Testosterone and oestradiol effects on sex differences in waxbill ornamentation

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Resumo

A investigação sobre ornamentação tem-se centrado principalmente nos machos, deixando os mecanismos subjacentes à ornamentação das fêmeas relativamente pouco estudados. As aves são especialmente adequadas para investigar estes mecanismos, devido ao facto de apresentarem frequentemente cores vibrantes e caraterísticas plásticas e flexíveis. A ornamentação nas aves pode surgir na forma de caracteres que sinalizam a aptidão para potenciais parceiros, e também para a competição. Sabe-se que os esteroides sexuais influenciam a exibição ornamental em muitas espécies, estando a testosterona geralmente associada à promoção da exibição de cores e o estradiol à sua inibição. O bico-de-lacre-comum (*Estrilda astrild*) é uma espécie com ornamentação mútua, em que ambos os sexos exibem bicos vermelhos vibrantes, embora em média, o bico dos machos possa apresentar uma saturação ligeiramente superior. A ornamentação do bico-de-lacre-comum é baseada em pigmentação por carotenoides, e a investigação tem mostrado que o bico das fêmeas desta espécie é uma caraterística plástica, capaz de mudar de acordo com as condições ambientais e influenciada por exposições prolongadas ao estradiol. No entanto, os efeitos da testosterona na cor do bico ainda não foram estudados nesta espécie.

O objetivo deste trabalho foi avaliar os efeitos das manipulações a curto prazo de testosterona e de estradiol na ornamentação da cor do bico, utilizando injeções em ambos os sexos, bem como os efeitos a longo prazo da testosterona, através de implantes, em ambos os sexos. As alterações na saturação da cor do bico foram monitorizadas, uma vez que esta é a métrica de cor que melhor descreve a concentração de pigmentos carotenoides e a que representa com maior precisão o dicromatismo sexual.

A exposição a curto prazo ao estradiol pareceu desencadear uma diminuição da saturação da cor do bico dos machos, mas esta acabou por ser neutralizada, e não afetou a saturação da cor do bico das fêmeas. Contrariando a primeira previsão, a testosterona não influenciou a ornamentação da cor do bico em nenhum dos sexos, nem no tratamento a curto prazo, nem no tratamento a longo prazo.

Os resultados deste trabalho, juntamente com descobertas de trabalhos anteriores sobre a ornamentação do bico-de-lacre-comum, sugerem que os machos tendem a exibir a ornamentação na sua capacidade máxima, enquanto a ornamentação das fêmeas é mais plástica e flexível a mudanças. Por conseguinte, conclui-se que a

cor do bico e o dimorfismo sexual do bico-de-lacre-comum parecem ser regulados principalmente pelas fêmeas.

Palavras-chave: Bico-de-lacre-comum, ornamentação, esteroides sexuais, Testosterona, Estradiol, Dimorfismo sexual, Saturação da cor do bico.

Abstract

Research on ornamentation has primarily focused on males, leaving the mechanisms behind female ornamentation relatively understudied. Birds are especially suitable to investigate these mechanisms due to their frequent display of vibrant colours and flexible plastic traits. Ornamentation in birds can serve as characters that signal fitness to potential mates and also in competition. Sex steroids are known to influence ornamental display in many species, with testosterone typically being associated with promoting colour display and oestradiol inhibiting it. The common waxbill (*Estrilda astrild*) is a mutual ornamented species where both sexes display vibrant red bills, with, on average, the males' bill presenting slightly higher saturation. Bill ornamentation in the common waxbill is based on carotenoid pigmentation, and research has shown that the female bill of this species is a plastic trait, capable of changing according to environmental conditions and influenced by long term exposure to oestradiol. However, the effects of testosterone on bill coloration have not been tested yet in this species.

The aim of this work was to assess the effects of short-term manipulations of testosterone and oestradiol on bill colour ornamentation using injections on both sexes as well as the long-term effects, through implants, in both sexes. Changes in bill colour saturation were monitored, since this colour metric best describes carotenoid pigment concentration and accurately represents sexual dichromatism.

Short-term exposure to oestradiol appeared to decrease male bill colour saturation, but it was eventually neutralized, and it did not affect female bill colour saturation. Going against the first prediction, testosterone did not influence bill colour ornamentation in either sex, neither in the short-term, nor the long-term treatments.

The results of this work, coupled with past research on the common waxbill ornamentation suggest that males tend to display ornamentation at full capacity, whilst female ornamentation is more plastic and flexible. Therefore, it's concluded that bill colour and sexual dimorphism in the common waxbill appears to be primarily regulated by females.

