stress-induced corticosterone levels. Adults presenting low baseline corticosterone levels during the breeding season had a higher probability of surviving than adults showing high levels. In contrast, individuals presenting high stress-induced corticosterone levels had a higher probability of surviving than individuals with a lower stress-induced response. Our results suggest that selection favors individuals with low baseline corticosterone levels and a strong acute stress response and that selection for low baseline levels may be stronger than for higher stress-induced levels.

**Introduction**

Glucocorticoids hormones, such as corticosterone, are crucial for the functioning of organisms as they regulate a number of important physiological processes, including food intake (Dallman *et al*, 1993; King, 1988), energy allocation (Landys *et al*, 2006; Sapolsky *et al*, 2000) and locomotor activity (Landys *et al*, 2006; Overli *et al*, 2002). Baseline glucocorticoids levels are responsible for maintaining energy homeostasis in relation to current and predictable changes in energetic demands (Sapolsky *et al*, 2000). To sustain these activities, glucocorticoids follow a diel and seasonal rhythm in response to the different energetic demands associated to daily and life-history stages (Carsia and Harvey, 2000; Landys *et al*, 2006; Romero, 2002). Glucocorticoids are also part of the adrenocortical stress response that allow the reallocation of resources to physiological functions essential to survival when the environment becomes unpredictably challenging (e.g. predator attack, food restriction, inclement weather condition). Glucocorticoids therefore play an important role in mediating trade-offs between different life-history traits and, as a consequence, could be associated to fitness-related traits regulated by glucocorticoids (Crespi *et al*, 2013; Hau *et al*, 2010; Ricklefs and Wikelski, 2002; Zera *et al*, 2007). They may also play an important role in selective processes underlying the expression of these traits given that variation in glucocorticoid levels has a heritable component (Brown and Nestor, 1973; Evans *et al*, 2006; Jenkins *et al*, 2014; Odeh *et al*, 2003; Pottinger and Carrick, 1999; Satterlee and Johnson, 1988). Although, baseline and acute stress-induced glucocorticoid levels can be associated with fitness components, such as reproductive success (Bonier *et al*, 2009b; Riechert *et al*, 2014; Schmid *et al*, 2013) and survival in adults and youngsters (Blas *et al*, 2007; Cabezas *et al*, 2007; Rivers *et al*, 2012; Romero and Wikelski, 2001), results are mixed (reviewed in Bonier *et al*, 2009a; Breuner *et al*, 2008). Moreover, our knowledge on the effect of glucocorticoids on fitness traits mostly comes from studies where glucocorticoids levels are experimentally elevated via implants and injection of glucocorticoids or an increase in stress (reviewed in Bonier *et al*, 2009a; Breuner *et al*, 2008; Crossin *et al*, 2016; Sopinka *et al*, 2015). Although these studies help us understand the effects of elevated glucocorticoid levels on different fitness components, these manipulations have common issues that limit their interpretation (Fusani, 2008) mainly because the experimental manipulations may not mimic perfectly natural situations. For

this reasons, it is important to investigate survival in relation to natural variation in glucocorticoid levels (e.g., Blas *et al*, 2007; Cabezas *et al*, 2007; Romero and Wikelski, 2001; Wey *et al*, 2015; Wilkening and Ray, 2016).

It is commonly assumed that low baseline glucocorticoid levels are beneficial because high and prolonged levels can impair the health of an individual (Bremner, 2007; Breuner *et al*, 2008; de Kloet *et al*, 1999; Martin, 2009; Sapolsky *et al*, 2000). A rapid rise in glucocorticoid levels, as a response to stressful events, is advantageous by allowing organisms to quickly redirect energy from body maintenance and reproductive activities towards physiological and behavioral processes that enhance survival (Almasi *et al*, 2008; Ouyang *et al*, 2012). However, the empirical evidence for these predictions are mixed with some studies having found positive, negative or no relation between glucocorticoids and fitness components (reviewed in Bonier *et al*, 2009a; Breuner *et al*, 2008). For instance, lizards (*Uta stansburiana* and *Lacerta vivipara*) with higher baseline corticosterone levels have a higher survival prospect (Comendant *et al*, 2003; Cote *et al*, 2006), whereas the opposite pattern has been reported in adult cliff swallows (*Petrochelidon pyrrhonota*) (Brown *et al*, 2005). In the mountain white-crowned sparrow (*Zonotrichia leucophrys oriantha*), individuals mounting higher stress-induced corticosterone responses showed higher survival (see also Cabezas *et al*, 2007; Patterson *et al*, 2014), whereas in nestling white storks (*Ciconia ciconia*) the opposite pattern was detected (Blas *et al*, 2007; and for review see Bonier *et al*, 2009a; Breuner *et al*, 2008; see also Romero and Wikelski, 2001). These inconsistencies between studies could result from variation in corticosterone levels across life-history stages and their different energetic demands, which might select for different levels depending on the stage and context in which individuals are sampled (Bonier *et al*, 2009a). For instance, selection may favour low corticosterone levels during the early stages of reproduction but higher levels at later stages to reallocate resources from body maintenance to parental care (Bonier *et al*, 2009b; Magee *et al*, 2006; Romero, 2002). Inconsistencies may also arise because of sex-specific roles in reproduction (Bonier *et al*, 2007) and individual differences in life-history strategies (Angelier *et al*, 2011; Lancaster *et al*, 2008; Schultner *et al*, 2013). Studying the regulation of glucocorticoids in natural population is inherently difficult as multiple environmental factors cannot be standardized, such as climatic conditions (Bize *et al*, 2010; Ouyang *et al*, 2015; Thierry *et al*, 2013), predation risk (Scheuerlein *et al*, 2001; Sheriff *et al*, 2009), food abundance (Jenni-

Eiermann *et al*, 2008; Kitaysky *et al*, 2007), population density (Dantzer *et al*, 2013; Glennemeier and Denver, 2002; Meylan and Clobert, 2004) and disease outbreaks (Dunn *et al*, 1989; Gustafsson *et al*, 1994; Love *et al*, 2016).

In the present study, we investigated whether baseline and stress-induced corticosterone levels predict adult survival in a free-living population of barn owls. We used captured-recapture data collected during 13 years in a breeding population of owls with corticosterone measurements collected during 11 years. This bird breeds in man-made sites, which may have profound consequences given that human presence and landscape structure around breeding sites affect corticosterone levels in barn owl nestlings (Almasi *et al*, 2015). It is however still unknown whether variation in corticosterone levels also predict survival in adult barn owls.

**Materiel and Methods**

*Study site and species*

The barn owl is a medium sized bird of prey that lives in open rural landscapes where it hunts on small mammals. In our study area, barn owls commonly breed in artificial nest boxes fixed to the wall of barns. From the middle of February to beginning of August, females lay 1 to 2 clutches per year containing each between 2 and 11 eggs (Beziers and Roulin, 2016). The present study was conducted between 2004 and 2016 in western Switzerland (46°49'N, 06°56'E) in a wild population of breeding barn owls. The females (n= 289 different individuals) and males (n= 166) were either captured at the end of incubation stage or during nestling provisioning. If an adult had not been ringed as a juvenile, its age was estimated with the molt pattern of wing feathers (Taylor, 1993). We distinguished females from males by their incubating behaviour and brood patch.

*Assessment of baseline and stress-induced corticosterone levels*

Using heparinised capillaries we collected blood samples by punctuating the brachial vein of breeding barn owls. Blood samples were directly centrifuged, plasma separated and stored in liquid nitrogen. Once back from the field, the samples were stored at -20°C until analysis within the next 6 months. Baseline samples (n= 695) were collected within 3 minutes (mean: 2'22'' ± 32'' (SD)) after capture of the bird. The increase in corticosterone level during the first 3 minutes after an acute stress have been showed to be marginal (Romero

and Reed, 2005; Roulin *et al*, 2010a). After having collected this blood sample, adults were weighed, the length of their wing measured to the nearest mm and we then placed adults in a cloth bag back until a second blood samples was taken 25 minutes (mean: 24'5'' ± 2'15'' (SD)) after capture to measure the stress-induced corticosterone response (n= 697). This time lapse corresponds to the peak of corticosterone response in the barn owl (mean baseline levels: 12.23 ± 11.41 ng/ml; stress-induced response levels: 63.49 ± 26.68 ng/ml).

*Total corticosterone assay*

Plasma corticosterone levels were extracted with dichloromethane and determined with an enzyme immunoassay (Munro and Stabenfeldt, 1984; Munro and Lasley, 1988) following (Müller *et al*, 2006). Ten microliters of plasma was added to 190µl water and extracted with 4ml dichloromethane. The solution was mixed for 30 minutes on a vortex machine, incubated for 2 hours. After separating the water phase, the dichloromethane was evaporated at 48°C and corticosterone was re-suspended in a phosphate buffer. The dilution of the corticosterone antibody (Chemicon; cross-reactivity: 11-dehydrocorticosterone 0.35%, progesterone 0.004%, 18-OH-DOC 0.01% cortisol 0.12%, 28-OH-B 0.02% and aldosterone 0.06%) was 1:8000. We used HRP (1:400 000) linked to corticosterone as enzyme label and ABTS as substrate. We determined the concentration of corticosterone in triplicate by using a standard curve run in duplicate on each plate. If the corticosterone concentration was below the detection threshold of 1ng/ml the analysis was repeated with 15µl or 20µl plasma. Plasma pools from chicken with a low and high corticosterone concentration were included as internal controls on each plate. Intra-assay variation ranges from 3 % to 14 % and inter-assay variation from 7 % to 22 %, depending on the concentration of the internal control and the year of analysis.

**Table 1.** Number of baseline and stress-induced corticosterone samples taken per year in breeding barn owls.

| *Type of samples* | 2004 | 2005 | 2006 | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 | 2015 | 2016 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Baseline* | | | 40 | 27 | 30 | 50 | 186 | 22 | 56 | 122 | 164 |
| *Stress-induced response* | 56 | 80 | 52 | 19 | 31 | 43 | 130 | 18 | 36 | 96 | 134 |

*Survival Analyses*

We used a multistate capture-recapture model to estimate the local survival of barn owl adults in relation to plasma baseline, stress-induced corticosterone levels and the increase in corticosterone levels from baseline to stress-induced levels (Lebreton *et al*, 2009). The model allows us estimate the probability of recapturing an individual and its survival from time *t* to time *t* +1, (φ) depending on its state. We modelled survival probability (φ) in relation to different covariates including sex, age, baseline and stress-induced corticosterone levels. Since the ability to mount a corticosterone stress-response is not entirely independent from its baseline corticosterone level we fitted a first model with only baseline, a second with only stress-induced corticosterone levels and a third one with baseline and stress-induced corticosterone as covariables. We also fitted a model with the increase in corticosterone levels from baseline to stress-induced levels to determine whether the effect of stress-induced response on survival is truly due to a high increase in corticosterone and not an indirect effect of baseline levels on the stress-induced levels. Since we did not have corticosterone measurements of each individual in each year we used multiple imputation (Little and Rubin, 2002). To do so, models for both baseline and corticosterone were integrated in the survival model within a Bayesian framework and Markov chain Monte Carlo simulation was used to fit the integrated model. For the baseline corticosterone model we included sex and mass of individual, date (*i.e.,* Julian date) and hour of sampling, and blood sampling time (*i.e.,* time lapse between capture and sampling) as predictors (supplementary material Table S1). All variables were normalized ((x-mean(x))/(2* standard deviation(x))) and the identity of the individuals and the year of sampling were added as random factors. Baseline corticosterone was log-transformed. The same variables, except sampling time, were used to model stress-induced corticosterone levels and the increase in corticosterone levels from baseline to stress-induced levels. Unfortunately, the Markov chains did not converge when the model for stress-induced corticosterone and the increase in corticosterone was integrated in the survival model. Therefore we used predicted values from a normal linear mixed model that we fitted in R using the function lmer from the package lme4 (Bates *et al*, 2015). With this approach we did not take into account the uncertainty of the stress-induced and the increase in corticosterone levels estimates to predict survival, which should be taken into account while interpreting the results.

Recapture probability was modelled according to the number of annual breeding pairs and the number of available nest boxes in our study area (*i.e.* the number of nest boxes varied from 124 to 274 between 2004 and 2016). We modelled the probability of recovering a dead individual as constant throughout the study period and used uninformative priors for the different parameters. In total, 98 adult males and 143 females ringed as juveniles and 154 females and 77 males ringed as adults were used in our analyses. From these 472 individuals 31 were recovered dead within the duration of the study. The number of baseline samples per individual ranged from 0 to 12, (mean: 1.47 + 1.44 (SD)) and stress-induced sample per individual ranged from 0 to 11 (mean: 1.47 + 1.62).

The model was performed in JAGS (Plummer, 2003) version 4.2, using the package RJAGS version 4-6 (Plummer, 2016) in R version 3.3.2 (R Core Team, 2016) . JAGS uses a Markov chain Monte Carlo (MCMC) procedure to estimate parameters. We ran the model in three parallel chains with 210'000 iterations for baseline-stress-induced model and 510'000 for other three models, with a burning phase of 10'000 iterations and a sampling interval of 100 iterations. We visually inspected the chains and used the R-hat statistics to assessed the convergence all chains (Gelman and Hill, 2006). R-hat values for each parameter were < 1.1, indicating convergence.

*Relationship between baseline, stress-induced and corticosterone increase levels*

We investigated the relationship between baseline and stress-induced corticosterone levels and between baseline corticosterone levels and the increase in corticosterone levels from baseline to stress-induced levels. The goal of these analyses is to determine whether individuals with high baseline corticosterone levels can hardly elevate corticosterone levels in response to a stressful situation compared to individuals showing low baseline corticosterone levels. To this aim, we ran a linear mixed effect model with stress-induced corticosterone levels as a dependent variable and baseline corticosterone levels, squared baseline corticosterone levels, sampling time (i.e., time difference between capture and blood sampling), date (i.e., Julian date), hour, sex and condition (i.e., mass of individuals divided by square of wing length) of individuals as independent fixed effects. The year and identity of individuals were added as random factors to account for annual variation in corticosterone levels (Almasi *et al*, 2009) and for individuals sampled more the once.

Models were run with the *lmer* function from *lme4* package in R. All models were verified for heterogeneity and normality.

**Results**

*Survival estimates*

Breeding barn owls presenting low baseline corticosterone levels showed a higher probability of annual survival compared to breeders with high levels of baseline corticosterone levels (Table 2, Figure 1a). In contrast, individuals presenting a high stress-induced corticosterone levels or a stronger increase from baseline to stress-induced levels had a higher probability of annual survival than individuals presenting a low stress-induced response (Table 2, Figure 1b and 1c). This effect was detected in the model considering only stress-induced corticosterone levels but not in a model where we also included baseline corticosterone levels (Table 2). Survival probability decreased also with age (Table 2) and the probability of recapturing an individual was lower in years with a high than low number of breeding pairs. In the other hand, the number of nest boxes was not associated to the probability of recapturing an individual (Table 2).

**Tables 2.** Survival estimates of breeding barn owls in relation with baseline, stress-induced corticosterone levels and the increase in corticosterone levels from baseline to stress-induced levels. Parameters are estimated from a multistate model applied to 13 years of capture-recapture data of adults monitored from 2004 to 2016. Are presented, the posterior mean with 95% Bayesian credible interval for a model with only baseline, stress-induced corticosterone levels as predictors, the increase in corticosterone levels from baseline to stress-induced levels and a model with both baseline and stress-induced corticosterone levels in the same model.

| | Baseline corticosterone model | Stress-induced corticosterone model | Baseline and Stress-induced corticosterone model | Corticosterone increase model |
|---|---|---|---|---|
| *Survival probability* | | | | |
| Intercept | **0.736 (0.173, 1.317)** | **0.912 (0.709, 1.122)** | **0.724 (0.156, 1.298)** | **0.901 (0.70, 1.106)** |
| Baseline coricosterone | **-13.32 (-18.55, -9.22)** | | **-13.144 (-18.59, -9.16)** | |
| Stress-induced corticosteorne | | **0.828 (0.119, 1.547)** | -0.241 (-1.724, 1.193) | **0.760 (0.150, 1.384)** |
| Age | **-0.156 (-0.238, -0.082)** | -0.116 (-0.175, -0.059) | **-0.116 (-0.175, -0.059)** | **-0.111 (-0.17, -0.052)** |
| *Recapture probability* | | | | |
| Intercept | **1.971 (1.704, 2.259)** | **2.08 (1.808, 2.374)** | **1.966 (1.705, 2.257)** | **2.069 (1.80, 2.358)** |
| Number of breeding pairs | **-1.979 (-2.654, -1.321)** | **-2.032 (-2.74, -1.341)** | **-1.973 (-2.63,-1.322)** | **-2.031 (-2.741, -1.362)** |
| Number of nest boxes | 0.307 (-0.275, 0.912) | 0.561 (0, 0.563) | 0.301 (-0.289, 0.895) | 0.539 (-0.031, 1.116) |
| *Recovery probability* | | | | |
| Recovery estimate | **-2.456 (-2.864, -2.077)** | **-2.474 (-2.873, -2.097)** | **-2.448 (-2.86, -2.065)** | **-2.478 (-2.888, -2.1)** |

*Relationship between baseline, stress-induced and corticosterone increase levels*

Survival was negatively associated with baseline corticosterone levels and positively with stress-induced corticosterone levels. Such opposite pattern could be a statistical artefact if these two measures of corticosterone are negatively correlated. However, this was not the case, since stress-induced corticosterone levels were associated to baseline corticosterone levels in a quadratic manner and the linear term was positive (Table 3, Figure 2a). Stress-induced corticosterone levels increased until the baseline threshold of 40 ng/ml and then stabilized. Stress-induced corticosterone decreased with date of sampling and body condition of individuals and tended to be higher in females than males (Table 3). Hour of sampling and sampling time did not predict stress-induced corticosterone levels. The increase in corticosterone levels from baseline to stress-induced levels was similarly associated to baseline corticosterone levels (Figure 2b), as well as the other fixed terms (Table 3).

**Table 3. Relation between baseline, stress-induced and the increase in corticosterone (CORT) levels from baseline to stress-induced levels in breeding barn owls.** Results from a linear mixed effect model for stress-induced and increase in corticosterone levels. Year and identity of individuals were added as random factors to correct for repeated measurements. The models were selected by backward elimination of non-significant variables ($P > 0.05$, two-tailed). Significant variables are highlighted in bold and all were significant in the full model.

| | Estimate | SE | *df* | *t*-value | *P* |
|---|---|---|---|---|---|
| *Stress-induced model* | | | | | |
| **Date of sampling** | **-0.077** | **0.023** | **434.3** | **-3.27** | **0.001** |
| Hour of sampling | 0.40 | 0.27 | 481.5 | 1.50 | 0.13 |
| Sampling time | 0.013 | 0.012 | 347.2 | 1.05 | 0.30 |
| Sex* | 5.64 | 3.2 | 407.0 | 1.76 | 0.079 |
| **Body condition** | **-10000** | **1783** | **476.7** | **-5.61** | **< 0.0001** |
| **Baseline CORT** | **2.02** | **0.25** | **509.0** | **8.07** | **< 0.0001** |
| **Baseline CORT$^2$** | **-0.02** | **0.01** | **501.6** | **-3.83** | **< 0.0001** |

* *Female-to-male difference*

| | Estimate | SE | *df* | *t*-value | *P* |
|---|---|---|---|---|---|
| *Corticosterone-increase model* | | | | | |
| ***Date of sampling*** | ***-0.08*** | ***0.023*** | ***434.3*** | ***-3.27*** | ***0.001*** |
| Hour of sampling | 0.40 | 0.27 | 481.5 | 1.50 | 0.13 |
| Sampling time | 0.013 | 0.012 | 347.2 | 1.05 | 0.30 |
| Sex* | 5.64 | 3.20 | 407.0 | 1.76 | 0.079 |
| ***Body condition*** | **-10850** | **1784.00** | **469.1** | **-6.08** | ***< 0.0001*** |
| ***Baseline CORT*** | ***0.82*** | ***0.26*** | ***507.7*** | ***3.23*** | ***0.001*** |
| ***Baseline CORT$^2$*** | ***-0.02*** | ***0.01*** | ***501.8*** | ***-3.23*** | ***0.001*** |

* *Female-to-male difference*

**Discussion**

In this study, we estimated whether variation in circulating corticosterone levels predicts survival in breeding barn owls using 11 years of corticosterone measurements and 13 years of capture-recapture records from 2004 to 2016. We showed that adult barn owls presenting low corticosterone baseline levels during the breeding season have a higher probability of survival than individuals with high baseline corticosterone levels (Figure 1a). We also found that individuals presenting a higher handling-stress corticosterone response and a stronger increase from baseline to stress-levels survived better than individuals showing a smaller handling-stress corticosterone response (Figure 1b). The relationship between stress-induced corticosterone levels and survival was less pronounced than for baseline levels (Figure 1). The opposite association between baseline and stress-induced levels with survival suggests that there may be an ongoing antagonistic selection process between baseline and stress-induced corticosterone levels. Because these two measures of corticosterone are positively correlated (Figure 2a), this pattern of antagonistic selection could help maintain genetic variation in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis and corticosterone levels. Indeed, we recently showed that part of the variation in baseline and stress-induced corticosterone levels was heritable and share a strong genetic basis (genetic correlation: 0.735) in barn owl nestlings (Chapter 1), with stress-induced response having a higher estimate of heritability ($h^2$: 0.441) than baseline levels ($h^2$: 0.199) suggesting that the regulation of the stress-induced corticosterone response to selection could potentially respond faster than baseline levels when exposed to similar selection pressure. Furthermore, selection exerted on one of the corticosterone trait may influence the evolution of the other. Our models showed that baseline corticosterone levels are strongly associated to survival suggesting that baseline may be more strongly selected compared to stress-induced

corticosterone response. If it is the case, this could explain why we found a lower heritability estimate for baseline compared to stress-induced corticosterone levels because natural selection is expected to deplete genetic variation (chapter 1).

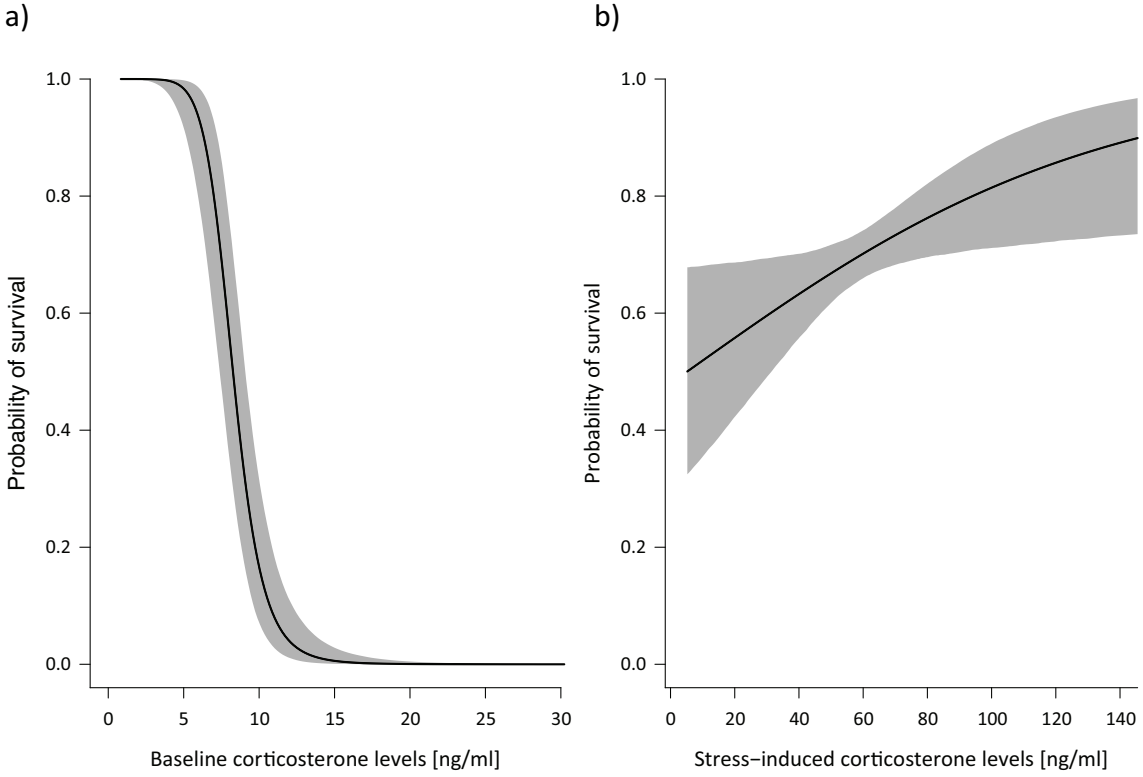
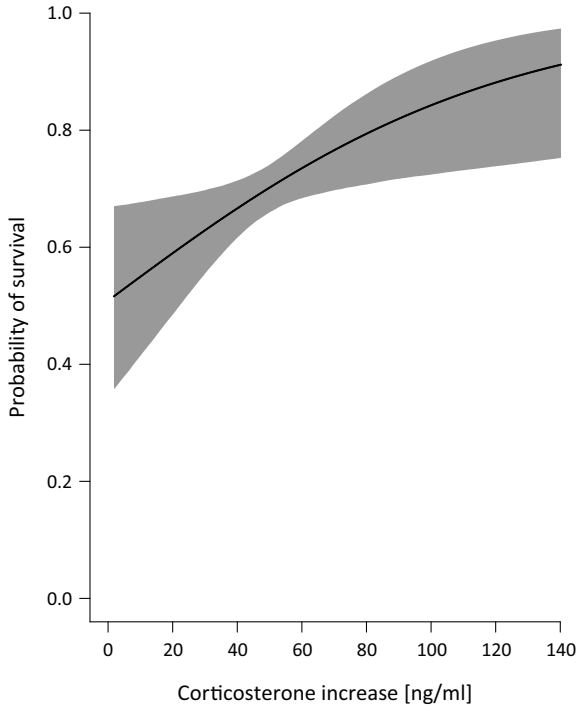



**Figure 1.** Estimated survival probability of breeding barn owls in relation to (a) baseline, (b) stress-induced corticosterone levels and (c) corticosterone increase from baseline to stress-induced levels. The black line represents the mean posterior distribution estimated from (a) the baseline stress-induced corticosterone model and (b) stress-induced corticosterone model, respectively. The shaded region represents the 95% Bayesian credible interval.