Keywords: Common waxbill, Ornamentation, Sex-steroids, Testosterone, Oestradiol, Sexual dichromatism, Bill colour saturation

Table of Contents

List of Tables

List of Figures

List of Abbreviations

CIBIO	CENTRO DE INVESTIGAÇÃO EM BIODIVERSIDADE E RECURSOS GENÉTICOS
FCUP	FACULDADE DE CIÊNCIAS DA UNIVERSIDADE DO PORTO
GnRH	GONADOTROPIN-RELEASING HORMONE
HPG	HYPOTHALAMIC PITUITARY GONADAL AXIS
LH	LUTEINIZING HORMONE
LMMs	LINEAR MIXED MODELS
UP	UNIVERSIDADE DO PORTO
UVS	ULTRAVIOLET SENSITIVE

1. Introduction

1.1 Secondary sexual characters

Reproductive success in both plants and animals is directly related to the diversity of primary and secondary sexual characters (Andersson, 1994; Willson, 1979). In *The Descent of Man* (Darwin, 1871), Darwin considered primary sexual characters as the organs directly required for reproduction, such as gonads, whereas secondary sexual characters, while not directly required for reproduction, are vital to the process, such as weaponry or ornaments (Andersson, 1994; Arnqvist and Rowe, 2005; Darwin, 1871; Ghiselin *et al.*, 2010). Although the evolution of sexual characters is driven by selective forces, Darwin made a distinction between the evolution of primary and secondary sexual characters (Darwin, 1871). Today, research regarding the evolution of sexual characters still focuses on complementing the theories first introduced by Darwin (Andersson, 1994; Arnqvist and Rowe, 2005; Ghiselin *et al.*, 2010; Leonard and Córdoba-Aguilar, 2010; Tobias *et al.*, 2012).

Secondary sexual characters arise from selection processes driven by competition, but the forces behind the development of armaments and ornaments differ according to their function (Andersson, 1994; Berglund *et al.*, 1996). In behavioural ecology, weapons or armaments include structures like antlers, horns, venomous fangs and claws (Emlen, 2008; Hill and Yasukawa, 2014). Although these attributes are not essential for foraging activities or reproduction, they can provide a competitive advantage in intraspecific competition over territory and resources, therefore, increasing chances of reproduction for the individuals who prevail (Andersson, 1994; Berglund *et al.*, 1996; Emlen, 2008; Hill and Yasukawa, 2014). An ornament is an attribute that enriches animals' appearance, enhancing their chances of being selected as a sexual mate (Berglund *et al.*, 1996; Hill, 2015). Ornaments include bright colours, elongated feathers, dewlaps, manes and thick wattles (Hill, 2015; Hill and Yasukawa, 2014) and across many species, males usually display more elaborate ornaments than females (Amundsen, 2000b; Dale *et al.*, 2015; Darwin, 1871). Research on ornamentation has focused primarily on males, but understanding the development and evolution of ornaments in both sexes is crucial for unraveling the complexities of sexual dichromatism and mutual ornamentation.

1.2 Ornamentation in birds

Birds are especially suitable to investigate the underlying mechanisms of sexual dimorphism, largely due to their frequent display of vibrant colours and the occurrence of mutual ornamentation (Kraaijeveld *et al.*, 2007; Moller and Pomiankowski, 1993). Their ornamental traits include not only extravagant and colourful feathers, such as the peacock's tail, but also elaborate crests, periorbital skin and bright legs and bills (Berglund *et al.*, 1996; Hill and Yasukawa, 2014). While many species can adjust their colours in response to changes in social status or environmental conditions, birds are generally unable to rapidly modify plumage-related signals due to unflexible moulting schedules (Hill and McGraw, 2006; Landmann and Kollinsky, 1995). In contrast, other secondary signals, such as skin coloration or bill and leg brightness, tend to be more plastic and adaptable to change (Hill and McGraw, 2006).