The association between baseline corticosterone levels and survival is in agreement with what we already know or predict about the costs and benefits of glucocorticoids hormones such as corticosterone (e.g., the "cort-fitness hypothesis", Bonier 2009a). Meaning that high and prolonged corticosterone levels can induce physiological deleterious effects that can subsequently affect survival. For instance, corticosterone has been showed to have immunosuppressive effects in different species (Bourgeon and Raclot, 2006; Fowles *et al*, 1993; Rubolini *et al*, 2005; Saino *et al*, 2003), including barn owl nestlings (Stier *et al*, 2009). Chronic stress is also viewed as an accelerator of ageing and has been associated to telomere length (Bauch *et al*, 2016; Choi *et al*, 2008; Haussmann and Heidinger, 2015; Monaghan, 2014), a biomarker of ageing and phenotypic quality in birds (Barrett *et al*, 2013; Bize *et al*, 2009; Haussmann and Heidinger, 2015) and humans

(Boonekamp *et al*, 2013). However, given that corticosterone is thought to mediate the trade-off between self-maintenance and reproduction (Wingfield and Sapolsky, 2003), these corticosterone variation may also reflect difference in life-history strategies between individuals. Indeed, corticosterone levels have been shown to increase during reproduction, in particular during nestling provisioning, an increase that may mediate the energetic demands related to parental care (Angelier *et al*, 2008; Beletsky *et al*, 1989; Bonier *et al*, 2009a; Bonier *et al*, 2009b; Comendant *et al*, 2003; Love *et al*, 2004; Love *et al*, 2014). Therefore, individuals expressing high levels of corticosterone may have a reduced survival compared to individuals with low corticosterone levels, but in the mean time, these individuals may have a higher reproductive success, as they may invest more effort in parental care than individuals with lower corticosterone levels (e.g., Beletsky *et al*, 1989; Bonier *et al*, 2009b; Love *et al*, 2004). Further studies investigating the relation between lifetime reproductive success and baseline corticosterone levels in the barn owl are necessary to examine whether there is a real fitness advantage of expressing low baseline corticosterone levels or if individuals differentially balance the costs and benefits of reproductive effort against survival with corticosterone mediating this trade-off.

c)



In contrast to baseline levels, we found that stress-induced corticosterone levels were positively associated to survival in adult barn owls. Although corticosterone secretion are both regulated by the HPA axis, the action and function of baseline corticosterone and the acute stress response are different (Romero, 2004). Baseline levels that vary in a daily and seasonal manner (Romero, 2002) are crucial in the homeostatic control of energy balance in face to the day-to-day predictable energetic demands. In contrast, the acute stress response plays an in important role in the case of emergency (Wingfield *et al*, 1998), as it induces a short and rapid adaptive change in behaviour and physiology in response to unpredictable fluctuations in the environment. For instance, elevated levels of corticosterone increase heart rate, blood pressure and peripheral blood supply, but also promote energy mobilization, foraging behaviour and food intake (reviewed in Sapolsky *et al*, 2000). As a result, the corticosterone response is thought to enhance short-time survival at the expense of other non-essential survival function such as reproduction or development (Meylan and Clobert, 2005; Rubolini *et al*, 2005; Wingfield *et al*, 1998). Considering that we found a positive link between stress-induced response and survival, barn owls with a higher stress-induced corticosterone response may survive better than individuals with lower stress corticosterone response, as they may be able to mobilize more resources and more rapidly during an emergency response. For instance, individuals with a higher stress-induced response may show a higher fight- or flight-response capacity than individuals with a lower stress-induced response (Cote *et al*, 2006; Overli *et al*, 2002). Alternatively, individuals with a low stress-induced response could have a lower survival because they already show high baseline corticosterone levels that may have induced a partial inhibition of the HPA axis and are hence unable to mount a correct stress-induced corticosterone response (Abe and Critchlow, 1980; Müller *et al*, 2009; Sapolsky *et al*, 1986). In this case, we would have expected to find that individuals with high baseline levels show a smaller corticosterone increase (i.e., difference between baseline and peak of stress-induced corticosterone levels) compared to individuals with low baseline levels rather than the contrary, which was not the case (Figure 2).

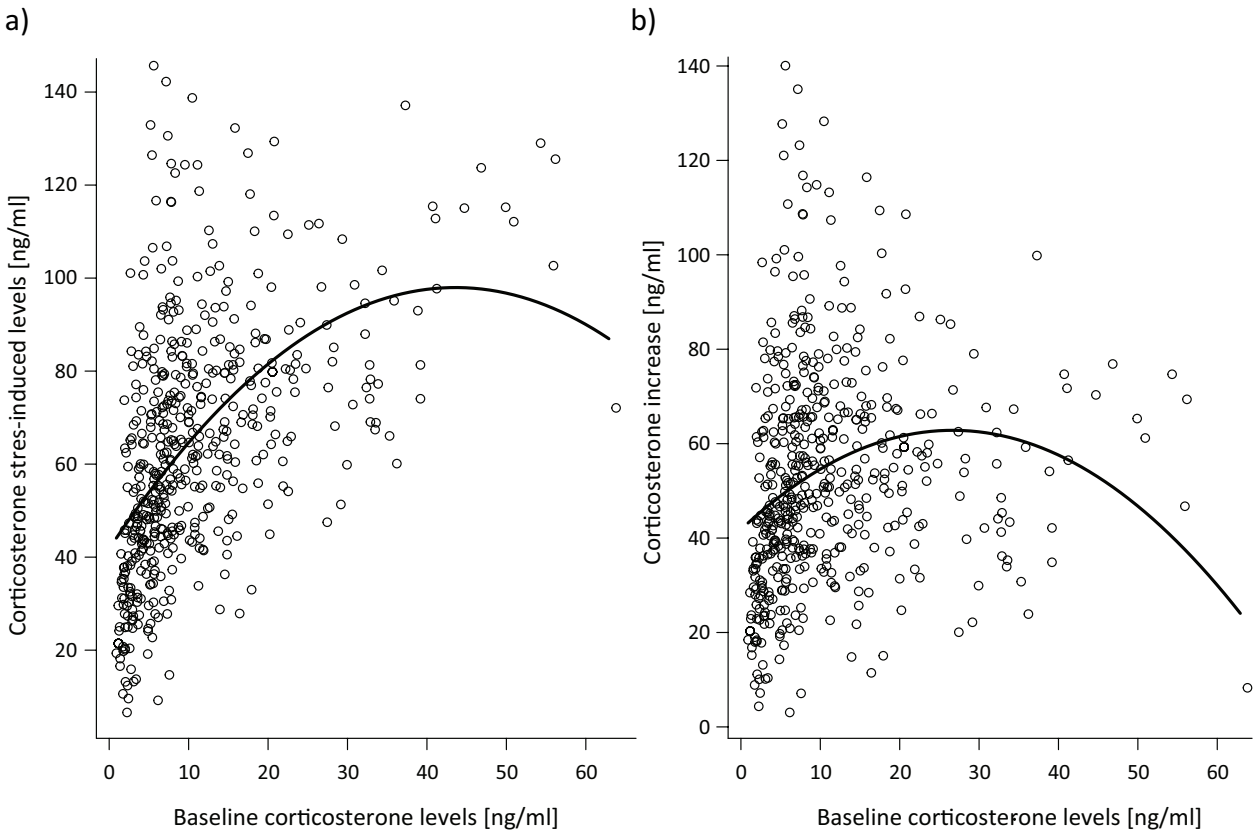



**Figure 2.** Relationship between baseline corticosterone levels and (a) stress-induced corticosterone levels and (b) the increase in corticosterone levels from baseline to stress-induced levels in breeding barn owls.

*Conclusion*

Our study indicates that baseline and stress-induced corticosterone levels are related to barn owl survival and that the strength and direction of selection on baseline and stress-induced corticosterone levels is different. This difference may have consequences in the evolution of the HPA axis given that baseline and stress-induced corticosterone levels share an important genetic basis. Therefore, further studies, investigating the pattern and mode of selection acting on these two components of the HPA axis will be necessary to better understand the capacity of the HPA axis and corticosterone levels to respond to selection.

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**Supplementary material**

**Table S1. Parameter estimates from a Bayesian model for baseline corticosterone levels measured between 2004 and 2016 on breeding barn owls.** The table shows the posterior mean, standard deviation and lower (2.5%) and upper (97.5%) limits of 95% Bayesian credible interval of fixed and random terms. These estimates were used to predict baseline corticosterone values in our multistate survival models.

| | Mean | SD | 2.5% | 97.5% |
|---|---|---|---|---|
| *Fixed effects* | | | | |
| Male - intercept | -0.052 | 0.031 | -0.116 | 0.006 |
| Female - intercept | -0.011 | 0.027 | -0.065 | 0.043 |
| Sampling time | 0.015 | 0.038 | -0.059 | 0.091 |
| Body mass | -0.157 | 0.043 | -0.241 | -0.073 |
| Date of sampling | -0.212 | 0.039 | -0.289 | -0.135 |
| Hour of sampling | 0.11 | 0.041 | 0.032 | 0.191 |
| *Random effects* | | | | |
| Individual identitiy | 0.018 | 0.012 | 0.001 | 0.045 |
| Year | 0.068 | 0.021 | 0.038 | 0.118 |

**Table S2. Parameter estimates from a linear mixed effect model for stress-induced corticosterone levels measured between 2004 and 2016 on breeding barn owls.** The table shows the fixed and random terms and the estimates used to predict stress-induced corticosterone values in our multistate survival models.

*Fixed effects*

| | Estimate ± SE | *df* | t - values | *P* |
|---|---|---|---|---|
| Intercept | -0.294 ± 0.13 | 35.3 | -2.26 | 0.030 |
| Sex | 0.157 ± 0.054 | 554.4 | 2.89 | 0.004 |
| Body mass | -0.354 ± 0.043 | 680.4 | -8.19 | < 0.0001 |
| Date of sampling | -0.19 ± 0.033 | 623.8 | -5.72 | < 0.0001 |
| Hour of sampling | 0.071 ± 0.032 | 662.2 | 2.18 | 0.030 |

*Random effects*

| | Variance | SD |
|---|---|---|
| Individual identity | 0.03548 | 0.1884 |
| Year | 0.06821 | 0.2612 |

# *Chapter 3*

## Expression of glucocorticoid and mineralocorticoid receptor genes in feathers co-vary with stress-related colour signal in barn owls

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**Abstract**

Glucocorticoid hormones, such as corticosterone, are important intermediates between an organism and its environment. They enable an organism to adjust his behavioural and physiological processes in response to environmental changes by binding to mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) expressed in many tissues, including the integument. The regulation of glucocorticoids covaries with melanin-based colouration in numerous species, an association that may result from the melanocortin system. Most studies have focused on the circulating levels of glucocorticoids disregarding the receptors that mediate their action and the proximate mechanisms underlying the covariation with melanin-based coloration. We investigated in nestling barn owls (*Tyto alba*) the association between melanin-based coloration and the expression levels of *GR* and *MR* in growing feathers. We also considered the expression levels of genes related to the melanocortin system and melanogenesis to better understand the origin of the link between the expression of receptors to which corticosterone binds and melanin-based coloration. Nestling barn owls displaying larger black feather spots expressed *GR* and *MR* to lower levels than did smaller-spotted individuals. Because the expression of the *GR* and *MR* genes were positively, rather than negatively, correlated with the expression of melanogenic genes, we suggest that the link between melanin-based coloration, GR and MR is not directly associated to melanogenesis. The finding that the expression of *GR* and *MR* was positively associated with the *PCSK2* gene that encodes the protein convertase responsible for post-translational modification of the proopiomelanocortin hormone, suggests that the melanocortin system may be implicated in the establishment of the covariation between melanism and the expression of receptors to which glucocorticoids bind. Together with previous studies, our results suggest that coloration is not only related to the production of glucocorticoids but also to the sensitivity of the integument to glucocorticoids.

**Introduction**

Hormones play an important role in translating external stimuli into physiological changes to enhance the survival prospects of an organism in the face of continuous changes in its environment (Ketterson and Nolan, 1992). The glucocorticoid hormones: corticosterone in amphibians, reptiles, birds and rodents and cortisol in non-rodent mammals and fishes, are under the control of the hypothalamic-pituitary-adrenal (HPA) axis and affect most cells in the body (Ballard *et al*, 1974; Lattin *et al*, 2012). Thus, variation in the levels of glucocorticoid hormones (hereafter referred to as "corticosterone") plays a major role in many physiological and behavioural functions (reviewed in Sapolsky *et al*, 2000; Wingfield *et al*, 1998), having important consequences on the development (Meyer, 1983; Ward, 1994), health (Costantini *et al*, 2011; Harris and Seckl, 2011), reproductive success (Bonier *et al*, 2009a; Breuner *et al*, 2008; Vitousek *et al*, 2014) and survival of an individual (Goutte *et al*, 2010; Rivers *et al*, 2012; Romero and Wikelski, 2001). Such multiple links to aspects of an individual's fitness have been proposed to favour the evolution of animal ornaments that reflect corticosterone levels or the capacity of an individual to withstand stressful conditions (Buchanan, 2000; Husak and Moore, 2008). However, although several empirical cases support the expected association between corticosterone levels and different types of ornaments (coloration and other visual signals, vocalizations, among others (see also Buchanan *et al*, 2016; Leary *et al*, 2006; Moore *et al*, 2016; Roulin *et al*, 2008; San-Jose and Fitze, 2013; Weiss *et al*, 2013), the proximate factors driving it remain obscure for most type of ornaments, particularly for those whose expression shows little environmental plasticity, such as colorations based on the deposition of melanin pigments (Roulin, 2016).

In many vertebrate species, corticosterone is associated with melanin-based colorations. Most studies that have investigated the covariation between melanin-based colour traits and the HPA axis have focused on glucocorticoid levels in plasma (e.g. Corbel *et al*, 2016; Kittilsen *et al*, 2009; Saino *et al*, 2013) or in integuments, such as feathers and hairs (Bennett and Hayssen, 2010; Ghassemi Nejad *et al*, 2017; Kennedy *et al*, 2013). Although the HPA axis is a complex system regulated at multiple levels and by multiple factors (Breuner and Orchinik, 2002; Funder, 2005; Holmes and Seckl, 2006; Romero, 2004), it is clear that the action of corticosterone is largely mediated in a dose-dependent manner by the amounts of glucocorticoid (GR) and mineralocorticoid (MR) receptors that bind to corticosterone in different target tissues (Iida *et al*, 1985; Vanderbilt *et al*, 1987; Yang *et al*, 1989). The nuclear mineralocorticoid (MR) and

glucocorticoid (GR) receptors are ligand-dependent transcription factors that influence the expression of numerous genes implicated in growth, metabolism, reproduction, resource allocation and immune functions (de Kloet *et al*, 2005; Le *et al*, 2005; Reddy *et al*, 2009). The nuclear MR has a ten-fold higher binding affinity to glucocorticoids than GR which are, however, more numerous and hence a higher carrying capacity than MR (de Kloet *et al*, 1990; Funder, 1997). These differences in properties are thought to mediate independently the action of baseline (MR) and stress-induced glucocorticoid levels (GR). Thus, MR is mainly thought to regulate the circadian variation of glucocorticoid levels while GR is mainly bound after an acute challenge or at the peak of ultradian rhythm when glucocorticoid concentration is high (Conway-Campbell *et al*, 2007), implying that MR are rapidly saturated while unbound GR are still numerous.

Glucocorticoid receptors are well distributed throughout the body with GR being more ubiquitous than MR (Ballard *et al*, 1974; Funder, 2005; Lattin *et al*, 2012; Lattin *et al*, 2013). Their distribution can show seasonal variations across tissues (Breuner and Orchinik, 2001; Lattin and Romero, 2015; Lattin *et al*, 2013), and be influenced or not by circulating hormone levels or stress (Breuner *et al*, 2003; Dickens *et al*, 2009; Lattin and Romero, 2015). More importantly, glucocorticoid receptors have been found to be expressed in different skin cells like melanocytes where melanin pigments are produced (Serres *et al*, 1996). Measuring variation in the expression of glucocorticoid receptors (GR, MR) is thus key not only to understand how the activity and action of the HPA axis is regulated, but also how the corticosterone levels influence the development of melanin-based colorations. Despite their importance in the regulation of the HPA axis and that they have been shown to relate to fitness (*e.g.*, receptors can vary in certain tissues between individuals adopting different reproductive tactics: Artbery, Deitcher & Bass 2010 ), only one study has investigated the link between melanin-based traits and the amount of glucocorticoid (GR) and mineralocorticoid (MR) receptors (Lattin and Romero, 2013). In this study, conducted in house sparrows (*Passer domesticus*), males with large black bibs showed a fewer amount of GR and MR in skin tissues than males with small bibs but the generality of this finding remains untested.

Additionally, it is still unclear by which means glucocorticoid (GR) and mineralocorticoid (MR) receptors and, thereby, corticosterone, could regulate the synthesis of melanin in the integument. The association between corticosterone and melanin-based coloration has been proposed to originate from the pleiotropic effect of the melanocortin system (Ducrest *et al*, 2008;

Roulin and Ducrest, 2011). This system is composed of five transmembrane melanocortin receptors that bind different melanocortin hormones and regulate specific processes, including melanin synthesis and stress response. On the one hand, binding of the melanocortin α-MSH (α-melanocyte-stimulating hormone) to the melanocortin 1-receptor (MC1R), induces the synthesis of melanin pigments in hair and feather follicles. On the other hand, binding of the melanocortin ACTH (adrenocorticotrophin hormone) to the melanocortin 2-receptor (MC2R) expressed in the adrenal glands activates the downstream signalling for the production of corticosterone. Both melanocortin hormones, α-MSH and ACTH, are derived from the cleavage of the proopiomelanocortin (POMC) prohormone by the protein convertases PC1/3 and PC2 (Chretien and Mbikay, 2016; Eipper and Richard, 1980; Pritchard and White, 2007). Thus, the link between melanin-based coloration and the HPA axis could potentially arise because α-MSH and ACTH derived from the same precursors and are both modulated by the same convertases. However, additional empirical knowledge is still needed to confirm this hypothesis.

In the present study, we examined the relation between melanin-based traits and the expression levels of *GR* and *MR* in growing feathers of nestling barn owls on the breast and belly, which can present very different colour patterns. The barn owl (*Tyto alba*) displays melanin-based traits that are associated to various life-history, morphological, behavioural and physiological traits (Roulin, 2004d). Barn owls displaying larger black spots on the feather's tip of their ventral body side are less sensitive to stress than smaller-spotted individuals, as they have a lower increase in blood corticosterone levels after a stressful event (Almasi *et al*, 2010), are less affected by an artificial increase in blood corticosterone levels during growth and breeding (Almasi *et al*, 2008; Almasi *et al*, 2012) and loose less weight under food depletion (Dreiss *et al*, 2010). In this case, given that large spotted individuals are thought to be less stress sensitive than small spotted individuals, we predict that large spotted individuals should express less *GR* and *MR* in their feathers than small spotted individuals. In parallel, we measured the expression levels of different melanocortin- and melanogenesis-related genes in order to identify the potential proximate mechanism or mechanisms linking melanin-based colour traits and the HPA axis activity. Assuming that genes involved in melanogenesis or those of the melanocortin system are involved in the association between melanin-based coloration and the expression levels of *GR* and *MR*, we expect to find a negative relation between the expression of melanin and melanocortin-related genes

with receptors involved in the binding of glucocorticoids (*GR, MR*), given the previously described negative relation between corticosterone and melanin-based traits in barn owls.

## Methods

*Study species and site*

The barn owl is a medium size raptor that lives in open landscape areas and preys on small mammals. Females lay two to eleven eggs at a rate of one egg every two to three days between February and the beginning of August. After an incubation of ca. 32 days, nestlings hatch asynchronously every two to three days, which leads to a within brood age hierarchy (Roulin, 2004b). Females stay in the nest box until the elder nestlings are about 3 weeks old and able to thermoregulate by themselves and feed without maternal help. Pennaceous feathers start growing around 10-15 days of age and plumage is completed at approximately 55 days of age, when nestlings are ready to fledge.

The study was carried out in an open rural landscape in western Switzerland (46°49'N, 06°56'E) and based on a sampling of 48 male and 35 female nestlings from 26 breeding pairs. Nestlings were visited for the first time at an early stage of feather development, when they were between 15 and 30 days old (mean ± SD: 21.81 ± 3.7 days). During this visit, we ringed the nestlings, plucked 3 to 5 feathers from their breasts and bellies, and took a blood sample for molecular sex determination following the protocol described by Py *et al* (2006). We plucked feathers at an early stage of development to try to pinpoint the time when nestlings produce the black eumelanic spot on the upper part of the feather. In this study, feathers were plucked at an earlier age compared to the previous study of San-Jose *et al* (2017) where spots were already formed. We re-visited the nestlings a second time when their feathers were fully developed (at ca. 55 days of age) in order to assess plumage coloration (see below for procedure). At each visit, we also weighed the nestlings and measured the length of their left wing in order to precisely determine their age. Most of the nestlings (75 out of 83) were cross-fostered before hatching between randomly chosen nests to allocate genotypes randomly among rearing environments and to avoid the potential confounding effect of the biological mother on the relationship between nestling spot size and the expression levels of *GR*, *MR*, melanogenic genes and genes belonging to the melanocortin system. We captured and measured the plumage coloration of adult females while they were incubating eggs or raising their chicks.

*Assessment of plumage coloration*

In the Swiss population of barn owls, the overall colour of ventral body side varies from white to rufous owing to differential deposition of pheomelanin whereas the tips of the ventral feathers are covered with a varying number of black spots, whose diameter and number also varies between individuals. On average, females are redder and display larger and more eumelanic spots than males (Dreiss and Roulin, 2010). Variation in the pheomelanin-based coloration is in part due to a valine (V)-to-isoleucine (I) substitution on the amino acid position 126 of the melanocortin 1-receptor (MC1R) (San-Jose *et al*, 2015), which is an important component of the melanin synthesis. This mutation was also shown to differently affect the expression of melanogenic-related genes (San-Jose *et al*, 2017). Individuals presenting the *MC1R* white allele ($MC1R_{White}$) express the *MC1R* and melanic genes at lower levels (*TYR*, *TYRP1*, *OCA2*, *SLC45A2*, *KIT*, *DCT*) than individuals carrying the *MC1R* rufous allele ($MC1R_{Rufous}$) responsible for the rufous coloration. The expression of these plumage traits is strongly heritable and weakly sensitive to the rearing environment (Roulin and Jensen, 2015). In this study, we focused only on the size of the black spots given that no associations have been found between spot number or the pheomelanin-based coloration with stress-related parameters in previous studies (Almasi *et al*, 2010; Almasi *et al*, 2008). We measured the diameter of 10 to 15 black spots to the nearest 0.1 mm using a slide calliper within a 60 x 40 $mm^2$ frame placed on a standard position of the breast and the belly of all nestlings and adults. We used the mean diameter of spots per body part for our statistical analyses. These methods have already been shown to be reliable (Roulin, 2004d).

*RNA extraction*

We extracted total RNA from the base of the developing feathers plucked from the breast and belly of the nestlings. We used breast and belly feathers because they can present very different colour patterns in terms of spots size. Additionally, by using feathers from two different body parts, we can assess whether an association between GR, MR and melanin-based traits is related to melanogenic genes (*MLANA*, *MITF-M* a melanocyte-specific isoform of *MITF, OCA2, PMEL, TYR, TYRP-1)* and to genes of the melanocortin system (*PCSK2*, *MC1R*) in a consistent manner between body parts. The feathers were collected within the hour following the capture of the nestlings and directly stored in liquid nitrogen before being stored at -80°C until gene expression analyses.

For each nestling and body part, two to five feather bases were grounded with a pestle in liquid nitrogen, resuspended in 600 μL of lysis buffer containing 10 mM DTT and passed through a spin filter column (InviTrap Spin Universal RNA Mini kit, Stratec, Berlin, Germany) to remove genomic DNA, barbs and barbules. Following the protocols (InviTrap Spin Universal RNA Mini kit) total RNAs were eluted in 50 μL of water. To further remove all possible genomic DNA contaminants, a second DNAse I treatment was applied by incubating the 50 μL of eluted RNA with 40 U of DNAse I (Roche diagnostics Ltd, Basel, Switzerland), 50 U of RNase Inhibitor (Promega AG, Dübendorf, Switzerland) and 1x Roche buffer for 30 min at 37°C, before rapidly freezing the solution at -80°C. The RNA was then purified and concentrated to 20 μL with the GeneJet RNA purification Kit (Thermo Fisher Scientific, Ecublens, Switzerland). For each series of RNA extraction (12 samples), one random sample was analysed with the Fragment analyser of Advanced Analytical (Labgene, Châtel-St-Denis, Switzerland) to assess RNA quality. All RQN (RNA quality number) values were above 8.0 (scale from 1 to 10), which indicate that the RNA samples were of high quality (Müller *et al*, 2016; Zhang *et al*, 2015). Finally, 1 μg of DNase I-treated total RNA was reverse transcribed in a final volume of 20 μL, using 4 μL of 5x VILO reaction buffer and 2 μL of 10x SuperScript Enzyme Mix reverse transcriptase (Thermo Fisher Scientific) at 25°C for 10 min, at 42°C for 60 min followed by reverse transcriptase inactivation at 85°C for 5 minutes. The cDNA was diluted 20x with 1x TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) to achieve a concentration of 100 ng of RNA and finally stored at -20 °C until quantification.

*Gene sequencing*

To explore the potential proximate mechanism linking melanin-based traits and the HPA axis activity, we choose to determine the relationship between genes involved in the HPA axis (*GR*, *MR*) with genes involved in the melanocortin system (*PCSK2*, *MC1R*) and the synthesis and development of melanin pigments (*MLANA*, *MITF-M* a melanocyte-specific isoform of *MITF, OCA2, PMEL, TYR, TYRP-1)*. We did not measure the expression of the POMC gene, a main actor of the melanocortin system, because in barn owl feathers we only detect its truncated form, which may not be functional (San-Jose *et al*, 2017). The convertase PC2 encoded by the gene *PCSK2* cleaves the POMC prohormone into different melanocortin peptides (e.g. ACTH, α-MSH) that bind to and activates different melanocortin receptors, including MC1R and MC2R (Chretien and Mbikay,

2016; Eipper and Richard, 1980; Gallo-Payet, 2016; Pritchard and White, 2007). Binding of the ligand α-MSH to the MC1R receptor triggers the melanogenesis process by activating the expression of the microphthalmia transcription factor (*MITF*) gene. MITF is a transcription factor that upregulates the expression of different genes involved in the synthesis of melanins (e.g. tyrosinase, *TYR*, tyrosinase-related protein 1, *TYRP-1*) (Du *et al*, 2003; Vachtenheim and Borovansky, 2010) and in the development and maturation of the melanosomes (e.g. protein melan-A, *MLANA*, Pre-MELanosome protein, *PMEL*, melanocyte-specific transporter protein, *OCA2*). MLANA is a transmembrane protein expressed on the surface of melanosomes and plays an essential role in melanosome biogenesis, vesicular trafficking and melanosome maturation and autophagy (Aydin *et al*, 2012; Giordano *et al*, 2009; Hoashi *et al*, 2005). The *PMEL* gene codes for a transmembrane glycoprotein that is regulated by multiple proteolytic processes resulting in an amyloid fibrillar matrix, which enables the deposition of melanin pigments and potentially prevents the toxic effect of melanin derivatives in the melanosome (Bissig *et al*, 2016). The *OCA2* gene, also known as the pink-eyed dilution protein or p-gene in human, plays an important role in the biogenesis of melanosomes and regulation of eumelanin content in melanocytes through the processing and trafficking of the tyrosinase enzyme (Hirobe *et al*, 2002; Orlow and Brilliant, 1999; Ozeki *et al*, 1995; Tamate *et al*, 1989).

The gene sequences of *GR* (*NR3C1)*, *MR* (*NR3C2)*, *MLANA*, *MITF-M, PMEL* and *TBP* were prepared from cDNAs following the protocols described in San-Jose *et al* (2017). We sequenced 8 exons of the glucocorticoid and mineralocorticoid receptors (*GR* and *MR*). The 2380 bp of *GR* included the start codon (position 1) and the stop codon (2322 bp). We found two synonymous mutations c.582C>A (I194, with 6 individuals of genotype *CC*, 1 of genotype *AA* and 1 heterozygous individual *CA*) and c.1557G>A (A519, with 6 homozygous individuals *GG*, 1 *AA* and 1 heterozygous *GA*) (for accession numbers see Table S1). For the *MR*, we sequenced 3108 bp. The sequence contained the start and stop codons located at 112 bp and 3055 bp, downstream the beginning of the sequence, respectively. We found four mutations, three of them synonymous: c.261C>T (C87, n=3 *CC*, 2 *TT* and 3 *CT* individuals), c.1353T>C (S451, n= 8 *TT*, 0 *CC* and 2 *TC*) and c.1707G>T (T569, n=7 *GG*, 0 *TT*, 3 *TG*), and one non-synonymous: 292C>G (Q98E, n=3 *CC*, 1 *GG* and 4 *CG*). We sequenced 590bp of 5 exons of *MLANA* (*MART-1*) with a start and stop codon at 69 and 410 bp, respectively. One non-synonymous mutation at position c.237C>T resulted in an arginine to threonine substitution (R56T, with 11 homozygotes *CC* and 1 heterozygous *CT* individuals). We

sequenced ten exons corresponding to 1977 bp of the Pre-MELanosome protein gene (*PMEL*), also called *gp100* or *SILV*. We also sequenced 1483 bp of the TATA-binding protein (*TBP*) and 1054 bp of the glyceraldehyde-3-phosphate deshydrogenase (*GAPDH*) which were used with the ribosome protein L13 (*RPL13*) sequenced in a previous study (San-Jose *et al*, 2017), as reference genes for qPCR. The sequence of TBP contained the start and the stop codons at position 135 and 1043 bp and includes 7 exons and the GAPDH sequence contains at least 10 exons but was missing the start codon. None of the mutations were related to barn owl coloration or to gene expression. Sequences of all the other genes (*MC1R, OCA2*, *PCSK2*, *RPL13*, *TYR*, *TYRP-1*) are described in a previous study (for details see San-Jose *et al*, 2017).

*Quantitative PCR*

The specific qPCR primers and dual labelled fluorescent probes were designed (FAM-BHQ1, fluorescein with BHQ1 quencher) with Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) and DNASTAR PrimerSelect software or directly by Microsynth (Balgach, Switzerland). Each primer and probe was blasted to verify its specificity before purchasing them from Microsynth (Balgach, Switzerland) and Eurogentec (Liège, Belgium). We then assessed the concentrations for each gene primer pairs and probes in order to reach an amplification efficiency of 95 to 105% (for details on efficiency see Table S2 in the supplementary material). Due to the low levels of gene expression of some genes (*MC1R, PCSK2*), we pre-amplified the cDNA with the TaqMan preamplification Master Mix kit (Thermo Fisher Scientific) for 14 cycles. We verified that the pre-amplification was uniform by calculating the delta–delta Ct values, which are ΔCt of the pre-amplified gene minus ΔCt of the cDNA (Ct values of the target genes minus Ct values of the reference gene *GAPDH*). The qPCR assays were run on a QuantStudio 6 real-time PCR system (Thermo Fisher Scientific) and each sample was run in triplicate with 2 μL of the diluted cDNA and 8 μL of master mix containing 1x qPCR MasterMix Plus Low ROX (Eurogentec, Liège, Belgium) or 1x TaqMan gene expression MasterMix (Thermo Fisher Scientific) and primers and probes (for more details on primers and probes see Table S2). To correct for interplate variation, each pre-amplified cDNA sample was arbitrarily assigned to a plate and three pools of different pre-amplified cDNAs were added to each plate and used to set up the threshold values between plates. To correct for any variation in cDNA content, CT scores of the candidate genes were normalized using three reference genes: the ribosome protein L13 (*RPL13*), glyceraldehyde-3-phosphate deshydrogenase (*GAPDH*) and TATA-

binding protein (*TBP*) (for accession numbers see Table S1). CT scores were imported into qBasePLUS software 1.3 (Biogazelle, Zwijnaarde, Belgium) and reference genes quality analysed with geNorm (Vandesompele *et al*, 2002). We calculated the mean relative quantities (RQs) per sample and used these values for our statistical analyses.

*Statistical analyses*

We used linear mixed models to test whether there is a relationship between the size of eumelanic spots displayed on the tip of ventral feathers and the expression of mineralocorticoid (*MR*) and glucocorticoid (*GR*) receptor genes. The models were conducted separately for *MR* and *GR*, which were considered as the dependent variables. We considered as fixed factors the spot diameter of the nestlings, the sex of the nestlings, the interaction between sex and spot diameter, the body part (*i.e.* part of the body where feathers were plucked, respectively the breast and belly) and its interaction with nestling spot diameter and age, and the date and time of the day (hour) when the feathers were sampled. The random effect of brood identity was considered in the models as well as the random effect of nestling identity in order to take into account for the repeated measurements taken on the nestlings (*i.e.*, the observed values at the two different body parts). The spot size of the biological mother was not included in the full models because preliminary analyses showed that this variable did not significantly influence the expression of *MR* and *GR* (not shown). To simplify the full model for each receptor type, we ran all the possible models contained within the full model (including the null model). For each receptor type, we ranked the models based on their AICc; Akaike's information criterion corrected for sample size and selected all the models that had a difference of AICc ($\Delta AICc$) < 4 from the model with the lowest AICc (Burnham *et al*, 2011). The estimates of the different parameters and *P*-values were determined by averaging all the models within a $\Delta AICc$ < 4 using the *model.avg* function as implemented in the MuMIn package (version 1.15.6).

To investigate the co-expression patterns of *GR* and *MR* with melanocortin and melanogenesis-related genes, and to investigate the degree of correlation between spot size and the expression of *GR* and *MR* with melanocortin and melanogenesis-related genes, we performed pairwise *Pearson*'s correlation tests among all the variables and, separately, for each body part (breast and belly). Given that the data was collected from full siblings and that our observations are therefore not statistically independent, we opted for a permutational approach to test for the

level of significance of the *Pearson'*s correlation coefficients (*r*) (Crowley, 1992). Within each of the 999 permutations conducted, the exchangeability of each observation was restricted within the nest of origin of each individual except for those cases where only one individual per nest was sampled (Winkler *et al*, 2014). These individuals' observations were randomly exchanged among them within each permutation. *P*-values were thereafter calculated as the proportion of permuted tests showing absolute *r* values equal or larger than the *r* value observed in the non-permuted data.

All the dependent variables were transformed to fulfil the normality assumptions of the parametric statistical tests and, to estimate the fit of best model, we calculated conditional and marginal $R^2$ values from the linear mixed models by following the method proposed by (Nakagawa and Schielzeth, 2013). All statistical analyses were done with R 3.0.2 (R Core Team, Vienna, Austria).

**Results**

*Glucocorticoid receptors, mineralocorticoid receptors and size of eumelanic spots*

Regarding the expression of the glucocorticoid receptor (*GR*) gene, four models were selected according to their *ΔAICc* values below 4 (accumulated weight, *w* = 0.85, Table 1). The model with the best fit included only the spot diameter of nestlings (*w* = 0.49), while the next three best models included none of the predictors (null model, *w* = 0.14), the additive effect of nestling spot diameter and sex (*w* = 0.12), and the effect of nestling sex alone, respectively (*w* = 0.10). Note that the first model had a larger weight and was 3.5 or more likely than the other three alternative models. The average model showed that nestlings displaying small spots expressed *GR* at higher levels than large-spotted individuals (Figure 1a, estimate for the slope of the size of eumelanic spots on *GR* expression: -0.16 ± 0.05, Z = 2.82, *P* = 0.005) and that female nestlings tended to express *GR* at lower levels than males (sex: -0.13 ± 0.07, Z = 1.93, *P* = 0.054). Spot diameter of nestlings explained 5.5 % of the total variance (estimated from the model with the lowest AICc), while neither the brood of origin nor the within-nestling variation between breast and belly feathers explained a substantial part of the variance in *GR* expression.

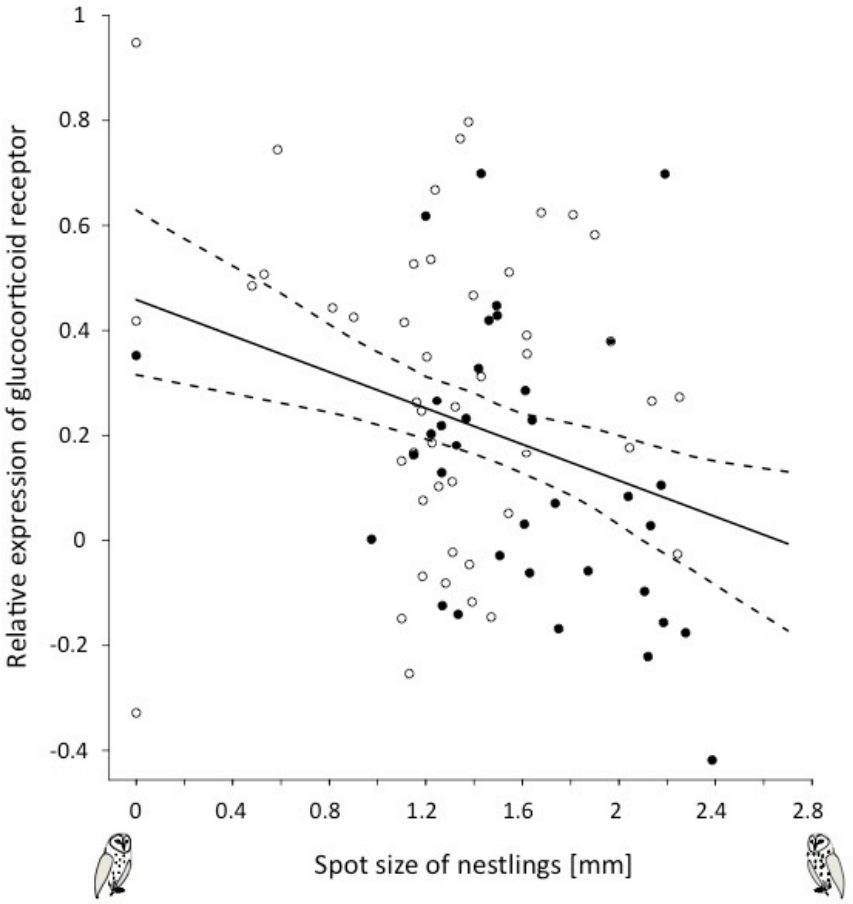


**Figure 1a.** Relation between the size of eumelanic black feather spots and the log-transformed expression of glucocorticoid receptors (GR) in feathers of nestling barn owls. The line represents the fitted values with the 95% confidence interval. The dots (females) and circles (males) represent the mean value of expression and spot diameter per individual. The relation was still significant ($P$ = 0.049) when removing individuals with zero spots from the analysis.

In relation to the expression of mineralocorticoid receptors (*MR*), two models were selected. The best model included the spot diameter of nestlings ($w$ = 0.55) and the second best model was the null model ($w$ = 0.25). Like for *GR*, *MR* were more expressed in small-spotted individuals compared to large-spotted individuals (Figure 1b, size of eumelanic spots: -0.16 ± 0.06, Z = 2.78, $P$ = 0.005). Spot diameter of nestlings explained 5.2 % of the total variance, while neither the brood of origin nor the within-nestling variation explained a substantial part of the variance in *MR* expression. There was no statistical evidence for differences between sexes in the expression of *MR* (the first model including sex had a delta AICc of 5.59). The models including nestling body

mass, age, body parts and the date and hour of sampling were all ranked with delta AICc values > 5.38 for the expression levels of the *GR* and 5.55 for the expression levels of *MR*. The expression levels of *GR* and *MR* were not correlated between body parts (all $r < 0.033$, all $P > 0.74$).

**Table 1. Expression levels of glucocorticoid (GR) and mineralocorticoid (MR) receptors from different body parts in relation to melanin-based traits in barn owl nestlings.** Results from a selection of competing models for glucocorticoid and mineralocorticoid receptors (log transformed) of 48 male and 35 female nestlings from 25 different broods. The nestling and brood identity were added as random factors to account for the lack of independence. The models were ranked on the Akaike's information criterion sizes (Burnham *et al*, 2011). The best models were selected according to a $\Delta AICc < 4$.

| Type of receptors | Predictors | *AICc** | Δ*AICc*** | weight† |
|---|---|---|---|---|
| *glucocorticoid receptors* | Size of eumelanic spots | 156.1 | - | 0.49 |
| | Null model | 158.7 | 2.53 | 0.14 |
| | Size of eumelanic spots + sex | 158.9 | 2.78 | 0.12 |
| | Sex | 159.3 | 3.14 | 0.10 |
| *mineralocorticoid receptors* | Size of eumelanic spots | 168.1 | - | 0.55 |
| | Null model | 169.7 | 1.6 | 0.25 |

Shown are the best models based on AICc (ΔAICc < 4) for each receptor.
*AIC value corrected for small simple sizes.
**Difference in a model's AICc to the best-ranked model's AIC.
†Model weight: probability of the model given the data.

*Co-expression of glucocorticoid and mineralocorticoid receptor genes with melanocortin and melanogenesis-related genes*

The glucocorticoid receptor (*GR*) gene was positively co-expressed with the melanocortin gene *PCSK2* in the feathers of both, breast and belly, and with the melanogenesis-related genes *MITF-M* (splice variant of *MITF*) and *PMEL* in the feathers of the belly and breast, respectively (Figure 2). In contrast, *GR* was not significantly co-expressed with the other melanocortin or melanogenesis-related genes: *TYR*, *TYRP-1*, *OCA2* and *MC1R*. The mineralocorticoid receptor (*MR*) gene was significantly co-expressed with the melanogenesis-related genes *MITF-M* and *MLANA* in all body parts and to *PCSK2* and *PMEL* on the belly (Figure 2). In contrast, *MR* was not significantly coexpressed with *MC1R*, *TYRP-1, OCA2* and *TYR*.

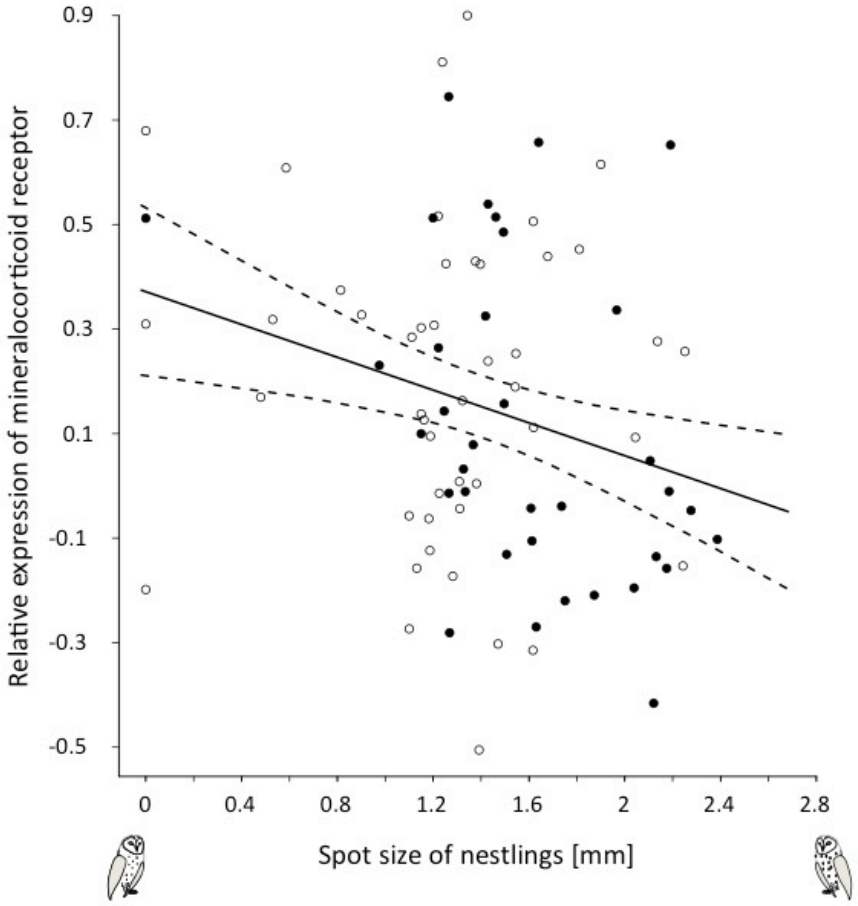


**Figure 1b.** Relation between the size of eumelanic black feather spots and the log-transformed expression of mineralocorticoid receptors (MR) in feathers of barn owl nestlings. The line represents the fitted values with the 95% confidence interval. The dots (females) and circles (males) represent the mean value of expression and spot diameter per individual. The association was still significant ($P$ = 0.029) when removing individuals with zero spots from the analysis.

The expression of *TYR* was significantly correlated with the spot diameter of nestlings in breast feathers ($r$ = 0.28, $P$ = 0.022) but not in belly feathers ($r$ = -0.03, $P$ = 0.85). The expression of the other melanogenic or melanocortin-related genes was not significantly correlated with spot diameter (all $|r| < 0.3$, all $P < 0.05$).

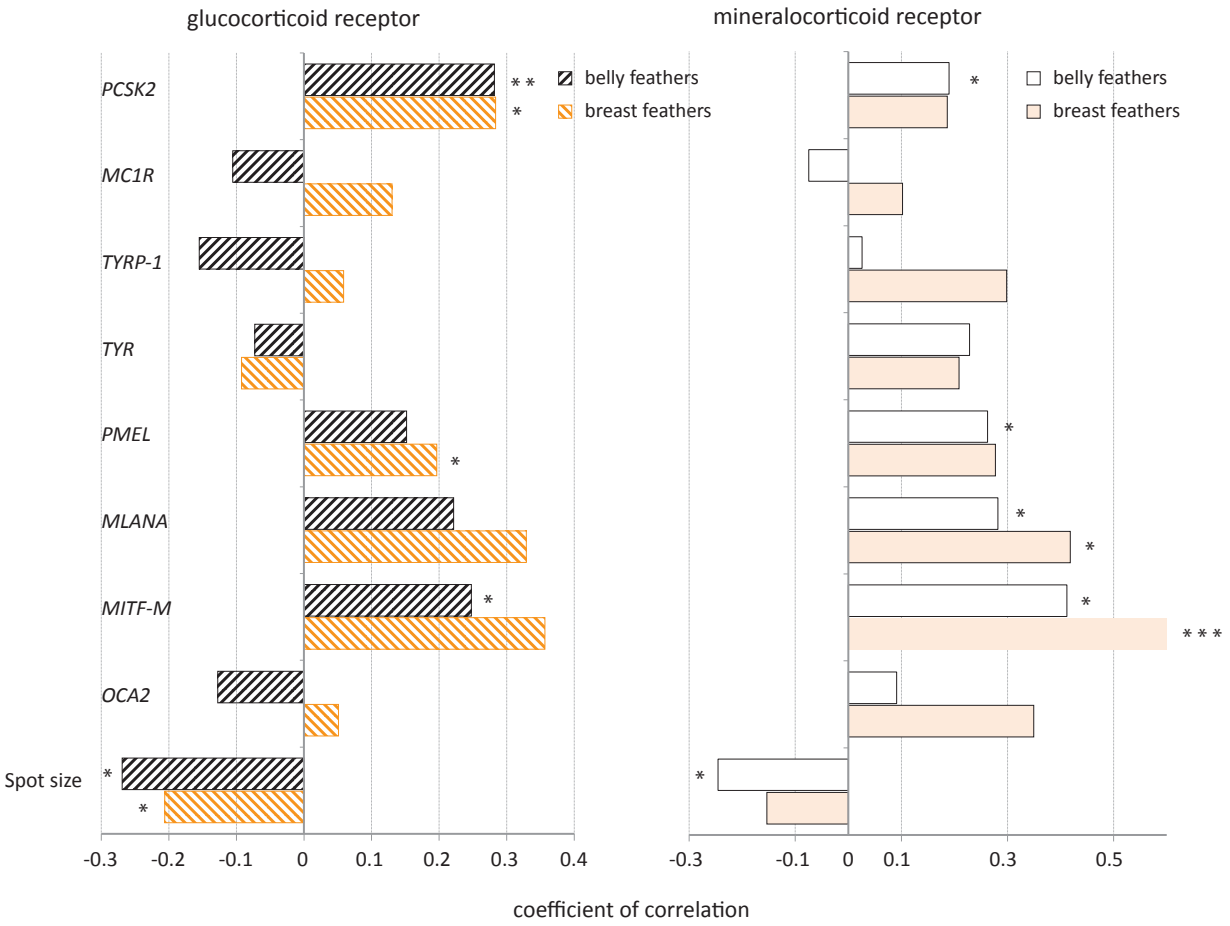


**Figure 2**. Co-expression of glucocorticoid (GR) and mineralocorticoid (MR) receptors with melanocortin- and melanogenesis-related genes measured in feathers plucked from the breast and belly of nestling barn owls. Bars represent the Pearson's correlation coefficient for GR, MR for the breast and belly (Table 2). Asterisks show the significance of the pairwise correlation (*$P \leq 0.05$, **$P \leq 0.01$, ***$P = 0.001$).