1.2.1 Ornamentation and sexual selection

In many bird species, female ornamentation is typically less complex when compared to male ornamentation (Darwin, 1871). Uncovering the pathways that lead to these differences and understanding how ornamental features evolve through time has been one of the oldest and most heated arguments in evolutionary biology. One possible explanation for the disparity between male and female ornamentation is sexual selection, i.e., the occurrence of selective forces due to competitive behaviours over reproductive opportunities, such as intrasexual competition for mates (Andersson, 1994; Tobias *et al.*, 2012). In species with conventional sex roles, female reproductive success is primarily influenced by physiological factors, whilst in males it depends on their ability to get multiple mates (Amundsen, 2000a; Andersson, 1994; Assersohn *et al.*, 2021; Dale *et al.*, 2015; Tsuboi *et al.*, 2012). Females often prefer to mate with more ornamented males (Hill, 2015) which makes males experience stronger levels of sexual selection (Andersson, 1994). According to the Handicap Principle, only high quality individuals have the capacity to afford the physiological costs of developing conspicuous ornamentation, and therefore, exaggerated ornamental traits are seen as honest signals of fitness (Zahavi, 1975). Hence, female preference for more ornamented males when choosing a mate is highly related with the fact that elaborated ornaments are a reflection of individual health, physiological condition and often a sign that a bird has a high capacity of thriving in its environment (Dunn *et al.*, 2010; Hill, 2015; Hill *et al.*, 2009; Pérez-Rodríguez *et al.*, 2013; Saks *et al.*, 2003), making males with these conspicuous

characteristics more likely to prevail in intrasexual competition over mates, and be chosen by females when It comes to mate choice and reproduction (Hill, 2006).

In mutually ornamented species, females can also exhibit conspicuous ornamental traits very similar to males' (Amundsen, 2000a; Clutton-Brock, 2009), which can also be explained by sexual selection. Although often overlooked, males, like female birds, can choose more ornamented females, since ornamental traits could signal fertility and better abilities for parental care (Jawor *et al.*, 2004). In species that present mutual ornamentation, mutual mate choice is a mechanism that can enhance reproductive success and offspring quality (Clutton-Brock, 2009; Lande, 1980). Another theory to explain the occurrence of mutual ornamentation, suggests that female ornamentation has evolved as a by-product of sexual selection acting on male ornaments. While males develop elaborate ornamental traits to attract mates, similar traits in females may emerge indirectly as a result of the selective pressures on males, due to genetic or developmental correlations (Amundsen, 2000b). Since both sexes share most of their genetic information, it is expected that females have the genetic information to display the same ornaments as males do (Amundsen, 2000a; Lande, 1980). However, sexual selection is not the only selective force that influences the development of secondary sexual characters like ornaments, and nonsexual social selection is also important to consider.

1.2.2 Ornamentation and social selection

Research has shown that in both sexes, challenging environmental conditions and stronger levels of competition for resources, highly influence the display of ornamental traits, often reducing their expression and sexual dichromatism (Vergara and Martínez-Padilla, 2012; Freitas *et al.*, 2021). This reduction presumably occurs because the physiological efforts of ornament display are reallocated into enhancing survival and reproduction, according to the individuals' surroundings (Funghi *et al.*, 2018; Smith, 1982; Smith and Harper, 2003; Vergara *et al.*, 2012a; Vergara *et al.*, 2012b).

In most bird species and for both sexes, beyond competing over access to mates, intra-sexual competition usually occurs over limited ecological resources, such as territory and food (Ligon et al., 1990), or to achieve a higher social status (Clutton-Brock, 2009; Pham *et al.*, 2014) and selective forces tend to favour the individuals with the most elaborate ornaments (Amundsen, 2000a; Tobias *et al.*, 2012). According to the Handicap principle, ornamental traits play an important role in intrasexual competition since they work as "badges of status", i.e., signals of competitive ability where the most ornamented

individuals are the ones presumed to prevail in competition, as ornamentation reflects physiological health (Dale, 2006; Laucht and Dale, 2012; Searcy William and Nowicki, 2005). Furthermore, the surrounding environmental conditions of an individual also influence ornamentation. For instance, previous studies on the common waxbill (*Estrilda astrild*) have shown that female investment in bill coloration is sensitive to nutrition and cold related stress (Freitas *et al.*, 2021). Additionally, differences in bill coloration between sexes, usually with higher saturation on males, disappear when the environmental conditions are more favourable (Funghi *et al.*, 2018).

Overall, sexual differences in ornamentation can be explained by the fact that males usually experience stronger selective forces, both sexual and social, making male ornamentation more complex and extravagant (Amundsen, 2000b; Andersson, 1994). The existence of mutual ornamentation in various gregarious species can be explained by the extensive social interactions that go beyond competition over mates and reproduction (Seyfarth and Cheney, 2012; Tobias *et al.*, 2012). In these species, elaborate ornamental traits in both sexes can provide many social advantages, such as protection against predators, enhanced foraging activities, and competition with other species over resources, which could explain why mutually ornamented species are often gregarious (Kraaijeveld *et al.*, 2007; Lyon and Montgomerie, 2012; Tobias *et al.*, 2012).