## Discussion

Large eumelanic spotted nestling barn owls showed lower expression of glucocorticoid (*GR*) and mineralocorticoid receptors (*MR*) genes in breast and belly feathers relatively to small-spotted conspecifics. These associations are in line with previous findings showing that the size of black feather spots is negatively related with the regulation of corticosterone, the hormone that binds to GR and MR in the barn owl (Almasi *et al*, 2010; Almasi *et al*, 2008) and other species (review in Ducrest *et al*, 2008; Roulin and Ducrest, 2011). Our results are also consistent with a previous study in house sparrows showing that males displaying large melanic bibs show less GR proteins in

the skin of their bib compared to males with small bibs (Lattin and Romero, 2013). However, as the null models were included within the selection of the best models, this may indicate that, as observed, the effect of spots on the expression of GR and MR may not be strong (respectively 5.5 and 5.2% of the total variance was explained by spot diameter) and that, although the effect is in line with the expectations of our study and previous findings, further studies may be needed to determine the robustness of the encountered associations.

The relationship between melanin-based traits and the expression of glucocorticoid receptors (*MR, GR*) could arise for different reasons. One possibility would be that this link arises through the melanocortin system and its multiple effects on the expression of melanogenic genes and the HPA axis. For instance, through the expression of the POMC prohormone or protein convertases (PC1/3, PC2) involved in the cleavage of the POMC prohormone into different active peptides (Pritchard *et al*, 2002). The relationship could arise because the proteins involved in the processing of POMC, or the downstream effector products regulated by the melanocortin system, affect simultaneously the expression of GR and MR and melanin-based traits. The cleavage of the POMC prohormone results in the adrenocorticotrophic hormone (ACTH) that induces the production of corticosterone in adrenal glands and in skin (Eipper and Richard, 1980; Slominski *et al*, 2005; Smith and Funder, 1988), which is itself known to affect the expression of glucocorticoid receptors (Karandrea *et al*, 2002; Nishimura *et al*, 2004; Paskitti *et al*, 2000) and therefore, influence the abundance of MR and GR in skin tissues. ACTH can be further cleaved by the protein convertase 2 (PC2) into corticotropin-like intermediate Peptide (CLIP) and α-melanocyte-stimulating hormone (α-MSH), which is involved in the synthesis of melanin pigments once bound to the melanocortin 1-receptor (MC1R). The coexpression of the upstream regulators PC2 (*PCSK2*) with *GR* and *MR* suggests that the melanocortin system and PC2 could be implicated in the link with melanin coloration owing to its role in the cleavage of ACTH into α-MSH and CLIP. This relationship might be the result of a trade-off between the production of ACTH and α-MSH by PC2. We can hypothesized that individuals expressing *PCSK2* at a higher level may produce less ACTH and therefore less corticosterone and receptors (*GR, MR*) but more α-MSH and therefore be more melanic, whereas individuals with low expression levels of *PCSK2* might produce more ACTH but less α-MSH. Although, we did not measure the corticosterone levels of nestlings in this study, this hypothesis is supported by previous studies in barn owls showing that melanin-based traits are associated to circulating corticosterone levels (Almasi *et al*, 2010) and to the expression of *PSCK2*

in older barn owl nestlings (Scriba *et al*, 2013). Finally, we cannot exclude that the expression of *GR* and *MR* are independent of the expression of *PCSK2* and that the relation between those genes may occur through a pathway that is independent of the melanocortin system.

Alternatively, the link between melanin-based traits and the expression of *MR* and *GR* could arise because the genes regulating the deposition of melanin pigments and these receptors are co-expressed, owing for instance to a common factor regulating the expression levels of melanogenic and the receptors binding glucocorticoids. In this case, we would have expected a negative association between the expression levels of melanogenic-related genes and *MR* and *GR* genes, given that the expression of the latter genes in this study and in a previous study in house sparrows is smaller in more melanised individuals (Figure 1). In our study, however, we did find significant correlations between glucocorticoids receptors (*MR, GR*) and genes involved in the production of melanin pigments (*MITF-M*) and in the biogenesis and development of melanosomes (*MLANA, PMEL*, Figure 2), genes coding for proteins involved in the early steps of synthesis, storage and transport of melanin pigments (Wasmeier *et al*, 2008). However, such associations were positive rather than negative, what we would have expected given the negative association between spot diameter and glucocorticoid receptors (*MR, GR*). This finding suggests that the covariation between melanin-based traits and the expression of *MR* and *GR* is not mediated by the melanogenic-related genes considered here.

The absence of evidence for a negative relationship between *MR* and *GR* with the expression of melanogenic-related genes could be due to the timing when we sampled the feathers. Although we tried to pinpoint the time when individuals produce the black spots, we do not have the certitude that all individuals were producing the spots at the time when we plucked feathers. This is supported by the fact that we found little evidence for a positive relationship between spot size and the expression levels of the melanogenic genes, except for *TYR* ($P = 0.02$), suggesting that the expression levels of melanogenic genes at the time when we collected the feathers is not yet entirely representative of the final colouration that the individuals will display. Future studies taking into account the temporal evolution of gene expression in feathers may help to better understand why we observed an association between the expression of *GR* and *MR* measured at an early age and melanin-based traits but not a similar association with the genes associated to the production of melanin pigments.

Although the potential for corticosterone to affect the expression of ornaments such melanin-based traits has been demonstrated in different studies (Jenni-Eiermann *et al*, 2015; Lattin *et al*, 2011; Roulin *et al*, 2008), we do not know how corticosterone affects the deposition of melanin pigments, including the role of MR and GR in this process. Assuming that a lower expression of *GR* and *MR* in the tissues corresponds to fewer receptors available for glucocorticoids, large-spotted individuals could reduce the long-term negative effect of high corticosterone levels (Jenni-Eiermann *et al*, 2015; Lattin *et al*, 2011; Roulin *et al*, 2008). However, the action of glucocorticoids (corticosterone in birds) in the skin or feather tissues may be limited or existing only when levels are elevated given that, in contrast to brain tissues where glucocorticoids can bind to MR and GR, the full occupancy of GR and MR by glucocorticoids in the skin is prevented by the intracellular metabolism of corticosterone by 11ß-hydroxysteroid dehydrogenase type 2 (11ß-HSD2) into inactive derivatives (Chapman *et al*, 2013; Joels *et al*, 2008). Thus, future studies should investigate to what extent corticosterone effects at the integumentary level are actually mediated by GR and MR receptors in order to clarify how corticosterone could be affecting colouration at the level of the integument *via* binding to its receptors. Interestingly, in humans, 11ß-HSD2 limits the access of GR and MR also to aldosterone, which regulates salt homeostasis, epidermal and hair development (Farman *et al*, 2010; Funder, 2005; Holmes and Seckl, 2006; Perez, 2011). This opens an alternative pathway for GR and MR receptors to affect melanin-based coloration via altering the development of the integument where melanins are deposited. GR and MR act as transcription factors that once bound to their ligands (aldosterone, corticosterone) are critical for the development of hair and skin cells. For instance, overexpression of *MR* and *GR* in keratinocyte of mice can induce skin atrophy resulting in the thinning of the upper layers of the skin causing them to be more fragile (Perez *et al*, 2001; Sainte Marie *et al*, 2007). Although in contrast to humans and mice (Farman *et al*, 2010; Perez, 2011) the roles of GR and MR in the development of feathers are unknown, variation in the expression of glucocorticoid and mineralocorticoid receptors in tissues of growing feathers might also have some effects on the development of feathers for instance in the rate of feather growth (O'reilly and Wingfield, 2001). These effects could induce structural differences in the feathers (Roulin *et al*, 2013) leading to differential expression of melanin-based traits, an hypothesis that could be addressed in future studies.

From a wider perspective, variability in the availability of corticosterone receptors in other tissues regulated by corticosterone may also have important physiological consequences. For instance, the availability of *GR* and *MR* in the hypothalamus have a crucial role in the sensitivity of HPA system as they determine the capacity of the system to come back to normal corticosterone basal levels (De Kloet *et al*, 1998) and to avoid the damaging effects of high and prolonged corticosterone levels. Individuals with lower *GR* and *MR* expression are assumed to have fewer available receptors and thus, have a reduced feedback signal when facing acute stress. A mitigated negative-feedback response implies that individuals will be exposed in general to higher concentration of corticosterone, which could have serious consequences on their health (Barden, 2004; Dickens *et al*, 2009; Marques *et al*, 2009). Differences in the expression levels of corticosterone receptors (GR, MR) and plasma levels during an acute stress between differently melanized individuals could indicate that barn owls have different strategies to cope with stress, which could be signal by colour traits (Korte *et al*, 2005). Thus, large-spotted barn owls may have higher peaks of corticosterone during an acute stress response but express glucocorticoid receptors at higher levels which enables them to recover from an acute stress faster than small-spotted individuals who, on the other hand, may produce less corticosterone during an acute stress. Although GR and MR are, to some extent, similarly expressed in different tissues throughout the body (Lattin *et al*, 2015), additional studies linking *GR* and *MR* expression levels in tissues involved in the mechanisms triggered by the HPA axis, including metabolic (i.e. liver, kidney, subcutaneous fat and muscles) and brain tissues, to melanin-based traits and melanocortin related genes will help us to better understand how differently melanized individuals modulate the HPA axis and how melanin-based coloration evolve as stress-related signals.

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*Author Contributions*
A.R. obtained funding. A.R.; P.B. and A.-L.D. conceived and designed the study; P.B. conducted fieldwork; P.B., A.-L.D. and C.S. conducted all the genetic analysis; P.B. and L.M.S.-J. conducted the

statistical analyses; P.B and A.R. wrote the manuscript with important contributions of L.M.S.-J. and A.-L.D. All authors read and provided input on the manuscript.

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## Supplementary material

**Table S1**. Sequenced genes used for quantitative PCR.

| Name | C* | Seq length (bp) | Exons | Start codon position (bp) | Stop codon position (bp) | Accession numbers |
|---|---|---|---|---|---|---|
| *GAPDH* | 1 | 1054 | 10 | ND | 1052 | KU712279 |
| *MC1R* | 11 | 945 | 1 | 1 | 945 | KR018388-92 |
| *MITF-M* | 12 | 1632 | 10 | 47 | 1534 | KU712283 |
| *MLANA* | Z | 590 | 5 | 69 | 410 | KY433283-4 |
| *GR* | 13 | 2380 | 8 | 1 | 2322 | KX108748-9 |
| *MR* | 1 | 3108 | 8 | 112 | 3055 | KX108754-7 |
| *OCA2* | 1 | 2627 | 23 | 72 | 2625 | KU712287-90 |
| *PCSK2* | 3 | 3273 | 13 | 79 | 1990 | KU712310-3 |
| *PMEL* | 33 | 1977 | 10 | ND | ND | KY433285 |
| *RPL13* | 11 | 642 | 5 | 7 | 640 | KU712291 |
| *TBP* | 3 | 1483 | 7 | 135 | 1043 | KY433286 |
| *TYR* | 1 | 1941 | 5 | 91 | 1678 | KU712302-5 |
| *TYRP-1* | Z | 1858 | 7 | 248 | 1858 | KU712300-1 |

* Position of gene on chicken chromosome, Exons: Number of detected exons; ND: not determined

**Table S2**. Primers and probes with concentration used for quantitative PCR

| Genes | Forward primers | Reverse primers | Probe | Primer pairs concentrations (nM) | Probe concentrations (nM) | PCR Efficiency % |
|---|---|---|---|---|---|---|
| GAPDH | TGCCAACCCCCAATGTCTC | AGCAGCCTTCACTACCCTC | GTGGACTTGACCTGCCGTCTGGAAAAA | 900/900 | 300 | 104 |
| MC1R | TCATCCTCATCGTCACCTG | ATGTGGAAGAGGTTGAAGTAAC | AACCCCTTCTGCACCTGCTTCTTC | 900/900 | 600 | 103 |
| MLANA | AGGAGGTTTGAGCAGGACCA | TTGCCAGCACCAAAATGAAG | TGCCCAGAAGAAACCACCATCCAGA | 300/300 | 150 | 98 |
| GR | TTCAAGCCCTGGAATGAGAT | CACCGTAATGACACCCAGAG | CGGGACCACCTCCCAAGCTC | 300/300 | 150 | 100 |
| MR | TGGTGAAGTGGGCAAAGATA | CCAACTCAAGGCAAATGATG | AGAAACTTGCCTCTCGAGGACCAAATT | 300/300 | 150 | 100 |
| MITF-M | TTTGAGATGATGCGGCAGTT | AGGATGCTCGGAATTGCTGT | TCCTTAAATGCTTCCCAAGAAATGGCGA | 300/300 | 300 | 100 |
| OCA2 | TGGGCAACCCTCCTTTTCT | AGAGGAGGCCAAAGCAGACAC | GTTTGTTCTCATGGAGGCTTTGGCTCATCT | 900/900 | 300 | 102 |
| PCSK2 | AGGCTAACTTGGACCTGAC | CAGCCCAACACCATTTCTAC | GCAGCATCTGACAGTGCTCACCTCAAA | 900/900 | 300 | 104 |
| PMEL | AAGCGCGGAAAATTCGTCTA | GTCCACCACCTGCCAGTAGC | CGTCTGGTGGACATGGGGGC | 300/300 | 200 | 101 |
| RPL13 | ATGCCTAAGAAGGGAGAC | CCGTTTGAAAACATTCTTGA | AACTCAAGATGGCGACTCAGC | 900/900 | 300 | 101 |
| TBP | CCCTCTCCAATGACTCCAAT | AAGATTCACGGTGGACACAA | AACACCGGCGTCCGAGAGCT | 300/300 | 300 | 98 |
| TYR | CCCAATTTACTCAGCCCAGCATC | TTGTGGCACTGCATAAAGCCTGT | CCTCATGGCAGGTAATTTGTACTCGGTCTGA | 300/300 | 150 | 100 |
| TYRP-1 | AACCATCTGCAACAGCAC | ACACCAACTTCCAAACACC | TGGTCCCATCAGAAGAAATCCTGCTGGAAA | 300/300 | 300 | 98 |

# *Chapter 4*

## Circulating testosterone and feather-gene expression of receptors and metabolic enzymes in relation to melanin-based coloration in the barn owl

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ABSTRACT

Knowledge of how and why secondary sexual characters are associated with sex hormones is important to understand their signalling function. Such a link can occur if i) testosterone participates in the elaboration of sex-traits, ii) the display of an ornament triggers behavioural response in conspecifics that induce a rise in testosterone, or iii) genes implicated in the elaboration of a sex-trait pleiotropically regulate testosterone physiology. To evaluate the origin of the co-variation between melanism and testosterone, we measured this hormone and the expression of enzymes involved in its metabolism in feathers of barn owl (*Tyto alba*) nestlings at the time of melanogenesis and in adults outside the period of melanogenesis. Male nestlings displaying smaller black feather spots had higher levels of circulating testosterone, potentially suggesting that testosterone could block the production of eumelanin pigments, or that genes involved in the production of small spots pleiotropically regulate testosterone production. In contrast, the enzyme 5α-reductase, that metabolizes testosterone to DHT, was more expressed in feathers of reddish-brown than light-reddish nestlings. This is consistent with the hypothesis that testosterone might be involved in the expression of reddish-brown pheomelanic pigments. In breeding adults, male barn owls displaying smaller black spots had higher levels of circulating testosterone, whereas in females the opposite result was detected during the rearing period, but not during incubation. The observed sex- and age-specific co-variations between black spottiness and testosterone in nestling and adult barn owls may not result from testosterone-dependent melanogenesis, but from melanogenic genes pleiotropically regulating testosterone, or from colour-specific life history strategies that influence testosterone levels.



## 1. Introduction

Secondary sexual traits often signal aspects of quality allowing sexually active individuals to secure the best mates (Andersson, 1994). Outside the breeding season non-sexually dimorphic phenotypes that vary between individuals can also function as signals. For instance, the so-called "badge of status", usually a large black patch, can be used to discriminate dominant from subordinate individuals to avoid fighting with highly competitive conspecifics (Rohwer, 1975). Several mechanisms can ensure that sexually selected traits and badges of status honestly signal quality (Acker et al., 2015). One long-debated hypothesis is that the expression of these traits relies upon the release of hormones that have detrimental effects on other aspects of the phenotype, such as immunocompetence (Folstad and Karter, 1992). Testosterone is central for this hypothesis (Roberts et al., 2004) because it is often essential for the full development of behavioural and morphological traits used in sexual contexts and social competition (Ketterson and Nolan, 1992).

Testosterone can induce the deposition of melanin pigments in secondary sexual characters, like the skin (Diaz et al., 1986; Edwards et al., 1941; Hamilton, 1939; Hamilton and Hubert, 1938; Noble and Wurm, 1940), hair (Hirobe et al., 2010) and feathers (Haase et al., 1995; Kimball, 2006; Lindsay et al., 2011). This observation often leads researchers to assume that, in any species, a co-variation between a melanin-based colour trait and testosterone, results from the fact that the full expression of an ornament is testosterone-dependent. Even if testosterone levels have been shown experimentally to induce the production of melanin pigments (reviews by Bokony et al., 2008; Kimball, 2006), it does

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not necessarily imply that all co-variations between melanin-based traits and testosterone arise from the melanogenic properties of testosterone. Indeed, such a co-variation can also occur if the display of an ornament modifies behaviour (Fargallo et al., 2014; Gonzalez et al., 2002; Safran et al., 2008), which in turn induces a change in hormone profiles, or if individuals expressing different colour traits display different life history strategies (Bokony and Liker, 2005; Emaresi et al., 2014). For instance, in many species, darker melanic individuals are socially more dominant and more often socially challenged than weakly melanic conspecifics (Bokony et al., 2008; Ducrest et al., 2008; Hill, 2006; Jawor and Breitwisch, 2003; Senar, 2006). Interestingly, Safran et al. (2008) showed that the manipulation of the reddish-brown colouration in American barn swallows (Hirundo rustica erythrogaster) induced a rise in testosterone in manipulated individuals. In that case, we would expect to see a relationship between colouration and testosterone at very specific time points, such as during reproduction or social encounters. An alternative explanation to the co-variation between melanin-based traits and testosterone, states that the genes involved in the production of melanin pigments pleiotropically regulate testosterone (Ducrest et al., 2008). Therefore, melanin-based colouration could be differentially related to testosterone in different contexts, such as during the period of melanogenesis (e.g. in birds when they moult), or during the breeding period when melanogenesis is reduced (e.g. most birds do not moult during the period of parental care).

A further complication, is that a given trait could also reflect different aspects of quality in males and females, or the same quality but to a different extent in the two sexes (Dakin, 2011; Jawor and Breitwisch, 2004; Jawor et al., 2004; Jawor and Winters, 2010; Kraak et al., 1999; Kunzler and Bakker, 2000; Møller, 1994; Sinervo and Lively, 1996; Sinervo et al., 2000). Due to sex-specific roles in reproduction, a given trait may reflect sex-specific qualities (e.g. fecundity in females and sexual behaviour in males) or similar qualities (e.g. competitiveness or immunocompetence), but at different times of the reproductive cycle. Given the role of testosterone on reproductive functions and behaviour, this hormone may have differential effects on the expression of male and female colouration, or it may differentially co-vary with colouration in each sex (Enstrom et al., 1997; Ketterson et al., 2009, 2005; O'Neal et al., 2008). Knowledge of whether secondary sexual characters co-vary with testosterone, and of the underlying mechanism, is important to fully understand the potential adaptive function of such traits.

The most common way to investigate the effect of testosterone on different phenotypic traits is to experimentally increase plasma testosterone levels (Ketterson et al., 2009; Lynn, 2008; Owen-Ashley et al., 2004; Quispe et al., 2015). However, the property of a tissue to respond specifically to a hormone is sometimes more important than the amount of hormones circulating in the blood (Ball and Balthazart, 2008; Grunt and Young, 1952). Indeed, the effect may depend on the affinity of testosterone with its carrier (sex hormone-binding globulin), as only the free fraction of testosterone can enter target cells and induce a physiological effect. It may also depend on the sensitivity of the target tissue (e.g. number of receptors) or the intracellular metabolism of the tissue. Although testosterone binds to intracellular receptors to trigger biological processes, testosterone can also be metabolized into estrogen by aromatase, or into dihydrotestosterone (DHT) by 5α-reductase, two hormones that mediate physiological functions (estrogen) other than testosterone, or the same functions but in a more potent way (DHT). Given that a significant part of testosterone effects are mediated by estrogen and DHT, the activity of the enzymes that transform testosterone can determine its action (Ball and Balthazart, 2008; Dessifulgheri et al., 1976). For this reason, it is not only important to manipulate or measure hormone levels, but also to investigate the enzymes and receptors involved in the hormone response, in target tissues where melanogenesis occurs (skin, feathers).

Here we examined whether melanin-based traits are related to circulating testosterone in the barn owl (*Tyto alba*). This bird displays a pronounced variation in the degree of reddish-brown pheomelanin-based colouration, and in the extent to which the plumage is marked with black spots (eumelanin-based trait) of varying size, located at the tip of ventral body feathers. Although both sexes can exhibit any plumage trait values, males are on average lighter reddish and display fewer and smaller black eumelanic spots than females. The expression of both traits is genetically controlled, with some genes being located on the sex chromosome, and others on autosomes (Roulin et al., 2010; Roulin and Jensen, 2015). Variation between a white and dark reddish plumage has a function in predator-prey interactions (Roulin, 2004a), whereas black spottiness experiences sexually antagonistic selection, with large spots being positively selected in females but negatively in males (Almasi et al., 2013; Roulin et al., 2010). To understand why black feather spots are differently selected in each sex, a key issue to tackle is to examine how testosterone and its associated metabolites and receptors co-vary with plumage spottiness in males and females. A possibility could be that hormones regulate variation in spot size, or inversely, the genes involved in the production of spot size regulate hormone levels in a sex-specific way. A link between testosterone and plumage traits is plausible because male and female barn owls that display a male-specific plumage (i.e. white plumage/small black spots) adopt male-specific behaviour by being more aggressive, less cooperative, and by dispersing shorter distances (Roulin et al., 2012; van den Brink et al., 2012a,b). Therefore, if plumage traits reflect the testosterone status, we predict light-reddish and/or small-spotted individuals of either sex to show higher blood-circulating testosterone levels than dark-reddish and large-spotted conspecifics. In contrast, the relationship between melanin-based traits and the expression of androgen and estrogen receptors, or the enzymes involved in the metabolism of testosterone (5α-reductase, aromatase), may be more complex. Since we do not know whether testosterone, or one of its metabolites (estrogen, DHT), is associated with the regulation of the colouration in this species. Moreover, there have been contradicting results on the effect of sex hormones on melanization. In some cases, estrogen has been found to increase melanization (Hirobe et al., 2010; Jian et al., 2011; Schwahn et al., 2005; Sun et al., 2017), while in other species, to inhibit melanin deposition (Kimball, 2006; Oribe et al., 2012), as observed also for testosterone (Hirobe et al., 2010; Kim et al., 2008; Kimball, 2006; Oribe et al., 2012; Tadokoro et al., 1997, 2003).

## 2. Methods

### 2.1. Field study and species

The barn owl is a medium-sized bird of prey (body mass varies from 250 to 400 g) that lives in the open landscape and preys upon small mammals. In our Swiss study area (46°49′N, 06°56′E), females lay two to eleven eggs per clutch between February and August. After an incubation of 32 days, the nestlings hatch asynchronously every two to three days, which leads to a pronounced within-brood age hierarchy (Roulin, 2004b). The mother broods and feeds her offspring until the first-born nestlings are about 3 weeks old, i.e., when they are able to thermoregulate and consume prey items without help. Fledging takes place at *ca.* 55 days of age.

The present study was carried out in 2007 on 27 breeding females (n = 29 testosterone samples) and 21 breeding males (n = 21 samples), and in 2012 and 2014 on 55 different breeding females (total n = 97 samples, 51 in 2012 and 46 in 2014) and 21 different breeding males in 2014 (n = 22 samples). A total of 106 nestlings (61 males and 45 females) from 30 different broods were also sampled for testosterone in 2014. In parallel, we measured the expression of sex hormone receptors (androgen and estrogen receptor) in feather tissues of 80 nestlings (42 males and 38 females) sampled in 2012, as well as the enzymes 5 α-reductase, which metabolizes testosterone to DHT, and aromatase, which metabolizes testosterone to estrogen.

To break down the potential correlation between plumage traits and rearing conditions that may influence the co-variation with testosterone metabolism, we cross-fostered eggs between nest pairs with similar laying dates. If clutch size between nest pairs was of similar size (±1 egg), we swapped all eggs, whereas if there was a difference of more than one egg, we cross-fostered the same number of eggs between the chosen nest pairs. In those cases, we made sure to keep the within-brood age hierarchy as prior to the manipulation, by swapping eggs that were at the same stage of incubation. We later verified the origin of each nestling using molecular markers (Ducret et al., 2016). Of the 106 nestlings we sampled, 94 (54 male and 40 female nestlings) were cross-fostered.

### 2.2. Assessment of plumage traits

The ventral body side of barn owls varies continuously, in pheomelanin-based colouration, from white to dark reddish-brown, and in both number and size of black feather spots. Although members of the two sexes exhibit the traits in the same range of possible values, females are on average redder and exhibit more and larger eumelanic spots than males (Dreiss and Roulin, 2010). The expression of these three plumage traits is more strongly heritable in males ($h^2$ for reddish colouration is 0.87 ± 0.12, 0.72 ± 0.14 for number of spots, and 0.81 ± 0.03 for spot diameter) than in females ($h^2$ for reddish colouration is 0.79 ± 0.13, 0.50 ± 0.04 for number of spots, and 0.58 ± 0.13 for spot diameter) and weakly sensitive to environmental conditions (Roulin and Jensen, 2015). For all nestlings and adults, we compared their degree of reddishness with eight colour standards ranging from −8 (white) to −1 (dark reddish). We counted the number of spots within a 60 × 40 mm frame placed on a standard position on the breast, and measured the size (diameter) of a representative number of spots to the nearest 0.1 mm with a slide calliper. For the statistical analyses, we used the mean diameter of all measured spots. These methods are highly reliable and correlated with objective measurements of colouration (Dreiss and Roulin, 2010; Roulin and Dijkstra, 2003).

### 2.3. Plasma testosterone levels

The blood samples were taken by punctuating the brachial vein immediately after capture, and collecting with heparinised capillaries. The blood was immediately centrifuged to separate the plasma from the blood cells and stored into liquid nitrogen in the field, and at −80 °C once at the laboratory. Adults were either sampled during the day when females were incubating eggs, or at night while they were feeding their offspring. In adults, sampling time had no significant effect on testosterone levels (*P*-values >0.25) and was therefore not considered in the final models.

In 2007, circulating testosterone levels were analysed using the competitive Salivary Testosterone Enzyme Immunoassay of Salimetrix (Labodia, Yens, Switzerland). 25 μL of plasma were analysed for testosterone in single (n = 43) or duplicate (n = 7), without plasma extraction. This kit used for saliva samples has a low detection limit of 6 pg/mL, which allows the detection of owl testosterone. The detected testosterone levels ranged between 14 and 2616 pg/mL (mean level: 386.6 pg/mL) and plasma samples with values above the highest standard 600 pg/mL were controlled by dilutions of the plasma 2–4 times in a second assay. The intra-assay variations ranged between 3.8% and 7.7% and the inter-assay was 9.5%. This assay has been validated by Washburn et al. (2007), in mourning doves (*Zenaida macroura*), white-eyed vireos (*Vireo griseus*), red-eyed vireos (*Vireo olivaceus*), and indigo buntings (*Passerina cyanea*). The cross-reactivity for progesterone, estrogen, dihydrotestosterone, 19-nortestosterone, androstenedione, and 11-hydroxytestoterone was below 2%, whereas for dihydrotestosterone (DHT) and 19-nortestosterone it was of 36.4% and 21.02%, respectively. This kit does not cross-react with corticoid derivatives. Due to the high cross-reactivity with DHT and 19-nortestosterone of the Salimetrix kit antibodies, the samples of 2012 and 2014 were extracted with dichloromethane, and analysed with a competitive radioimmunoassay kit (Testo-CT2 kit, Cisbio Assays, France). 5–100 μL of plasma were added to 100 μL of water, and extracted twice with 4 mL of dichloromethane, incubated overnight, dried and resuspended in 50 μL of PBS buffer. Following the manufacturer protocol (Testo-CT2 kit, Cisbio Assays), we determined the concentration of plasma testosterone in duplicate. The intra-assay variation ranged between 1.6% and 2.8%, and inter-assay variation was 19.5%. The recovery after extraction was of 78.9% and the cross-reactivity with other hormones was below 2.7 % (for dihydrotestosterone, 17β-estradiol, progesterone, androstenedione) and 0.2% for other steroids. The detected testosterone levels ranged between 10 and 4046 pg/mL (mean level: 202 pg/mL) for adults and 10 –1003 pg/mL (mean level: 177 pg/mL) for nestlings with a detection limit of 10 pg/mL. Hereafter, the plasma testosterone is denoted "testosterone". Testosterone levels measured in 2007 presented a similar range as the levels measured in 2012 and 2014 in both sexes (females levels varied from 14 to 1030 pg/mL in 2007 and from 0 to 1176 pg/mL in 2012 and 2014; in males testosterone levels varied from 49 to 2616 in 2007, and from 0 to 4046 in 2014 with two males above 2600 pg/mL). For statistical analyses, we combined all the data and added the year to our models as a fixed factor to control for a possible effect of the assays on testosterone measurements.

### 2.4. RNA extraction and gene sequencing

In 2012, total RNA was extracted from the base of two to three feathers at blood quill stage (*i.e.* the growing feather is wrapped in a shaft and contains the vascularized pulp tissue), plucked from the breast of 42 male and 38 female nestlings at an age of 34 to 55 days (mean ± SD: 46.5 ± 4.5 days), from 27 broods. The feathers were taken within an hour after the nestlings had been captured, directly stored in liquid nitrogen and transferred at −80 °C until gene expression analyses. The extraction of RNA was made following the procedure described in San-Jose et al. (2016).

To obtain the sequences of the different genes, cDNA was prepared by reverse transcribing 1 μg of total RNA, as described in San-Jose et al. (2016). PCR primers were designed using the Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) on conserved regions between *Gallus gallus* and *Taeniopygia guttata* sequences. PCR fragments of the expected length were purified (Minelute Kit, Qiagen, Hombrechtikon Switzerland) and sequenced directly, or TA-cloned (Promega, Dübendorf, Switzerland) and sequenced using Big Dye V 3.1 terminator chemistry on ABI3130XL Genetic Analyzer (Life Technologies, Zug, Switzerland). Parts of the PCR fragments or bacterial clones were sequenced directly by Microsynth (Microsynth, Balgach, Switzerland). Gene sequences were edited and aligned with CodonCode Aligner 3.7.1.2 (CodonCode Corporations).

We sequenced the whole coding sequence of the androgen receptor (*AR*) gene that consists of 8 exons (n = 6 individuals). We found a polymorphism of 3–6 glutamines in the N terminal part of the gene (accession number: KX108735-7). This glutamine polymorphism was further genotyped through fragment length analysis, with genomic DNA extracted from blood samples of 84 individuals, using the DNeasy Tissues kits and the Biosprint Robot 96 (Qiagen, Hombrechtikon Switzerland). The following conditions were used: 500 nM of each primers: AR_60Fw (5′-CGCGGGGCCTT TCAGAGCTT-3′), and AR_293Rev labeled with fluorescein in 5′ (5′-GGCGCAGGCGAAGGGAGAG) with 0.2 U of kappa 2G Robust (Labgene, Châtel-St-Denis, Switzerland), 1× Kapa buffer A, 1× Enhancer, 4% DMSO, 200 μM dNTPs and 10 ng of genomic DNA in 20 μl denatured at 95 °C for 5 min followed by 30 cycles at 95 °C for 30 s, 60 °C 15 s, 72 °C 30 s and then run on the ABI3100 (Life Technologies). The number of glutamines was not related to *AR* gene expression, testosterone level or barn owl plumage traits. For the estrogen receptor (*ESR1*), we found a splice variant with missing exon 4 which may be non-functional, because the protein may not bind steroid with DNA. This splice variant was not further investigated (accession number: KX108738 for the wild-type form and KX108739 for the exon 4 missing form). We also sequenced the 5α-reductase (*SRD5A2*) gene (accession number is KX108740) consisting of 5 coding exons. We did not detect any polymorphisms in this gene. For the aromatase gene (*CYP19A1*), we found one synonymous (c.72A>G) and one non-synonymous mutation (c.1167T>G, N389K) in the 1403 bp of the coding sequence of 9 individuals (accession number: KX108750-2). The non-synonymous mutation was further investigated in the genomic DNA of 80 more individuals using the following primer pairs: CYP19A1_1054Fw (5′-TGCCAAACTTGAAAACCGTGGA-3′) and CYP19A1_1243Rev (5′-ACTCATTCGGCTTTGGGAAGAA-3′), and found not to be related to melanin-based traits. Due to its low expression level in the feathers even after qPCR preamplification, we did not record its expression.

### 2.5. Quantitative PCR

Specific qPCR primers and dual labelled fluorescent probes (FAM-BHQ1, fluorescein with BHQ1 quencher) were designed with the software Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) and DNASTAR PrimerSelect. Each primer and probe was blasted, in order to check its specificity before ordering from Microsynth (Balgach, Switzerland) and Eurogentec (Liège, Belgium). Primer and probe concentrations were tested to get a qPCR efficiency between 95 and 105 % (see Table S1 in Supplementary material). Due to low gene expression in the feather base, we added a preamplification step prior to qPCR assays. For this purpose, we preamplified 10 μL of cDNA for 14 cycles in a thermal cycler (Biometra TProfessional 96) with TaqMan PreAmp Master Mix (Life technologies, Zug, Switzerland). Preamplification uniformity was assessed with the delta-delta Ct values that are deltaCt of the preamplified cDNA – deltaCt of the cDNA (Ct values of the target genes – Ct values of a reference gene). Values were between 0 ± 1.5 as expected. The pre-amplified cDNA was then diluted 10× with TE 1×. The qPCR assays were performed in duplicates as follows: 2 μL of the diluted cDNA was added to 18 μL of master mix containing 1× qPCR MasterMix Plus Low ROX w/o UNG (Eurogentec, Liège, Belgium), primers and probes (for concentration and sequences see Table S1 in Supplementary material), and run on the ABI7500 (Life Technologies) in 96 wells. When the Ct value difference of a duplicate was bigger than 0.25 Ct, the sample was controlled in a supplementary run. In order to correct for inter-plate variation, three pools of different pre-amplified cDNAs were introduced into each plate and used to set up the threshold values. To correct for any variation in cDNA content, CT scores of the candidate genes were normalized using the two reference genes, the ribosome protein L13 (*RPL13*) and the elongation factor 1-alpha 1 (*EEF1α1*) (accession numbers: KU712291 and KU712278, respectively). CT scores were imported into qBasePLUS software 1.3 (Biogazelle) and control genes quality analysed with geNorm software 3.4 (Vandesompele et al., 2002). Mean relative quantities (RQs) for each sample were calculated and further Box-Cox transformed to fulfil the normality assumptions of the parametric statistical tests.

### 2.6. Statistical analyses

To investigate the association between testosterone levels and plumage traits in nestling barn owls (data collected in 2014), we performed a linear mixed effect model (*nlme* and *lme4* packages in R) (Team, 2015) with testosterone (log transformed) as a dependent variable. The full model included spot diameter and pheomelanin-based colouration as independent variables, as well as sex (and its interaction with colour traits), sampling date, time of the day (*i.e.*, hour when blood was sampled) and nestling age. The number of spots was not included in the models as it was not significant in preliminary analyses. Other variables such as "sampling time" (*i.e.* the time lag between the moment nestlings were first disturbed, and blood sampling), rank in the within-brood age hierarchy, brood size, and body condition of nestlings were not considered in the full model, as preliminary analyses showed that these factors were not significantly associated with testosterone (not shown). We modelled the random effect of brood of origin to account for the lack of independence between measurements taken on siblings.

For breeding barn owls, testosterone levels from males and females were analysed separately, given that for females we had repeated measurements within and between years, whereas most males (41 out 42) were sampled only once. With the data collected on males in 2007 and 2014, we carried out a linear model with testosterone level (log transformed) as a dependent variable and spot diameter and pheomelanin-based colouration as independent variables; since there was only one male sampled twice, we did not run a linear mixed model. The full model also included sampling year (and its interaction with plumage traits), age class (i.e. yearlings vs. older individuals), date, time of the day, and "reproductive stage" (i.e. the number of days between the date when we collected blood samples and the date when their first egg had been laid).

A similar model was conducted to analyse testosterone levels (log transformed) in breeding females for samples taken in 2007, 2012 and 2014, but for this dataset, to account for repeated measurements, we performed a linear mixed effect model with female identity as a random factor. The three-way interaction between sampling year, reproductive stage, and plumage traits were not considered in the full model, given that preliminary analyses showed a non-significant association between the interaction and testosterone (not shown).

To investigate the relationship between melanin-based traits and the expression levels of genes related to androgen (5α-reductase [*SRD5α2*] and estrogen alpha [*ERα*]), and androgen receptors [*AR*]), we performed linear mixed effect models with nest of origin as a random factor (because siblings sharing the same nest have been sampled). All three genes were standardized (value – mean divided by the standard deviation), to compare effect sizes, and transformed with a Box-Cox function to fit a normal distribution. These genes were used as dependent variables in separate models. As independent variables, we included the same terms as for the analysis on nestlings' testosterone described above.

For all models, the independent variables were standardized and the final models were simplified by backward selection of all non-significant interactions ($P > 0.1$) and non-significant variables

($P > 0.05$, two-tailed). All the significant variables presented in our models were already significant in the initial models, and all models were verified for normality and heterogeneity. To evaluate the significance of the associations within interactions, we used *simple slopes* tests with the *contrasts* function in the *stats* package (Quinn and Keough, 2002). Finally, to estimate the model fit, we calculated conditional and marginal $R^2$ values by following the method proposed by Nakagawa and Schielzeth (2013).

## 3. Results

### 3.1. Testosterone in relation to melanin-based traits in nestling barn owls

Nestling males had more testosterone than nestling females (estimate ± SE testosterone levels 199.2 ± 1.4 pg/mL and 40.3 ± 1.6 pg/mL, respectively) and testosterone was associated with eumelanic spot diameter in interaction with sex (Table 1). Small-spotted males had more testosterone than large-spotted males (Fig. 1; −0.24 ± 0.10, $t_{74} = -2.31$, $P = 0.024$), whereas in females the relationship with spot diameter was not significantly positive (0.22 ± 0.13, $t_{74} = 1.62$, $P = 0.11$). The origin of the brood explained 70% of the total variance, whereas the spot diameter in males explained only 2.14% of the variation in testosterone levels. Pheomelanin colour was not significantly associated to testosterone in barn owl nestlings, and remained non-significant (*P-values* > 0.7) after excluding "spot diameter" from the model to account for the co-linearity between both traits in males and females (Roulin and Jensen, 2015). Testosterone was not significantly associated with date, time of the day or age (Table 1).

### 3.2. Testosterone in relation to melanin-based traits in breeding barn owls

Testosterone was associated with spot diameter in breeding male barn owls (Table 2). Small-spotted males had more testosterone than large-spotted males (Fig. 1b; −0.49 ± 0.21, $t_{40} = -2.38$, $P = 0.022$). Spot diameter explained 10 % of the total variance of testosterone levels and was still significant when we removed pheomelanin colour from the full model. Pheomelanin colour was not significant alone (Table 2) or when we removed spot diameter from the model (*P*-values > 0.7). Date, time of the day, body mass, age class (yearlings *vs.* adults), or sampling year were not significantly associated with testosterone (Table 2).

In breeding females, testosterone was associated with eumelanin-based spottiness in interaction with the reproductive stage (Table 3). Females had more testosterone at the end than at the beginning of the rearing period ("reproductive stage": 0.39 ± 0.12, $t_{120} = 3.34$, $P = 0.001$), and during offspring care, testosterone circulated at a higher concentration in large- than in small-spotted females (Fig. 3, *simple slopes tests*, 0.78 ± 0.32, $t_{118} = 2.43$, $P = 0.017$), a relationship that did not prevail in females sampled at the end of the incubation stage (*simple slopes tests*, −0.42 ± 0.32 $t_{115.4} = -1.3$, $P = 0.19$). Testosterone levels were higher in 2007 than in 2012 and 2014 (Table 3). The year explained 12.1% of the total variance in testosterone levels, whereas spot diameter and reproductive stage explained 10.6% of the variance. The random factor "identity" explained less than 1% of the total variance. Pheomelanin-based colour was not significantly associated to testosterone alone, or in interaction with spot diameter (*P*-values > 0.25). Date, time of the day, body mass or age class (yearlings *vs.* adults) were not significantly associated with testosterone (Table 3).

### 3.3. Melanin-based traits and expression of 5α-reductase (SRD5α2), estrogen receptor alpha (ERα) and androgen receptor (AR) in nestling feathers

The expression of estrogen receptor alpha (*ERα*) in growing feathers in nestlings was associated with nestling spot diameter in interaction with sex (Table 4). Large-spotted females tended to express *ERα* at higher levels than small-spotted females (0.34 ± 0.17, $t_{50} = 1.97$, $P = 0.054$), whereas in males, there was a tendency for small-spotted males to express *ERα* at a higher level than large-spotted males (−0.24 ± 0.15, $t_{50} = -1.68$, $P = 0.10$). Spot diameter explained 11.7% of the total variance in the expression of *ERα* in females, and 6% in males. Pheomelanin colour was not significant when included in the same model as spot diameter. However, in a separate model without spot diameter, nestling pheomelanin colour was associated with the expression of *ERα* in interaction with nestling sex (lme with Brood ID as random factor, sex: −0.45 ± 0.25, $t_{50} = -1.82$, $P = 0.075$, pheomelanin: −0.04 ± 0.14, $t_{50} = -0.28$, $P = 0.78$, interaction: 0.55 ± 0.27, $t_{50} = 2.07$, $P = 0.044$). Light-reddish females expressed *ERα* at lower levels than dark-reddish females (Fig. 2, *simple slopes tests*, 0.51 ± 0.22, $t_{20} = 2.28$, $P = 0.027$), a relationship that was not significant in males (*simple slopes tests*, −0.04 ± 0.14, $t_{50} = -0.28$, $P = 0.78$). Date, time of the day and nestling age were not associated with the expression of *ERα* (Table 4).

The mRNA expression levels of 5α-reductase (*SRD5α2*), an enzyme that metabolizes testosterone into DHT, were higher in dark than in light reddish individuals, independently of sex (Table 4, Fig. 3). The origin of the brood explained 18.2% of the variation in the expression of 5α-reductase, whereas pheomelanin nestling colour explained 8.2%. Spot diameter, date, time of the day, age and sex were not associated to the expression of *SRD5α2*. Similar models with the expression of androgen receptors (*AR*) revealed no association with melanin-based traits (Table 4). *AR*

**Table 1**
Testosterone levels measured in 2014 in nestling barn owls in relation to their eumelanin and pheomelanin plumage traits. Results from a linear mixed effect model on the testosterone levels (log transformed) of 61 male and 45 female nestlings from 29 different broods for which the identity was added as a random factor. The models were simplified by backward elimination of non-significant two-way interactions ($P > 0.1$) and non-significant variables ($P > 0.05$, two-tailed). Significant variables are highlighted in bold and the terms that have been dropped out of the full model are written in italics.

| Parameters | Estimate† ±S.E | *df* | t-value | *P* |
|---|---|---|---|---|
| *Date of sampling* | *0.36 ± 0.2* | *27.41* | *1.81* | *0.081* |
| *Hour of sampling* | *0.22 ± 0.21* | *26.34* | *1.04* | *0.31* |
| *Nestling age* | *−0.03 ± 0.08* | *80.81* | *−0.38* | *0.71* |
| **Nestling sex*** | **−0.32 ± 0.14** | **78.76** | **−2.42** | **0.018** |
| *Pheomelanin* | *0.09 ± 0.08* | *79.89* | *1.09* | *0.28* |
| **Spot diameter** | **−0.24 ± 0.1** | **83.8** | **−2.31** | **0.023** |
| *Sex* × pheomelanin* | *−0.24 ± 0.21* | *78.7* | *−1.17* | *0.25* |
| **Sex* × spot diameter** | **0.45 ± 0.154** | **77.86** | **2.94** | **0.004** |

† Estimate from standardized data.
* Female-to-male difference estimate.

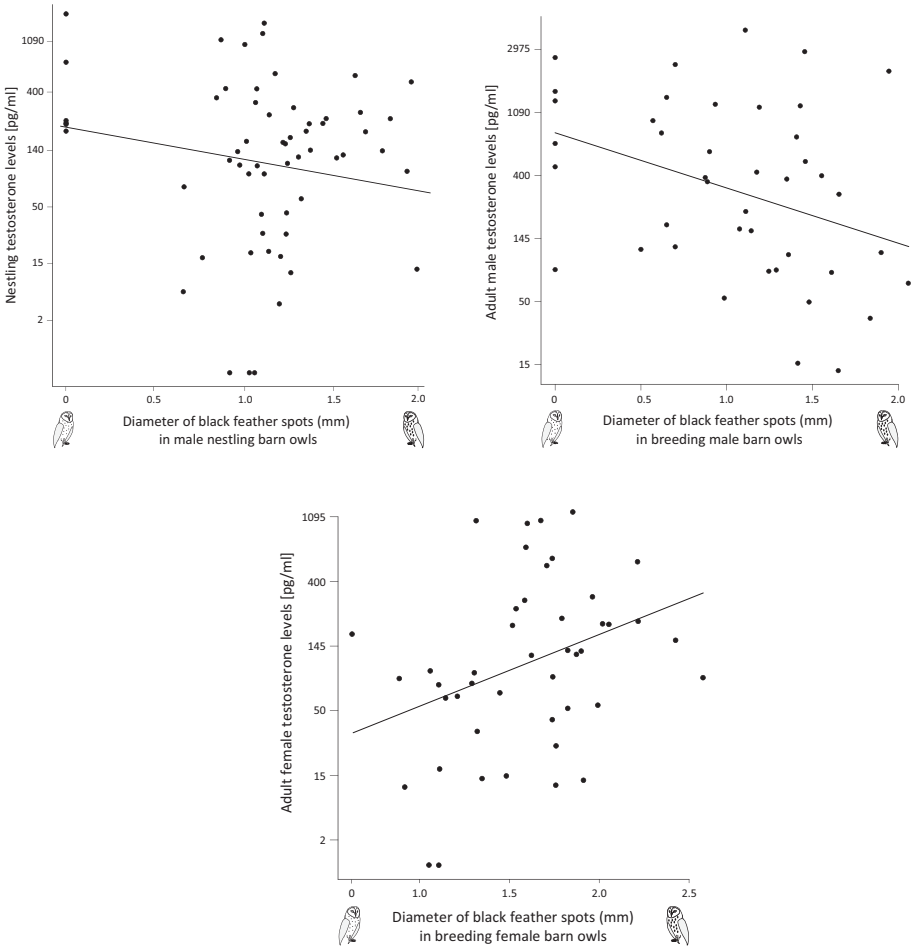


**Fig. 1.** Relationship between circulating testosterone levels and the size of black feather spots in (a) male nestlings, (b) breeding males and (c) breeding female barn owls. The testosterone levels measured in females were sampled while females were taking care of their nestlings. The lines are based on the estimate of the models (Tables 1–3).

**Table 2**
Testosterone levels measured in 2007 and 2014 in breeding male barn owls in relation to eumelanin and pheomelanin traits. Linear models based on the testosterone levels (log transformed) measured in 43 samples taken in 42 different males. The models were simplified by backward elimination of non-significant two-way interactions ($P > 0.1$) and non-significant variables ($P > 0.05$, two-tailed). Significant variables are highlighted in bold and the terms in italics have been dropped out of the full model.

| Parameters | Estimate† ±S.E | *df* | t-value | *P* |
|---|---|---|---|---|
| *Year** | *0.09 ± 0.51* | *31* | *0.17* | *0.87* |
| *Date of sampling* | *−0.05 ± 0.22* | *32* | *−0.23* | *0.82* |
| *Hour of sampling* | *−0.005 ± 0.26* | *30* | *−0.02* | *0.99* |
| *Reproductive stage* | *−0.14 ± 0.23* | *36* | *−0.59* | *0.56* |
| *Body mass* | *−0.15 ± 0.28* | *33* | *−0.54* | *0.59* |
| *Age class* ** | *0.36 ± 0.25* | *39* | *1.48* | *0.15* |
| *Pheomelanin colour* | *−0.08 ± 0.22* | *35* | *−0.38* | *0.71* |
| **Spot diameter** | **−0.49 ± 0.21** | **40** | **−2.38** | **0.022** |
| *Year × pheomelanin colour* | *−0.07 ± 0.54* | *28* | *−0.14* | *0.89* |
| *Year × spot diameter* | *0.38 ± 0.49* | *29* | *0.77* | *0.45* |

† Estimate from standardized data.
* Year 2014 to 2007 difference estimate.
** Yearling-to-adult difference estimate.

was expressed more in females than in males and more in older than in younger nestlings. 20.3% of the total variance in *AR* expression levels was explained by the origin of the brood, 11.7% by age and 7.1% by sex of nestlings. Date and time of the day were not associated to the expression of *AR* (Table 4).

## 4. Discussion

Previous studies in the field of evolutionary biology suggest that testosterone may induce the production of melanin (reviews by Bokony et al., 2008; Kimball, 2006). However, the exact role of testosterone on melanogenesis is unclear. Indeed, even if researchers inject testosterone in animals during the period of melanogenesis, other hormones processed from testosterone (e.g. estrogen, DHT) may causally influence melanin-based colouration. As discussed below, this may explain why although many studies reported positive associations between melanin-based colouration and testosterone (reviews by Bokony et al., 2008; Kimball, 2006), a number of studies reported negative relationships (Atwell et al., 2014; Fargallo et al., 2007; Garamszegi et al., 2004; McGlothlin

**Table 3**
Testosterone levels measured in 2007, 2012 and 2014 in breeding female barn owls in relation to eumelanin and pheomelanin traits. Linear mixed effect models on testosterone levels (log transformed) measured in 126 samples taken in 83 different females and for which the identity was added as a random factor. The models were simplified by backward elimination of non-significant two-way interactions ($P > 0.1$) and non-significant variables ($P > 0.05$, two-tailed). Significant variables are highlighted in bold and the terms in italics have been dropped out of the full model.

| Parameters | Estimate† ±S.E | *df* | t-value | *P* |
|---|---|---|---|---|
| **Year 2012** | **−1.23 ± 0.31** | **120** | **−4.01** | **<0.0001** |
| **Year 2014** | **−1.14 ± 0.31** | **120** | **−3.67** | **<0.001** |
| *Date of sampling* | *0.09 ± 0.12* | *118* | *0.76* | *0.45* |
| *Hour of sampling* | *0.11 ± 0.12* | *115* | *0.91* | *0.36* |
| **Reproductive stage** | **0.39 ± 0.12** | **120** | **3.34** | **0.001** |
| *Body mass* | *−0.03 ± 0.15* | *113* | *−0.2* | *0.84* |
| *Age class** | *−0.32 ± 0.26* | *119* | *−1.26* | *0.21* |
| **Spot diameter** | **0.21 ± 0.119** | **120** | **1.73** | **0.09** |
| *Pheomelanin colour* | 0.07 ± 0.14 | 112 | 0.52 | 0.61 |
| **Reproductive stage × spot diameter** | **0.3 ± 0.13** | **120** | **2.39** | **0.019** |
| *Reproductive stage × pheomelanin* | *−0.09 ± 0.12* | *111* | *−0.76* | *0.45* |

† Estimate from standardized data.
* Yearling-to-adult difference estimate.

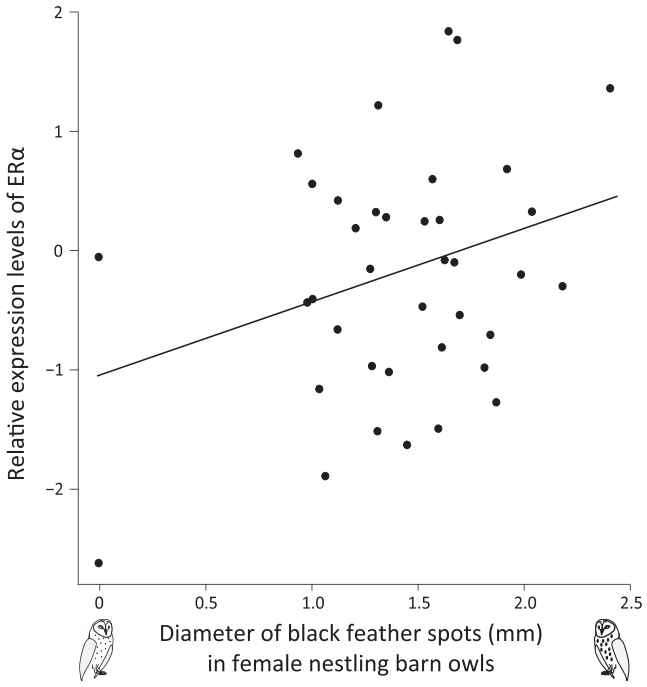


**Fig. 2.** Expression of estrogen receptor alpha (ERα) in relation with the size of black feather spots in female nestling barn owls. The line is based on the estimate of the model (Table 4).

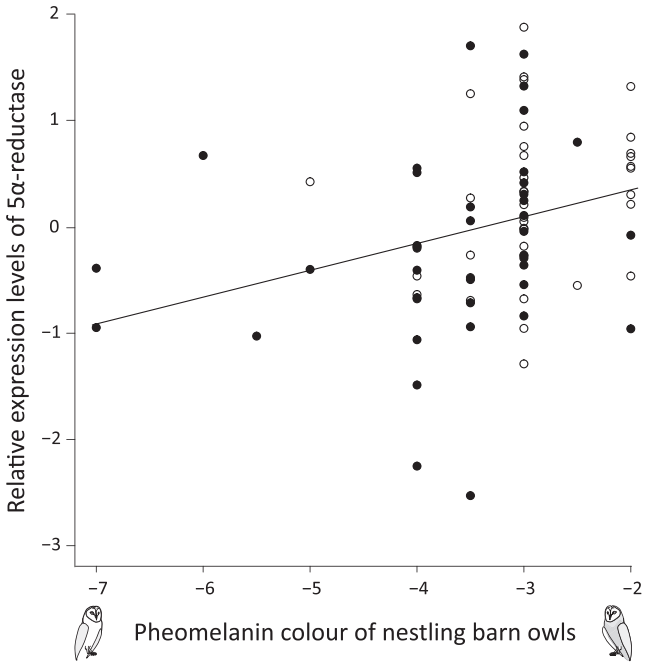


**Fig. 3.** Expression of 5α-reductase in relation to pheomelanin coloration of feathers in nestling barn owls. Black dots represent male nestlings and circles female nestlings. The line is based on the estimate of the model (Table 4).

et al., 2008; Moreno et al., 2014; Siefferman et al., 2013; Spinney et al., 2006; Swett and Breuner, 2009). It is therefore unclear how testosterone should co-vary with melanism, and this statement is particularly true in species where males are less melanic than females. Hence, a key question remains open. Is testosterone related to the production of melanin itself, or to the production of male-specific phenotypes, such as those based on melanin pigments?

### 4.1. Blood circulating testosterone

Our study of the barn owl, a species in which males are less melanic than females, showed that in males, a male-specific phenotype (i.e. small black spots) is associated with higher circulating levels of testosterone in both adults and nestlings (Fig. 1a & b). While breeding females displaying a deep melanic plumage trait (i.e. large black spots which is female-specific) had more testosterone (Fig. 1c) during the offspring rearing period, but not during incubation. Given that large-spotted females tended to express estrogen receptors in feathers to a larger extent than small-spotted females, we speculate that in feathers of darker melanic females, testosterone is metabolized into estrogen to a larger extent. This may explain why the association between spottiness and testosterone levels was contrary in males and females. In other words, small-spotted males that display a typical male-plumage may use more testosterone than large-spotted males, whereas large-spotted females that display a typical female-plumage may have more testosterone than small-spotted females, in order to metabolize it into estrogen. This statement is however speculative because we did not detect aromatase expression in feathers, and the expression of estrogen receptor alpha (*ERα*) was measured in nestlings but not in adults. Although it is unclear how these genes are expressed in adulthood, and at different life-history stages, this hypothesis is worth being considered in future studies.

**Table 4**
Expression of 5α-reductase [*SRD5α2*], androgen [*AR*] and estrogen receptors [*ERα*] in growing feathers in relation to eumelanin and pheomelanin traits in nestling barn owls. Linear mixed effect models on the expression of *SRD5α2, AR* and *ERα* (Box-Cox transformed) measured in 42 males and 38 females from 27 broods for which the brood identity was added as a random factor. The models were simplified by backward elimination of non-significant two-way interactions ($P > 0.1$). Significant variables are highlighted in bold and in italics are the terms that have been dropped out of the full model.

| Parameters | *Expression of 5α-reductase [SRD5A2]* | | | | *Expression of estrogen receptor [ERα]* | | | | *Expression of androgen receptor [AR]* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Estimate* ± S.E | *df* | *t* | *P* | Estimate* ± S.E | *df* | *t* | *P* | Estimate* ± S.E | *df* | *t* | *P* |
| Intercept | *−0.01 ± 0.11* | *50* | *−0.07* | *0.94* | *0.07 ± 0.16* | *45* | *0.43* | *0.67* | *−0.3 ± 0.15* | *51* | *−1.94* | *0.057* |
| Date | *0.002 ± 0.11* | *45* | *0.02* | *0.99* | *−0.03 ± 0.12* | *46* | *−0.24* | *0.81* | *−0.08 ± 0.12* | *49* | *−0.64* | *0.53* |
| Time of the day | *0.12 ± 0.11* | *47* | *1.07* | *0.29* | *0.04 ± 0.11* | *47* | *0.35* | *0.73* | *0.06 ± 0.13* | *48* | *0.48* | *0.63* |
| **Age** | *−0.14 ± 0.1* | *49* | *−1.49* | *0.14* | *−0.1 ± 0.11* | *48* | *−0.86* | *0.40* | **0.34 ± 0.10** | **51** | **3.48** | **0.001** |
| **Sex**** | *0.25 ± 0.2* | *48* | *1.26* | *0.21* | −0.26 ± 0.22 | 50 | −1.19 | 0.24 | **0.53 ± 0.19** | **51** | **2.73** | **0.009** |
| **Pheomelanin** | **0.24 ± 0.09** | **50** | **2.72** | **0.009** | *0.11 ± 0.13* | *49* | *0.87* | *0.39* | *0.07 ± 0.11* | *50* | *0.65* | *0.52* |
| **Spot diameter** | *0.05 ± 0.1* | *46* | *0.51* | 0.61 | **−0.24 ± 0.15** | **50** | **−1.68** | **0.099** | *−0.04 ± 0.12* | *47* | *−0.37* | *0.71* |
| Sex × pheomelanin | *−0.01 ± 0.25* | *43* | *−0.05* | *0.96* | *0.44 ± 0.29* | *45* | *1.49* | *0.14* | *0.12 ± 0.27* | *45* | *0.42* | *0.67* |
| **Sex × spot diameter** | *−0.04 ± 0.19* | *44* | *−0.2* | *0.84* | **0.59 ± 0.23** | **50** | **2.59** | **0.012** | *0.27 ± 0.21* | *46* | *1.3* | *0.20* |

* Estimate from standardized data.
** Female-to-male difference estimate.

The finding that small-spotted male nestlings were more aggressive when handled than large-spotted males (Peleg et al., 2014; van den Brink et al., 2012a) is consistent with the present study, showing that small-spotted nestling and adult male barn owls had higher circulating levels of testosterone than large-spotted male conspecifics (Fig. 1a & b). Therefore, the hypothesis stating that dark melanic individuals should be more aggressive and present higher testosterone levels than their less melanised conspecifics has to be clarified (Bokony et al., 2008; Ducrest et al., 2008; Jawor and Breitwisch, 2003). Indeed, a number of studies found that less melanic individuals can have higher plasma testosterone levels, or be more aggressive than highly melanic individuals (Atwell et al., 2014; Fargallo et al., 2007; Jones, 1990; McGlothlin et al., 2008; Moreno et al., 2014; Siefferman et al., 2013; Spinney et al., 2006; Swett and Breuner, 2009; Tuttle, 2003). For instance, in male collared flycatchers (*Ficedula albicollis*) the white forehead patch size is positively associated to testosterone levels (Garamszegi et al., 2004).

The finding that the association between testosterone and spot size is contrary in males and females suggests that blood-circulating testosterone is independently regulated from the mechanism underling the expression of black feather spots. The finding that in adults testosterone levels varied between years to a large extent, whereas the expression of black spottiness is under strong genetic control and hence static, further suggests that the co-variation between plumage spottiness and testosterone levels is behaviourally, or environmentally mediated, at least in adults. Regardless of whether testosterone has blocking or induction effects on melanogenesis, we propose that regulation of blood-circulating testosterone in the barn owl might not be the result of a causal effect of testosterone on the expression of melanin-based colouration, but rather from pleiotropic genes regulating both melanogenesis and testosterone metabolism (Ducrest et al., 2008), or from melanin-dependent behaviour (Safran et al., 2008). The latest may explain the observed variation in testosterone levels between years, as testosterone levels can rapidly change in relation to environmental factors, including social interactions. A non-mutually exclusive hypothesis for the observed co-variation between melanism and testosterone levels is that the regulation of this hormone depends on the condition or quality of individuals (e.g. Muck and Goymann, 2011), which is related to plumage spottiness in the barn owl. Indeed, females displaying large feather spots regulate energy balance differently from small-spotted females, which explains why they are on average heavier (Almasi and Roulin, 2015; Roulin, 2009), while large-spotted male barn owls tend to be less fit than small-spotted males (Almasi et al., 2013; Roulin et al., 2010). Alternatively, steroid hormones, such as testosterone and estrogen, have both activational and organizational actions (Arnold, 2009; Phoenix et al., 1959) which can have long-term or permanent effects that can influence the expression of secondary sexual traits (Crews, 1998; Moore et al., 1998; Strasser and Schwabl, 2004; Vaillant et al., 2003). For instance, testosterone treatment during early development in house sparrows influences the expression of the breast black badge in males but does not affect female colouration (Strasser and Schwabl, 2004). Thus, differences in testosterone secretion or exposure during critical phases of development could influence, later in life, the expression of the genes involved in the production of melanin pigments in a sex specific manner. This may be mediated by complex interactions between testosterone, its metabolites (estrogen and DHT) and receptors, which could result in reduced pigmentation in males and increased pigmentation in females. Whatever the mechanism accounting for the co-variation between melanin-based colouration and testosterone in the barn owl, melanin-based traits may not have the same signalling value in each sex, at least for those traits regulated by testosterone.

### 4.2. Testosterone metabolism in feathers

In the southern California junco males (*Junco hyemalis thurberi*), testosterone response to gonadotropin-releasing hormone injection was positively associated to the proportion of white colouration on the tail and to the proportion of dark colouration on the head (Atwell et al., 2014). Therefore, in the same species testosterone can be positively correlated with the degree of melanism of one body part, and negatively with respect to another body part. This observation clearly suggests that measuring blood-circulating testosterone gives only a partial idea of the exact impact of testosterone in the tissues where melanin is produced. In order to have a complete picture we need to consider testosterone metabolism at the exact place where melanogenesis is taking place.

Assuming that testosterone regulates the expression of melanin-based traits, as most evolutionary ecologists consider, we would have expected a tight association between melanin-based traits and the receptors to which testosterone binds, or steroid enzymes that metabolize testosterone. If melanin-based colouration is testosterone-dependent, melanogenesis should be more pronounced in feathers that are particularly sensitive to testosterone, i.e. feathers with a larger number of androgen receptors (AR). Alternatively, if metabolites of testosterone causally regulate melanogenesis, we should expect feathers of darker coloured individuals to transform testosterone into new active metabolites at higher rates, i.e. their feathers should show higher levels of 5α-reductase to produce DHT from testosterone, or higher levels

of aromatase to transform testosterone into estrogen. Potential feather sensitivity to testosterone, as estimated by the relative quantity of mRNA for androgen receptors to which testosterone binds, did not co-vary with plumage spottiness. However, female nestlings expressed in their feathers more androgen receptors than males, suggesting that male feather tissues are potentially less sensitive to testosterone, a statement that requires further investigation.

Regarding testosterone metabolism, the expression of the enzyme 5α-reductase (SRD5α2) that transforms testosterone to its more potent metabolite DHT (which binds to the same androgen receptors as testosterone) was not related to plumage spottiness, but to the degree of pheomelanin-based colouration in nestlings (Fig. 3). Individuals with higher levels of 5α-reductase were darker reddish, potentially indicating that the production of reddish pheomelanin pigments is DHT-dependent. Testosterone can also be metabolized into estrogen by aromatase in target cells, and affect the deposition of melanin pigments in feathers (Kimball, 2006). Although aromatase expression levels in growing feathers was on the limit of detection (expression of aromatase gene was still very low after pre-amplification), the expression of *ERα* tended to be higher in large- than small-spotted females. This finding suggests that feather tissues in dark melanic females may be more sensitive to estrogen than in light melanic females. The low levels of aromatase expression suggest that little estrogen is metabolized from testosterone in such tissues, which could indicate that aromatase is a limiting factor compared to ERα. However, during growth, feathers are still well vascularized and thus, we cannot exclude that ERα in such tissues may bind to circulating estrogen rather than to estrogen metabolized from testosterone. Further studies combining sex hormones manipulation and the expression of genes and enzymes involved in the production of melanin traits, may help us to better understand the role of sex hormones in the expression of melanic traits in the barn owls.

## 5. Conclusion

The present study indicates that the relationship between circulating testosterone and melanin-based traits is not necessarily positive but can also be negative. We propose that a key factor to predict the sign of the co-variation between testosterone and colouration depends on which sex is the most melanic. Furthermore, not taking into consideration testosterone metabolism in the tissues where melanogenesis is taking place can only give a partial understanding about the potential role of testosterone on melanogenesis. A further complication is that independently of the causal effect of testosterone (or its metabolites) on the expression of melanin pigments, an association between the regulation of testosterone and colouration can arise from several different mechanisms including pleiotropy, social interactions, and colour-specific variation in body condition. The endocrinological aspect of melanin-based traits requires further detailed studies, if we want to understand the exact adaptive function of these colour traits. This also appears to be important to define the role of androgens in sexually antagonistic selection, with males being selected to be dark melanic (or weakly melanic as in the barn owl) and females to be weakly melanic (or deeply melanic in the barn owl).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2017.04.015.

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# *Chapter 5*

## Double brooding and offspring desertion in the barn owl

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# Double brooding and offspring desertion in the barn owl *Tyto alba*

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Many bird species produce two annual broods during a single breeding season. However, not all individuals reproduce twice in the same year suggesting that double brooding is condition-dependent. In contrast to most raptors and owls, the barn owl *Tyto alba* produces two annual clutches in most worldwide distributed populations. Nevertheless, the determinants of double brooding are still poorly studied. We performed such a study in a Swiss barn owl population monitored between 1990 and 2014. The annual frequency of double brooding varied from 0 to 14% for males and 0 to 59% for females. The likelihood of double brooding was higher when individuals initiated their first clutch early rather than late in the season and when males had few rather than many offspring at the first nest. Despite the reproductive benefits of double brooding (single- and double-brooded individuals produced $3.97 \pm 0.11$ and $7.07 \pm 0.24$ fledglings, respectively), double brooding appears to be traded off against offspring quality because at the first nest double-brooded males produced poorer quality offspring than single-brooded males. This might explain why females desert their first mate to produce a second brood with another male without jeopardizing reproductive success at the first nest. Furthermore, the reproductive cycle being very long in the barn owl (120 d from start of laying to offspring independence), selection may have favoured behaviours that accelerate the initiation of a second annual brood. Accordingly, half of the double-brooded females abandoned their young offspring to look for a new partner in order to initiate the second breeding attempt, 9.48 d earlier than when producing the second brood with the same partner. We conclude that male and female barn owls adopt different reproductive strategies. Females have more opportunities to reproduce twice in a single season than males because mothers are not strictly required during the entire rearing period in contrast to fathers. A high proportion of male floaters may also encourage females to desert their first brood to re-nest with a new male who is free of parental care duties.

Breeding multiple times in the same breeding season is relatively frequent in birds (Geupel and Desante 1990, Alker and Redfern 1996, Fargallo et al. 1996, Jamieson 2011, Jacobs et al. 2013, Tarwater and Beissinger 2013, Carro et al. 2014, Hoffmann et al. 2015). Although breeding twice allows individuals to produce more offspring than single-brooded conspecifics (Geupel and Desante 1990, Nagy and Holmes 2005a, b, Husby et al. 2009, Carro et al. 2014, Hoffmann et al. 2015), not all of them opt to do so because the costs associated with double brooding are substantial. For instance, breeding twice during a single season may depend on experience (age), body condition and past investment in various activities such as body maintenance and reproduction (Geupel and Desante 1990, Verhulst and Hut 1996, Jacobs et al. 2013, Hoffmann et al. 2015). The decision to produce a second annual brood may also depend on the mate's ability to pursue reproductive activities over a longer period of time, and if he or she is not able to reproduce again, this decision will depend on the availability of non-breeders or conspecifics that failed a previous reproductive attempt and are willing to produce a replacement brood. Accordingly, in the Kentish plover *Charadrius alexandrines*, females initiate more often a second clutch than males because the operational sex ratio is biased towards males, offering to females more opportunities to re-mate (Szekely et al. 1999). Because in species such as raptors and owls, the male is essential to feed the progeny, females have more occasions to abandon their brood in the middle of the rearing period to start a second breeding attempt with a new mate as shown in the Tengmalm's owl *Aegolius funereus* (Eldegard and Sonerud 2009, Korpimaki et al. 2011). In other species like the treecreeper *Certhia familiaris*, breeding partners raise their first and second annual clutches together (Kuitunen et al. 1996). There is thus ample intra- and interspecific sex-specific variation in the likelihood of breeding twice in the same season.

Producing a second brood also depends on life history traits such as the duration of the breeding season, nestling development time and timing of the first clutch. Several studies have indeed shown that individuals breeding early in the season have a higher probability of producing a second annual clutch than late breeders (Geupel and Desante 1990, Verhulst and Hut 1996, Eldegard and Sonerud 2009, Jacobs et al. 2013, Carro et al. 2014, Hoffmann et al. 2015). The quality of the breeding territory and the abundance of resources have also a profound impact on the likelihood of breeding twice in the same season (Beissinger 1986, Marks and Perkins 1999, Moore and Morris 2005, Nagy

and Holmes 2005b, Eldegard and Sonerud 2009, 2012, Carro et al. 2014). For instance, female black-throated blue warblers *Dendroica caerulescens* experimentally fed with extra food were more likely to initiate a second brood than unfed females (Nagy and Holmes 2005a). Reproducing twice in the same season may also affect the quality and survival of the offspring at the first nest, as parents will have less time to invest in parental care (Geupel and Desante 1990, Eldegard and Sonerud 2009). Accordingly, parental care at the first annual breeding attempt was shown to be of lower quality in double-brooded than single-brooded parents (Geupel and Desante 1990, Verhulst et al. 1997, Eldegard and Sonerud 2009). This review about the various determinants of producing a second annual brood emphasizes the possibility that reproducing twice in the same year is traded off against self-maintenance and reproductive success at the first brood.

Most species of raptors and owls produce a single annual brood, especially in large species which have long breeding cycles with extended post-fledging parental care (Newton 1979). Double brooding is usually a rare event as observed in the American *Falco sparverius* and European kestrels *Falco tinnuculus* (Stahlecker and Griese 1977, Toland 1985, van Heerden et al. 1994, Fargallo et al. 1996, Steenhof and Peterson 1997), the Tengmalm's owl *Aegolius funereus* (Eldegard and Sonerud 2009, Korpimaki et al. 2011, Eldegard and Sonerud 2012), the burrowing owl *Athene cunicularia* (Millsap 1990) and the long-eared owl *Asio otus* (Marks and Perkins 1999). In these species, producing two annual clutches occurs only in years with exceptional breeding conditions. Frequent double brooding has been reported in only a few raptors, including the pale chanting goshawk *Melierax canorus*, (annual frequency of double brooding ranges from 0 to 30%) (Malan et al. 1997), the crested caracaras *Caracara cheriway* (11–19%) (Morrison 1998), and the black sparrowhawk *Accipiter melanoleucus* (Curtis et al. 2005). The barn owl *Tyto alba* is particularly interesting in this respect because it has a high reproductive potential with individuals being able to produce up to three broods in a single year, each clutch comprising sometimes more than 10 eggs (Stopper 1983, Lenton 1984a, b, Baudvin 1986, Lander et al. 1991, Muller 1991, Andrusiak 1994, Marti 1997, Martinez and Lopez 1999, Debrot et al. 2001, Shawyer 2003, Kniprath and Stier 2008). Furthermore, the barn owl has one of the longest breeding cycle (Curtis et al. 2005) with incubation lasting ca 32 d, nestlings staying ca 60 d in their nest and post-fledging parental care extending to 30 d (Bunn et al. 1982, Courtney and Debus 2006). Even in the largest *Tyto* species such as the masked owl *Tyto novaehollandiae* and sooty owl *Tyto tenebricosa* in which post-fledging parental care can last 3 months, double brooding can occur if food conditions are exceptional (Debus 1994, 1997). Although a number of studies have reported double brooding in different barn owl populations across the world, few of them have evaluated the factors contributing to its occurrence (Baudvin 1986, Marti 1994, Kniprath and Stier 2008). In these studies, second annual clutches were shown to be more frequent in years with abundant food resources and when most breeding pairs laid their first clutch early in the season.

In the present study, our aim is to examine the frequency of double brooding in a Swiss population of barn owls *Tyto alba* between 1990 and 2014, and identify the causes and consequences of double brooding. More specifically, we investigated whether the probability of producing a second annual brood is associated with the timing of laying and reproductive success at the first brood, and with age of the breeders. We examined which factor (age, laying date and reproductive success at the first nest) predicts whether breeders use different sites to produce the first and second annual clutch. Finally, we studied which of these factors predict whether a female changes mate between the first and second annual brood.

## Methods

The data were collected during 25 breeding seasons between 1990 and 2014 in a population of barn owls located in western Switzerland (46°49′N, 06°56′E) in a study area of 1070 km$^2$ at an altitude of 420–730 m. In 1990 and 1991, 110 nest-boxes were fixed to the external wall of barns to progressively reach 134 boxes in 2005; from 2006 to 2014 we fixed new boxes to reach 350 units. Nest-boxes were regularly visited between March and October to record breeding parameters (laying date, clutch size, number of hatchlings and brood size at fledging). We also measured body mass (to the nearest g) and wing length (to the nearest mm) of nestlings at each of our visits. Nestling age was determined soon after hatching (0–25 d) (Roulin 2004), and hence wing length measured at a later stage was sensitive not only to age but body condition. A blood sample was taken to identify nestling sex (Roulin et al. 1999).

Between 1990 and 2014, we recorded 1177 first annual clutches and captured 1169 breeding females and 1030 breeding males. Out of 145 recorded second annual clutches, we captured 144 breeding females and 78 of the breeding males; for 127 double brooding females we could identify the identity of their male at the first and second annual broods. From the 18 cases where we captured the breeding male at only the first or second clutch, we could deduce that the same male produced the two successive annual clutches in three cases because the female stayed in the same nest-box to produce her second annual clutch and because the second clutch was laid long before the first offspring were independent from their father. In 5 cases, we deduced that it was a different male because the distance between breeding sites of the first and second annual clutches were too far (more than 3.5 km) for the father to assume the two nests simultaneously. All females were captured while they were incubating the eggs and males were either captured at the same time as the females or later when feeding their offspring. Of the 1997 breeding adults, 969 had been ringed as nestlings and hence their age was known with precision; the age of 1028 other individuals ringed as adult was estimated from the moult pattern (Taylor 1993). We classified birds in the age class 'yearling' (i.e. individuals in their first-year of life) and if older as 'adult'. An individual was considered as 'double-brooder' if it successfully produced a first brood (i.e. at least one nestling fledged) and a second clutch in the same or different site. The term 'site' refers to the barn where a pair produced a clutch. Some barns have two nest-boxes, which enables females to produce a second annual clutch in the same site. The 236 individuals that failed to produce

any fledglings and laid a replacement clutch were not considered in the present study. Forty-one polygamous males who were rearing two broods simultaneously were considered as polygamous but not as double-brooders, and hence they are not considered here.

### Statistical procedure

The analyses were performed with R Studio (ver. 0.98.501) (R Core Team 2004) and the libraries lme4 (mixed models), sim and arm (to compute confidence intervals) and pbkrtest (model selection). We ran generalized linear mixed models (GLMMs, function glmer) when the data followed binomial and Poisson distributions, and linear mixed models (LMERs, function lmer) for normally distributed variables. Because a number of individuals bred in more than one year, we implemented individual identity and year as random variables to avoid pseudo-replication. We analysed nestling body mass and fledging success in order to investigate the potential fitness effects of double brooding. We ran linear mixed models (function lmer, package lme4) to test whether offspring body mass differed between single- and double-brooded parents. We first examined whether nestling body mass (measured between 0 and 32 d of age) predicted whether parents produced a second brood later on; 32 d is before mothers start to desert their brood to produce a second brood with another partner (Roulin 2002). This analysis is therefore useful to examine whether parents are more likely to produce a second brood if rearing conditions are good (i.e. if their offspring are heavy rather than light). In a second model, we specifically investigated whether producing a second annual clutch negatively affected nestling body mass at the first annual nest. For this model, we considered body mass measured between 45 and 60 d of age, which corresponds to the period when the mother can already abandon the nest to produce a second clutch. These models were performed for male and female parents separately because the probability of producing a second brood differs between males and females. For all analyses, the initial full models included nestling age in days, age$^2$, age$^3$, wing length (mm), time of the day (hour), laying date, brood size, rank of nestling in the within-brood age hierarchy and the number of annual clutches (i.e. 1st or 2nd brood). To account for repeated-measurements of nestling body mass as well as for repeated reproductive events of the parents within or across years, we included nestling and parent identities as random factors. Brood identity and year were also included as random factors.

To evaluate whether the total annual breeding success (i.e. number of fledglings at the first and second nests) is related to the number of clutches produced in a year, we ran a GLMER with a binomial error structure and logit link function (function glmer, package lme4); we introduced the total number of fledglings who survived and died as a dichotomous variable.

All models were simplified using a backward selection procedure where each model is compared against a simpler nested model. We simulated 200 times a set of response values from the null model and calculated the likelihood ratio between the alternative and null model for each response values (function PBmodcomp, package pbkrtest). From these likelihood ratios we estimated a p-value for each response value with the bootstrap method (Faraway 2006) and used them to reduce the model until it contained only significant variables ($p \leq 0.05$, two tails). Likewise, when the random variables did not explain any significant part of the variation, we removed them from the model to improve its convergence. Assumptions of all statistical tests were verified. The estimates reported in the results section are extracted from the models. For logistic reasons, the number of individuals can vary between analyses for instance because we did not record all reproductive parameters in all individuals. Means are quoted ± SE.

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.9sk18> (Béziers and Roulin 2015).

## Results

### Frequency of second annual broods

Between 1990 and 2014, we monitored 1006 males (487 different individuals) producing a first annual brood of which 888 (440 different individuals) raised at least one fledgling (88%). Of these 888 males, 78 (66 different individuals) produced a second annual brood (8%). Concerning females, we monitored 1131 first annual broods (664 different individuals) of which 950 (572 different individuals) produced at least one fledgling (84%). Of these 950 females, 145 (115 different individuals) produced a second annual brood (13%), a proportion that was significantly higher than in males (chi-squared test: $\chi^2_1 = 14.20$, $p < 0.0002$). Up to 59% of females and 38% of males produced two broods in the same year (Table 1) with some individuals double brooding more than once during the 25 year-long study period: 92 females and 54 males double brooded only once, 20 females and 12 males twice and 3 females three times. The number of first clutches produced in a year was not associated with the proportion of second annual clutches (GLM: $Z = -0.20$, $p = 0.84$); the model was corrected for the year (fixed factor) as the number of nestboxes increased during the study period.

### Factors associated with the probability of producing a second annual clutch

Males were more likely to produce a second annual clutch if their first brood had been laid early than late in the season (laying date at first annual breeding attempt of single- and double-brooded males is 24 April ± 4.1 d and 24 March ± 5.8 d, respectively; GLMER: $LR_1 = 53.23$, $p_{boot} = 0.005$) and if the number of fledglings at this first breeding attempt was low ($LR_1 = 12.71$, $p_{boot} = 0.005$, number of fledglings at first annual breeding attempt of single- and double-brooded males is 4.15 ± 0.07 and 3.75 ± 0.23, respectively); age class (adult vs yearling) did not predict whether a male produced a second annual clutch ($LR_1 = 1.27$, $p_{boot} = 0.25$). In a similar model, where we replaced the number of fledglings by clutch size at the first breeding attempt, clutch size did not predict whether males produced a second annual brood ($LR_1 = 2.28$, $p_{boot} = 0.13$). Males who initiated a second annual clutch in a different breeding site than the one of

Table 1. Frequency of first and second annual clutches in a Swiss barn owl population.

| | Number of 1st annual clutches | | Number of 2nd annual clutches | | Number of individuals producing a 2nd annual clutch with another partner than at the 1st annual clutch | |
|---|---|---|---|---|---|---|
| Year | Females | Males | Females | Males | Females | Males |
| 1990 | 39 | 8 | 2 (5%) | ? | ? | ? |
| 1991 | 26 | 8 | 1 (4%) | ? | ? | ? |
| 1992 | 25 | 23 | 0 | 0 | – | – |
| 1993 | 43 | 42 | 12 (28%) | 9 (21%) | 4 (33%) | 1 (11%) |
| 1994 | 57 | 54 | 3 (5%) | 4 (7%) | 0 | 1 (25%) |
| 1995 | 58 | 58 | 7 (12%) | 3 (5%) | 4 (57%) | 1 (33%) |
| 1996 | 67 | 61 | 9 (13%) | 4 (7%) | 6 (67%) | 0 |
| 1997 | 34 | 34 | 2 (6%) | 1 (3%) | 1 (50%) | 0 |
| 1998 | 44 | 40 | 6 (14%) | 4 (10%) | 3 (50%) | 1 (25%) |
| 1999 | 41 | 35 | 0 | 0 | 0 | 0 |
| 2000 | 54 | 45 | 1 (2%) | 1 (2%) | 1 (100%) | 1 (100%) |
| 2001 | 52 | 45 | 1 (2%) | 0 | 1 (100%) | 0 |
| 2002 | 77 | 65 | 1 (1%) | 0 | 0 | 0 |
| 2003 | 50 | 45 | 0 | 0 | 0 | 0 |
| 2004 | 30 | 40 | 13 (43%) | 3 (8%) | 10 (77%) | ? |
| 2005 | 47 | 51 | 9 (19%) | 2 (4%) | 7 (77%) | 1 (50%) |
| 2006 | 30 | 23 | 0 | 0 | 0 | 0 |
| 2007 | 59 | 60 | 21 (36%) | 10 (17%) | 14 (67%) | 4 (40%) |
| 2008 | 76 | 73 | 0 | 0 | 0 | 0 |
| 2009 | 19 | 18 | 2 (11%) | 1 (6%) | 1 (50%) | ? |
| 2010 | 35 | 36 | 10 (29%) | 6 (17%) | 3 (30%) | 1 (17%) |
| 2011 | 40 | 34 | 14 (35%) | 13 (38%) | 4 (29%) | 3 (23%) |
| 2012 | 95 | 74 | 18 (19%) | 14 (19%) | 7 (39%) | 4 (29%) |
| 2013 | 11 | 9 | 0 | 0 | 0 | 0 |
| 2014 | 22 | 25 | 13 (59%) | 3 (8%) | 8 (62%) | 1 (50%) |
| **Total** | **1131** | **1006** | **145 (13%)** | **78 (8%)** | **70 (48%)** | **18 (23%)** |

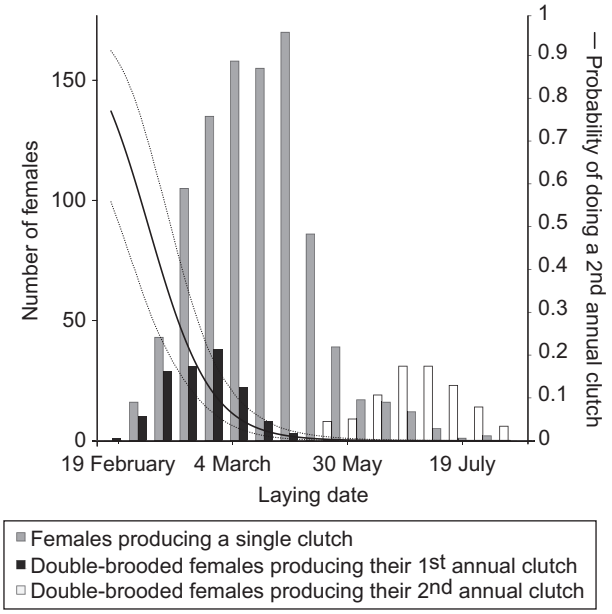


Figure 1. Distribution of laying dates of female barn owls producing a single clutch and of laying dates of the first and second clutch of double-brooded females. The line represents the likelihood of double brooding in relation to laying date of first clutches. The dashed lines represent the 95% confidence intervals.

the first brood initiated their second annual clutch earlier (85.0 ± 2.3 d between laying date of first and second annual clutch; range: 44 to 117 d; LMER: $LR_1 = 7.1$, $p_{boot} = 0.01$) than males who stayed in the same breeding site (99.5 ± 4.2 d; range: 54 to 139 d). The time interval between laying the two successive annual clutches was not related to laying date at the first breeding attempt ($LR_1 = 3.08$, $p_{boot} = 0.10$), male age ($LR_1 = 0.45$, $p_{boot} = 0.49$), the distance between the sites where the two clutches were deposited ($LR_1 = 1.25$, $p_{boot} = 0.30$) and whether males changed partner between first and second annual clutch ($LR_1 = 2.53$, $p_{boot} = 0.12$).

We performed a similar model for females because 50% of them changed mate to produce a second annual breeding attempt, implying that potentially different factors could predict double brooding in males and females. The probability that females produced a second annual clutch increased if the first clutch had been produced early than late in the season (Fig. 1; laying date at first annual breeding attempt of single- and double-brooded females is 22 April ± 4.5 d and 28 March ± 3.1 d, respectively; $LR_1 = 69.01$, $p_{boot} = 0.005$); number of fledglings, clutch size and age class of females were not significant ($p_{boot} > 0.10$).

Body mass measured between 0 and 32 d of age in nestlings at the first breeding attempt was not related to whether their mother or father later produced a second annual clutch (GLMER, male: n = 9041 measurements of body mass in 4285 nestlings, $LR_1 = 0.87$, $p_{boot} = 0.30$; female: n = 9289 measurements of body mass in 4650 nestlings, $LR_1 = 0.91$, $p_{boot} = 0.33$).

## Probability of changing site to produce a second annual clutch

Of the males, 38% (30 out of 78, Fig. 2) produced their second annual clutch in the same site as the one where they produced their first annual clutch while only 20% of females (29 out of 145) (chi-square test: $\chi^2_1 = 4.97$, $p = 0.026$). When considering only the individuals who produced two annual broods in the same site, 27% of the males (8 of 30) changed mate but none of the 29 females ($\chi^2_1 = 6.93$, $p = 0.008$). Among the individuals who changed site to produce their second annual brood, 60% of the females (70 out of 116) changed mate but only 21% of the males (10 out of 48) ($\chi^2_1 = 8.34$, $p = 0.0038$).

The probability that a female changed breeding site between the first and second annual clutches (29 females stayed in the same breeding site and 116 changed breeding site) was neither related to her age (GLMER: $LR_1 = 3.33$, $p_{boot} = 0.11$), laying date ($LR_1 = 0.30$, $p_{boot} = 0.60$) and the number of fledglings at the first annual breeding attempt ($LR_1 = 0.62$, $p_{boot} = 0.53$). Although females who changed site laid their second clutch significantly earlier than females who stayed in the same breeding site (24 June ± 2.4 d vs 8 July ± 3.8 d; GLMER: $LR_1 = 11.96$, $p = 0.002$), these two groups of females produced a clutch and brood of similar sizes at the first and second breeding attempts ($LR_1 = 0.11$, $p_{boot} = 0.88$ and $LR_1 = 0.64$, $p_{boot} = 0.42$, respectively).

Females who changed partner and breeding site to produce their second annual brood travelled longer distances than females who changed breeding site but stayed faithful to their first partner (4.6 ± 0.5 km (range: 0.9 to 29.1 km) vs 1.2 ± 0.1 km (range: 0.2 to 3.0 km); LMER with log transformed distance between the two sites: interaction 'sex by faithfulness' $LR_1 = 13.35$, $p_{boot} = 0.005$). Unlike females,

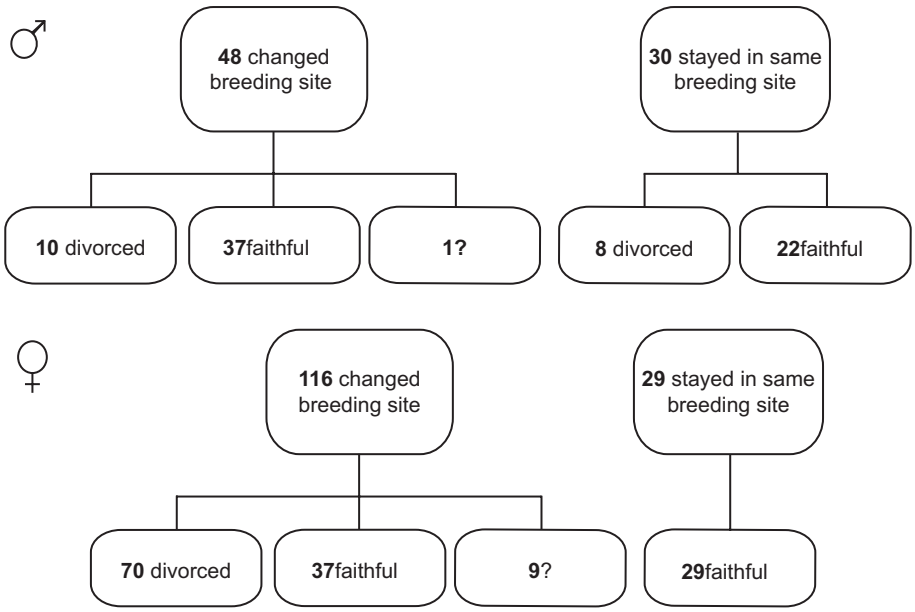


Figure 2. Flow-chart representing the number of females and males who stayed or changed breeding site and those who were faithful or who divorced between first and second annual clutch.

the 10 males who changed partner and breeding site did not move longer distances than the 37 males who changed site but remained faithful to their female partner (1.5 ± 0.3 km (range: 0.1 to 3.3 km) vs 1.1 ± 0.2 km (0.2 to 2.7 km).

## Partner at the second breeding attempt

Among 136 double-brooded females for which we knew the identity of their mate at the first and second breeding attempts, 66 produced their two annual broods with the same partner and 70 had different partners. A female was more likely to change partner if the first clutch had been laid relatively late than early in the season (Fig. 3; GLMER: $LR_1 = 5.26$, $p_{boot} = 0.03$; laying date at first annual breeding attempt of females who were faithful or changed partner is respectively 24 March ± 2 d and 31 March ± 2.4) and if brood size at the first nest was large rather than small (Fig. 4; $LR_1 = 5.68$, $p_{boot} = 0.03$; brood size at first annual breeding attempt of females who were faithful or changed partner is 3.80 ± 0.22 and 4.54 ± 0.21). Age of the first male did not predict whether females divorced to produce a second annual breeding attempt ($LR_1 = 0.0002$, $p_{boot} = 0.99$).

At the first nest, females who stayed with the same partner to produce a second brood were more often paired with an adult than a yearling (50 out of 60 females were paired with an adult male, 83%; for 6 females age of their partner was not known) while the male partner at the second annual brood of females who changed partner was a yearling in 50% of the cases (30 out of 60 females were paired with a yearling male; GLMER: $LR_1 = 15.76$, $p_{boot} = 0.005$). In most cases the new mate of double-brooded females was producing his first annual brood in the study area (46 out of 60, 77%; $\chi^2_1 = 17.06$, $p < 0.0001$), in 11 cases this was his 2nd annual brood, in 2 cases a replacement clutch and in one case no information was available. Because few males changed partner to produce their second annual brood (18 out of 78, 23%), similar analyses were not possible.

The number of days separating the laying of the two annual clutches tended to be higher in faithful females who produced larger first broods compared to faithful females who had small broods ($LR_1 = 3.13$, $p_{boot} = 0.065$), while there was no such a difference in females who divorced (average time interval between 1st and 2nd clutch of females who were faithful or divorced was 94.52 ± 2.04 and

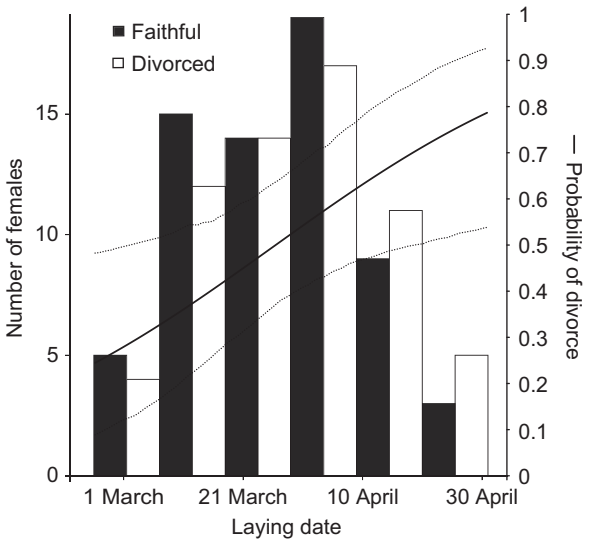


Figure 3. Distribution of laying dates of female barn owls who were faithful and those who were divorced between the first and second annual breeding attempts. The line represents the likelihood of changing partner to produce a second annual clutch in relation to laying date of first clutches. The dashed lines represent the 95% confidence intervals.

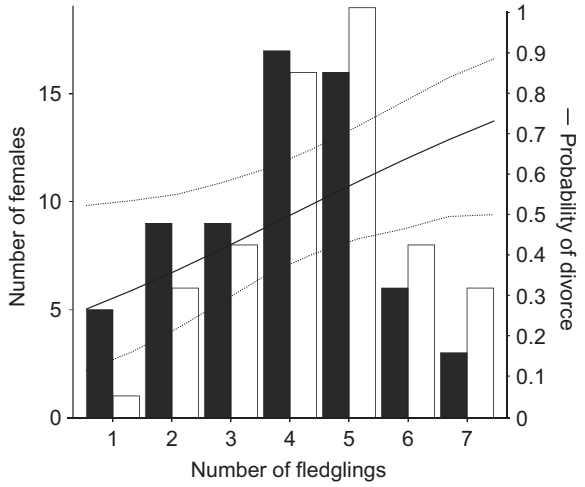


Figure 4. Distribution of the number of fledglings at first annual clutch of female barn owls who were faithful and those who divorced between the first and second annual breeding attempts. The line represents the likelihood of changing partner to produce a second annual clutch in relation to the number of fledglings at the first nest. The dashed lines represent the 95% confidence intervals.

85.04 ± 1.69 d, respectively). In females who changed mate, the number of days between laying the first and second annual clutches was significantly lower when females bred late in the season rather than early in the season. In contrast, in faithful females the time interval between laying dates of the first and second annual clutches was not affected by laying date of their first clutch (Fig. 5; GLMER with log-transformed number of days between the two annual clutches, n = 127, interaction between 'laying date of 1st clutch by divorce': $LR_1 = 6.47$, $p_{boot} = 0.02$).

At the first nest, nestling body mass measured between 0 to 32 d of age (i.e. before their mother laid the second annual clutch) was not different whether their mother stayed with her partner or divorced to produce the second annual clutch (GLMER: n = 66, $LR_1 = 1.2$, $p_{boot} = 0.35$).

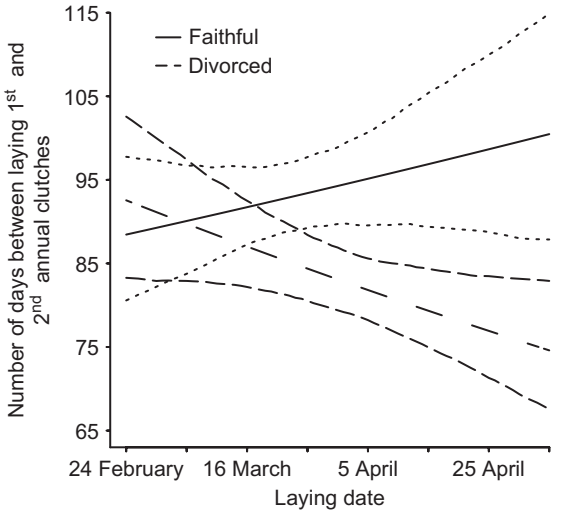


Figure 5. Number of days between first and second clutches in relation to laying date in females who were faithful and in females who divorced between the first and second annual breeding attempts.

## Potential fitness effects of double brooding

The total number of offspring produced in the same year was higher in double-brooded than in single-brooded females (7.34 ± 0.23 (range: 1–15 fledglings) and 3.92 ± 0.12 (0–9 fledglings), respectively; (LMER: n = 923, $LR_1 = 202.58$, $p_{boot} = 0.005$). Similar results apply to double-brooded males (6.79 ± 0.25 fledglings) compared to single-brooded males (4.06 ± 0.11 fledglings) (LMER: n = 850, $LR_1 = 144.53$, $p_{boot} = 0.005$). In these analyses, we statistically controlled for laying date of the first annual clutch and age (yearling vs adult).

When considering only second annual clutches, clutch size decreased along the season (LMER with identity of female and year as random variables: n = 103, $LR_1 = 12.31$, $p_{boot} = 0.005$). The size of the second annual clutch was positively associated to the size of the first clutch ($LR_1 = 18.2$, $p_{boot} = 0.005$) but did not differ between adult and yearling females ($LR_1 = 0.28$, $p_{boot} = 0.70$) or between adult and yearling males ($LR_1 = 0.70$, $p_{boot} = 0.55$). Furthermore, second annual clutches were larger than first annual clutches after controlling for laying date of the first clutch ($LR_1 = 7.3$, $p_{boot} = 0.01$, 6.0 ± 0.2 eggs (range: 4 to 12 eggs) and 5.43 ± 0.24 eggs (range: 2 to 12 eggs), respectively). Females who produced a second annual clutch produced a larger first clutch the following year compared to individuals who produced a single annual clutch the year before (LMER: n = 377, $LR_1 = 8.11$, $p_{boot} = 0.01$, 6.28 ± 0.19 and 5.88 ± 0.08 eggs). Adult females compared to yearling females (GLMER: n = 1050, $LR_1 = 5.75$, $p_{boot} = 0.02$) and females who produced two annual clutches compared to a single annual clutch ($LR_1 = 9.29$, $p_{boot} = 0.004$) had a higher probability of being recaptured as a breeder the following year. In males, adults were more often recaptured as breeders the following year than yearlings (GLMER: n = 955, $LR_1 = 12$, $p_{boot} = 0.002$), whereas the number of clutches produced in year X did not predict the probability of being recaptured in year X + 1 (GLMER: n = 955, $LR_1 = 0.13$, $p_{boot} = 0.72$).

Breeding success at the second annual reproductive attempt was higher when the second clutch had been laid earlier in the season (GLMER: n = 93, $\chi^2_1 = 10.21$, p = 0.01). In the same model, breeding success was not associated with female age ($\chi^2_1 = 0.36$, p = 0.6) or male age ($\chi^2_1 = 2.32$, p = 0.23). Similarly, changing site between the first and second breeding attempts ($\chi^2_1 = 1.6$, p = 0.23), the time interval between laying the first and second annual clutches ($\chi^2_1 = 2.1$, p = 0.21), the number of fledglings at the first clutch ($\chi^2_1 = 0.51$, p = 0.54) or changing partner between first and second annual clutches ($\chi^2_1 = 0.28$, p = 0.72) did not predict breeding success at the second reproductive attempt. However, at the first nest nestlings were lighter in body mass between 45 and 60 d of age if their father produced a second brood in the same year (Fig. 6; GLMER, 45–60 d: n = 3106 measurements of nestling body mass, $LR_1 = 5.71$, $p_{boot} = 0.02$); in a similar model, nestling body mass was not related to whether their mother produced a second annual clutch (n = 3139, $LR_1 = 0.05$, $p_{boot} = 0.8$).

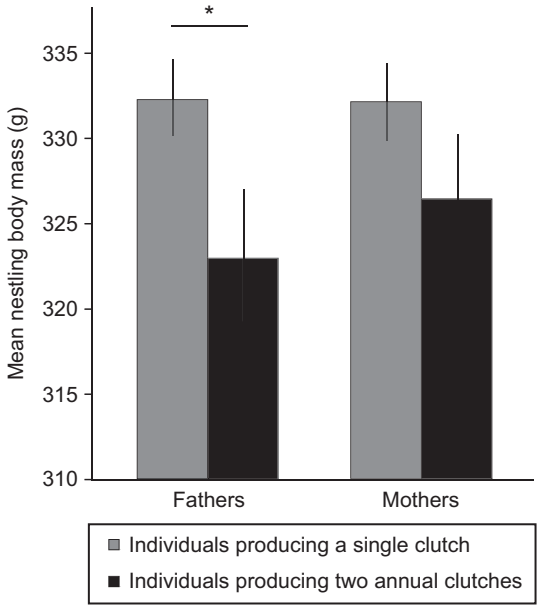


Figure 6. Mean body mass of nestling measured between 45–60 d old in relation to fathers and mothers who produce one or two annual clutches. The bars represent standard error.

Producing a second annual clutch did not predict the breeding success of females the following year (GLMER: $n = 367$, $\chi^2_1 = 0.22$, $p = 0.64$) nor did female age ($\chi^2_1 = 0.38$, $p = 0.52$) or laying date ($\chi^2_1 = 0.2$, $p = 0.65$). In a similar model for males, these factors were not related to the breeding success the following year (number of annual clutches: $\chi^2_1 = 2.04$, $p = 0.16$; male age: $\chi^2_1 = 1.8$, $p = 0.21$).

## Discussion

### Determinants of double brooding

Double brooding was common in our population of Barn owls and varied between years and sexes with on average 13% of the females and 8% of the males producing a second brood (Table 1). The likelihood of producing a second annual clutch was higher if the first clutch had been laid early rather than late in the season (Fig. 1) and if males had few offspring at their first annual clutch. The effect of an early initiation of reproductive activities on the probability of producing a second annual brood is in line with previous studies in various species (Beissinger 1986, Geupel and Desante 1990, Ogden and Stutchbury 1996, Monroe et al. 2008, Townsend et al. 2013, Carro et al. 2014, Hoffmann et al. 2015) including the barn owl (Baudvin 1986, Marti 1994).

Variation in the propensity to breed twice in the same year could be explained by individual-specific life history strategies, with individuals differentially trading self-maintenance against the number of reproduction events (Saino et al. 1999). An alternative hypothesis is that the production of a second annual brood is related to body condition (Jacobs et al. 2013, Hoffmann et al. 2015), hunting ability or territory quality (Nagy and Holmes 2005a). Mainly individuals in prime condition may produce a second annual brood or those breeding in high quality territories, and they may show a high survival prospect because they are in good condition rather than because they produce a second annual brood. Because this result was verified for females but not for males, we propose that individual quality (in females) rather than territory quality explains why double-brooders have a higher survival prospect.

The fact that in females brood size did not predict double brooding but divorce suggests that the male presence is mandatory to complete parental duties up to offspring independence. If brood size is large, the father has to invest substantial effort to feed his offspring giving him little time to invest in the preparation of a second nest (e.g. copulation, courtship feeding). This may induce the female to search for another male who is free of parental care duties as shown by our data: 77% of the new partners of deserting females were not observed breeding in the study area the same year and 3% of them had failed their first annual clutch. Deserting females will therefore save time by quickly producing their second brood instead of waiting for their first male to become free from paternal duties to initiate a second breeding attempt with him. This is supported by the fact that deserting females double-brooded earlier than faithful females. Alternatively, because double brooding males produced poorer quality offspring at the first nest, females may desert their first brood to prevent males from double brooding and impairing the quality of their offspring.

As the success of second broods decreases along the season, females should desert early in the season but only if their first mate can assume parental care duties alone. However, this may not be the only reason as females having an early first clutch deserted their offspring at an older age than late breeders. If deserting young offspring may accelerate the production of a second brood, this behaviour may negatively affect the last-born offspring who still require the presence of their mother. Therefore, deserting young offspring may be an option only late in the season because fledging success at the second annual breeding attempt decreases along the season. Another non-mutually exclusive explanation is that rearing the first brood may be more costly early than late in the season and for this reason females may desert their offspring at an older age early in the season in order to assist their mate for longer. The fact that females who divorced re-nested farther from their first brood compared to faithful females who stayed in the proximity of their first brood suggests that they were no longer contributing to parental care at the first nest.

### Cost and benefits of double brooding

Similarly to the Tengmalm's owl (Korpimaki et al. 2011), double-brooded females produced on average more fledglings (7.34) per year than individuals producing a single annual brood (3.92 fledglings). Because brood size at the first nest did not predict double brooding in females, we conclude that in females the adaptive function of double brooding is not to compensate a low reproductive success at the first annual breeding attempt but rather to increase the overall reproductive success, if environmental conditions are sufficiently good to allow the production of a second annual brood. Double-brooded males produced also more offspring (6.79) than single brooded males (4.06). However, the fact

that males with fewer offspring at their first brood had a higher propensity to produce a second brood could also suggest that males compensate a low reproductive success at the first breeding attempt with a second annual brood, implying that they have still enough energy to breed again. Alternatively, producing a second annual brood may impair the survival of fledglings at the first annual clutch (Eldegard and Sonerud 2009). As mentioned above, however, another explanation is that males, for whom the first brood is large, do not have the time to start a second breeding attempt. Accordingly, the negative impact of brood size on double brooding has already been reported in other bird species (Verboven and Verhulst 1996, Nagy and Holmes 2005a, Parejo and Danchin 2006, Eldegard and Sonerud 2009).

At the first nest, nestling body condition decreased if their father, but not mother, produced a second annual clutch. This further emphasises the claim that the female presence is often not mandatory to complete parental care duties in contrast to the male presence (Eldegard and Sonerud 2012). Thus, the cost of paternal double brooding is in part paid by the offspring, as shown in the snow bunting *Plectrophenax nivalis* (Smith and Marquiss 1995), whereas the cost of maternal double brooding may be paid by the male who has to pursue rearing activities alone. Nevertheless, double brooding did not appear to translate into a reduction in adult survival, since double brooding in a given year did not impair reproductive success or the probability of reproducing the following year, which contrasts with a study in the spotted owl *Strix occidentalis occidentalis* (Stoelting et al. 2015). We are aware that in correlative studies such as in our case, it might be difficult to identify the costs of double brooding paid by the parents as shown experimentally in the great tit *Parus major* (Verhulst 1998). Accordingly, in the barn owl we found that double-brooded females produced larger clutches the following year than single-brooded females and they were more often recaptured. This may indicate that high quality individuals are more likely to produce two broods than poor quality conspecifics.

### Conclusion

Double brooding is an adaptive strategy as double-brooded barn owls produced in total more fledglings than single-brooded conspecifics. Although the timing of laying the first clutch predicted double brooding in both males and females, each sex adopts different reproductive strategies. Females should pair with high quality males to produce large first broods and ensure that their partner feeds the progeny at a high rate. Even if producing many offspring at the first nest may force males to invest in parental care rather than in the production of a second annual brood, females have still the possibility to desert their first nest to pair with a new partner. Because the success of second broods decreases throughout the season, females should desert early in the season but only if their first mate can assume parental care duties alone. If the female strategy to produce two annual broods is clearly advantageous, in males the situation is more complex. Males who raise a small number of offspring at the first breeding attempt are more likely to produce a second annual brood and have in total more offspring than single-brooded males. However, because double-brooded males produce lower quality of offspring at the first nest raises the question of whether males should better produce two annual brood or a single large brood.

With its ability to produce several large clutches in a single breeding season, the barn owl adopts a reproductive strategy similar to passerines rather than the conventional reproductive strategy observed in most raptors and owls (Newton 1979). In temperature regions, it may allow the barn owl to compensate for frequent population crashes due to pronounced variation in food supply and harsh winters (Henny 1969, Marti and Wagner 1985, Muller 1991, Altwegg et al. 2006). Double brooding in the barn owl is also common at low latitudes (Muller 1991) where environmental conditions are more stable, and hence where fluctuations in population sizes may be less pronounced than in temperate regions. The factors that determine the propensity to produce multiple broods in a single season may therefore differ between populations emphasizing the need of studying the reproductive biology of this bird at different latitudes. A comparative study across bird species may also be useful to determine the relative importance of ecological, behavioural and life history traits in the determinism of double brooding.

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## General discussion

In the present thesis, I investigated different aspects associated with the endocrinological system of the barn owl, including the degree of heritable variation (heritability) underling the regulation of baseline and stress-induced corticosterone secretion by the HPA axis and the association of baseline and stress-induced corticosterone levels to survival in adult barn owls. I also explored the link between steroid hormones and melanin-based traits and their potential implication in the expression of melanin-based colour ornaments in the barn owl. Hormones are ubiquitous in the body of an organism where they target a variety of cells and tissues. Therefore, hormones have a crucial and large impact in many facets of the phenotype of an individual, including its behaviour, morphology, physiology and life history (Phoenix et al., 1959, Landys et al., 2006, Zera et al., 2007, Norris, 2006, Hau et al., 2010, Ricklefs & Wikelski, 2002). Their relevance is evidenced by the fact that, in certain species, hormone profiles are signalled by ornamental colour traits (e.g., Saino et al., 2013, Jawor & Winters, 2010, Bokony et al., 2008, Almasi et al., 2010, Moreno et al., 2014), being directly implicated in the expression of these colour traits in some species (reviewed in Kimball, 2006). To understand the role and function of hormones and the mechanisms by which such traits may have evolved, it is necessary to understand the genetic basis of endocrinological system and the proximate mechanisms regulating these phenotypic traits, including the fitness components associated to these phenotypic variations.

### *Heritability of corticosterone levels*

The aim of evolutionary biology is to understand the biological diversity observed between species, populations and individuals and how and why such diversity has evolved. The variation underlying the expression of a trait can be determined by genetic and non-genetic causes, but only the variation caused by heritable factors can be used by selection as the raw material for evolution. Therefore, determining the contribution of genetic variance to the expression of a trait is essential in assessing to what extent the proportion of the expression of a phenotypic trait may be transmitted from one generation to another and therefore its potential to respond to selection and evolve. Glucocorticoids, such as corticosterone, are important given that they regulate energy homeostasis, metabolism and behaviour of an organism. Corticosterone is also a key component in the physiological stress response as it enables organisms to cope with fluctuations in their environment. Although the adrenocortical stress response is adaptive, chronic stress or prolonged

exposure to high levels of corticosterone can have permanent or transient damaging effects on the health of an organism (Sapolsky et al., 2000, Martin, 2009, de Kloet et al., 1999, Bremner, 2007, Breuner et al., 2008). Therefore, to better understand how the secretion of corticosterone by the HPA axis may respond to perturbations in the environment and evolve adaptive mechanisms, it is necessary to determine the genetic basis of the variation in the expression of corticosterone levels.

To assess the potential of corticosterone secretion to respond to selection, I estimated the heritable variation underlying the difference in baseline and stress-induced corticosterone secretion and their genetic correlation. I used 7 years of corticosterone measurement taken on a free-living population of nestling barn owls combined with a long-term pedigree record to estimate the heritability of baseline and stress-induced corticosterone levels. This study showed that baseline and stress-induced corticosterone levels had, respectively, low ($h^2$= 0.199) to moderate ($h^2$=0.441) heritability. This suggests that secretion of corticosterone by the HPA axis could potentially evolve, and that, given the same intensity of selection, the acute corticosterone stress response can be expected to respond quicker to selective processes than baseline levels. This study is also the first to show that baseline and stress-induced corticosterone secretions share a strong genetic basis (genetic correlation: 0.735), suggesting that if selection is acting on baseline corticosterone levels, indirect selection would affect the expression of the corticosterone stress response and inversely, selection acting on the corticosterone stress response will indirectly affect the evolution of baseline corticosterone levels.

Although this study was built on a deep pedigree (20 years) and a large data set measured from a wild population, the uncontrolled environment can have potentially induced some bias in our estimates of heritability, particularly for baseline corticosterone levels, which can be influenced by numerous internal (Dunn et al., 1989, Gustafsson et al., 1994, Love et al., 2016, Carsia & Harvey, 2000, Lanfranco et al., 2006) and external factors (Romero, 2002, Monclus et al., 2005, Almasi et al., 2015, Partecke et al., 2006, Bize et al., 2010, Jenni-Eiermann et al., 2008). Moreover, corticosterone stress response was measured, as it is often the case in this type of studies, using a standard handling-restrain protocol; a manipulation that may not necessarily perfectly mimic a natural stressful factor. Moreover, they may be very different neural pathways and responses towards different types of stressors (e.g., to a predator attack, lack of food, environmental condition). Therefore, determining to what extent stress-response levels differ when triggered by different stressful factors will be necessary to make more general conclusions

on the heritability differences between baseline and stress-induced corticosterone levels. To give stronger support to our findings, further studies on the selective environments would be also necessary to determine how corticosterone levels respond to specific selective processes. Moreover, the secretion of corticosterone by the HPA axis is a complex set of neuroendocrine interactions, which involve the perception, integration and processing of internal and external signals into chemical messengers, which enable an organism to adopt the appropriate morphological, physiological and behavioural reactions to cope with environmental perturbation and life-history trajectories. Therefore, further studies exploring the genetic basis of other components of the HPA axis may be necessary to fully understand how the HPA axis may respond to selection processes and therefore how animals evolve stress-coping mechanisms. Furthermore, given that glucocorticoids regulate and respond to a large number of physiological and behavioural traits, we may expect glucocorticoids to be genetically correlated with a larger number of other traits, which in turn may constrain the evolution of the HPA axis. Investigating the genetic correlation between corticosterone levels and corticosterone-dependent traits such as metabolic and immune functions, as well as, other steroid hormones, could give more general insights in to the evolution of the HPA axis.

*Are corticosterone levels a reliable fitness proxy?*

Since the advent of field endocrinology, researchers have tried to interpret the meaning of different hormonal patterns and determine the mechanisms by which such patterns have evolved (Bonier & Martin, 2016). In particular, glucocorticoid hormones, such as corticosterone, have been extensively studied in the laboratory and field ecology given that they play a crucial role in regulating energy allocation and in enabling organisms' to cope with unpredictable stressors in their environments. Therefore, glucocorticoids levels have been thought to be used as a physiological index to assess the health of populations or individuals and, thereby, to predict population or individual fitness (Bonier et al., 2009, Breuner et al., 2008). Despite the fact that an increasing number of studies have tried to link glucocorticoid levels to variation in fitness-related traits, it is still unclear whether glucocorticoids, including corticosterone, can be considered as a reliable fitness proxy. A potential reason is that corticosterone levels are labile as they can be affected by a number of intrinsic (Dunn et al., 1989, Gustafsson et al., 1994, Love et al., 2016, Carsia & Harvey, 2000, Lanfranco et al., 2006) and extrinsic factors (Romero, 2002, Monclus et al., 2005, Almasi et al., 2015, Partecke et al., 2006, Bize et al., 2010, Jenni-Eiermann et al., 2008).

Therefore, the relationship between glucocorticoid levels and fitness components may be context-dependent, depending on the life history stage at which an individual is sampled, and on what components of the HPA axis (e.g., stress induce response levels, the sensitivity of the response or the rapidity to induce a feedback response) or fitness metrics are used.

To evaluate the potential fitness value of corticosterone, I investigated whether variation in baseline and stress-induced corticosterone levels predict adult survival in barn owls. With this aim, I used 13 years of capture-recapture data with 11 years of corticosterone measurements taken on breeding barn owls to evaluate the association between corticosterone variation and survival. Our study showed that individuals with low baseline corticosterone levels and high stress-induced corticosterone levels survived better than individuals with high baseline corticosterone and low stress-induced corticosterone levels. These findings suggest that natural selection strongly selects against high baseline corticosterone levels, which is consistent with the idea that elevated corticosterone levels can entail survival costs (Sapolsky et al., 2000, Martin, 2009, de Kloet et al., 1999, Bremner, 2007, Breuner et al., 2008). These results also suggest that corticosterone levels may be used as physiological indices to assess the condition or health of our barn owl population.
It is nevertheless important to consider that the survival cost associated with the corticosterone titre might be counterbalanced *via* reproduction. For instance, and given that elevated corticosterone levels during the breeding season is thought to support the energetic demand associated with rearing offspring, barn owls might differently balance survival against the cost of reproduction (Bonier et al., 2009, Wingfield & Sapolsky, 2003, Beletsky et al., 1989, Love et al., 2004). Further studies are therefore necessary, to determine whether individuals with low baseline and high stress-induced corticosterone levels show a better lifetime reproductive success and, therefore, a better fitness than individuals with high baseline and low stress-induced corticosterone levels. Interestingly, we show that baseline and stress-induced corticosterone levels were differently associated with survival and in the previous chapter (chapter 1), that part of the variation in corticosterone levels is heritable and strongly genetically correlated. This might restrain the evolution of the HPA axis and corticosterone levels, if the contrary selection forces acting on baseline and stress-induced levels cancel each other out. However, this should depend on the strength and stability of selection acting on baseline and stress-induced levels.

Again, because of the complexity and the number of components involved in the regulation of corticosterone secretion, further studies, investigating the link between corticosterone or other components of the HPA axis (e.g., rapidity of feedback loop, number of glucocorticoid receptors)

and different fitness components such as reproductive success should be performed to examine whether corticosterone can be used as a reliable fitness proxy in the barn owl.

*Link between the hypothalamic-pituitary-adrenal (HPA) axis and the melanocortin system*

Melanin-based traits are present in a wide range of species, where they may take different forms and functions (Majerus, 1998). Many studies have shown an association between melanin-based traits and suites of different morphological, behavioural and physiological traits (Roulin & Ducrest, 2011), including hormones such as corticosterone. These associations have been proposed to arise because the genes involved in the processing of the proteins underlying the expression of melanin pigments affect a number of other physiological and behavioural functions, including the secretion of steroid hormones (Ducrest et al., 2008). Thus, a key issue to understand how such associations may arise is to identify the underlying gene(s).

In this context, I chose a set of candidate genes based on their function and known role in melanogenesis and the regulation of corticosterone physiology, in order to identify the potential mechanisms linking melanin-based traits to the HPA axis. I found that the expression of *GR* and *MR,* receptors involved in the action of corticosterone, were both positively associated with the *PCSK2* gene that encodes the protein convertase responsible for post-translational modification of the proopiomelanocortin hormone (POMC). This suggests that the melanocortin system and *PCSK2* may be implicated in the establishment of the covariation between melanism and the HPA axis or at least with the expression of receptors to which corticosterone binds. This study was explorative and thus, further studies, especially focused on the tissues directly involved in the HPA axis (e.g., adrenal glands, brain tissues) are needed to clearly identify the proximate mechanisms linking melanin-based traits and the HPA axis. For instance, ACTH release is controlled by the cleavage of the POMC prohormone by protein convertases (Figure 5), including PC2, in the anterior pituitary gland (Smith & Funder, 1988). Determining the expression of POMC and protein convertase involved in the cleavage of POMC into ACTH and α-MSH in the anterior pituitary glands might give a better insight into the proximate mechanism linking melanin-based traits and the HPA axis. Additionally, our results showed also a difference in the expression levels of GR and MR with melanin-based traits suggesting that light and dark melanic individuals may differently regulate the HPA axis or resist differently to stress. Assessing the HPA axis feedback loop capacity of individuals between different melanin-based traits individuals may also be interesting to

determine whether eumelanic individuals are really more resistant to stress or whether differently melanised individuals modulate differently the HPA axis.

*Melanin-based traits and sex hormones*

Secondary sexual traits, such as colourful ornaments, are often used to signal the quality of an individual (Zahavi, 1975, Grafen, 1990, McGraw, 2006b, Hill, 1991, Hill, 1990). These signals of quality are considered to be honest because their production entails costs that only the fittest individuals are able to withstand. In certain species, these traits are associated to sex hormones, like testosterone, which can be immunosuppressive and metabolically costly at high levels (Hau, 2007, Wingfield et al., 2001). These relationships may occur if testosterone regulates the expression of sexual traits, if the display of an ornament triggers behavioural response in conspecifics that induce a rise in testosterone, or if genes involved in the elaboration of a sexual trait pleiotropically regulate testosterone physiology. Understanding how and why secondary sexual characters are associated with sex hormones is, thus, essential to determine their signalling function and the selective processes that led to their evolution.

To address this alternative hypothesis, I measured blood testosterone levels and, in growing feathers, the expression levels of the androgen and estrogen receptors to which sex hormones bind and the enzyme *5α*-reductase that metabolizes testosterone into the more potent hormone dihydrotestosterone (DHT). Adult and nestling male barn owls displaying a male-specific plumage (i.e., small spots) had higher levels of circulating testosterone. In breeding females, individuals displaying a female-specific plumage (i.e., large spots) had more testosterone, potentially to be metabolized into estrogen in feathers where estrogen receptors tended to be more expressed in large- than smaller-spotted females. In contrast, the enzyme 5*α*-reductase, that metabolizes testosterone to DHT, was more expressed in feathers of reddish-brown than light-reddish nestlings. This is consistent with the hypothesis that testosterone might be involved in the expression of reddish-brown pheomelanic pigments (Kimball, 2006, Bokony et al., 2008, McGraw, 2006a). These results suggest that testosterone is not necessarily more produced or used in this form in darker melanic organisms as usually observed (Ducrest et al., 2008). The sign of the covariation between testosterone and melanism may be then linked to whether males are more melanic than females. This finding has important consequences on our understanding of the adaptive function of melanin-based coloration and more generally of secondary sexual characters. As it shows that males and females may advertise the same phenotypic qualities with the same

phenotypic traits but in a different manner. In our case, melanin-based feather spottiness advertises testosterone-dependent behaviours and physiology processes in an opposite way. Further experimental studies, including hormone manipulation with genetic analyses are needed to clearly define the role of sex hormones, including DHT, in the expression of melanin-based traits in the barn owl. Moreover, hormones, including testosterone, can be locally metabolized into estrogen or DHT in tissues such as the skin. Therefore, unless enzyme inhibitors, receptor blockers and hormones are not experimentally injected locally, none of these studies will be able to finally elucidate the role of sex hormones in the elaboration of melanin-based ornaments.

*Final conclusion*

In the present thesis, I presented new information on the selective and adaptive value of steroid hormones and their link with melanin-based traits, a colourful trait that is associated to different morphological, physiological and behavioural functions and that may be under sexual selection in the barn owl. Although, we know much about the physiology of hormones, little is still known about how selection may act on endocrine traits, including steroid hormones, and how these traits may respond to different selection processes (e.g., natural and sexual selection). Moreover, hormones such as steroid hormones regulate many traits (e.g., growth, development, metabolism, sexual characters) and can be affected by many factors (e.g., health, body condition, environmental condition), which may cancel each other out and thereby, restrain the evolution of endocrine traits. Therefore, future studies determining the genetic link between hormone-dependent or -regulated traits with hormone levels are necessary to determine whether such traits restrict or drive the evolution of endocrine traits. In addition, future studies may consider investigating more tissue-specific and combined studies where we analyse simultaneously several components, determinant in the hormone pathway and action (enzymes, binding protein, receptors), instead of general blood measurements of hormones, to draw general conclusion of hormones on a specific trait. Not only is it important to determine the mechanism involved in the expression and the regulation of hormone mediated traits but it is also required to understand the genetic link and selective force driving these traits.

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