1.3 Carotenoid-based ornamentation

Ornamentation in animals can either be structural or pigment-based, with pigment-based ornamentation being the most studied origin of animal coloration. In nature, pigments, both organic and inorganic, absorb certain wavelengths of light, resulting in the perception of colour (Britton and Britton, 1983). In birds, living tissues like the bill, snoods, skin surrounding the eye and wattles are vascularized, and their coloration is directly influenced by blood circulation and pigmentation levels (Lucas, 1972; Pérez-Rodríguez, 2008; Rosenthal *et al.*, 2012). One of the main types of pigmentation in birds is carotenoid pigmentation. Carotenoids are responsible for many bright colours, like red, orange and yellow (Moller *et al.*, 2000). More than 750 carotenoids have been described, and based on their molecular structure, they can be classified as xanthophylls or carotenes (Fox, 1976; Moller *et al.*, 2000). Despite this diversity, all carotenoids comprise a chromophore, a structure of alternating single and double bonds in the central region of the molecule, making them effective in absorbing the energy from light at wavelengths from 400–500 nm (blue-green), appearing yellow,

orange or red to the human eye when reflected (Brush, 1990; Svensson and Wong, 2011).

Carotenoids cannot be naturally synthesized by animals and must be obtained directly through diet, mainly through the consumption of plants, or by transforming the structure of ingested carotenoids. After intake and metabolic transformations, carotenoids circulate in the bloodstream and can be stored or transported and deposited into various tissues, where they carry out many different functions (Brush, 1990; Moller *et al.*, 2000; Navara and Hill, 2003; Svensson and Wong, 2011). Birds usually tend to accumulate xanthophylls, such as lutein, zeaxanthin and canthaxanthin over carotenes such as β-carotene (Brush, 1990). In the common waxbill, the most abundant carotenoids in blood circulation are yellow carotenoids, specifically lutein, zeaxanthin, β-cryptoxanthin, 2', 3'-anhydrolutein and 3'-dehydrolutein (McGraw, 2004). Before getting deposited into the integument for instance, into the bill, originating colour ornamentation, these carotenoids can be converted by metabolic processes into the red carotenoids astaxanthin, α-doradexanthin, adonirubin and canthaxanthin (Brush, 1990; McGraw, 2004; McGraw and Schuetz, 2004).

For animals in general, other than their important role in generating and maintaining ornamentation, carotenoids are also essential to various physiological functions. For instance, they are important precursors to vitamin A and have important antioxidant functions due to the structure of the chromophore, i.e., acting as molecules that supress the damaging effects essential biomolecules caused by oxidants (K. McGraw *et al.*, 2006; McGraw and Schuetz, 2004; Pérez-Rodríguez, 2009; Svensson and Wong, 2011). Carotenoids are also very important to the immune system: when microorganisms invade the system, leukocytes produce peroxides and radicals that attack the invading microorganism (Chew and Park, 2004). By suppressing the damaging effects of these radicals, carotenoids protect both the white blood cells and the surrounding tissues (Bendich, 1989; Chew and Park, 2004; Navara and Hill, 2003). In birds specifically, carotenoids also play an important part in reproduction, for the development of embryos: eggs contain high concentrations of carotenoids, which, due to their antioxidant activities, can be important when protecting the embryo from the damaging effects of oxidants during development (Karadas *et al.*, 2005; Svensson and Wong, 2011).

When it is necessary to reallocate carotenoids, to support the immune system (Bendich, 1989; Chew and Park, 2004), protect eggs (Karadas *et al.*, 2005; Pérez-Rodríguez *et al.*, 2013; Saino *et al.*, 2003), or even to fight parasite infections (Hõrak *et*

al., 2001), there may be carotenoid trade-offs between ornamentation and physiological systems (Figure 1). For instance, if carotenoids are needed, the reallocation of carotenoids from tissues like the bill, can result in less ornamented individual, or vice-versa (Blount and McGraw, 2008; Svensson and Wong, 2011). These trade-offs may affect female ornamentation more intensely since females reallocate carotenoids to protect both their own physiological systems and to protect developing embryos (Hipfner *et al.*, 2010), which can be another explanation as to why females are often less ornamented than males (Blount *et al.*, 2002; Rosenthal *et al.*, 2012; Saino *et al.*, 2003; Svensson and Wong, 2011).

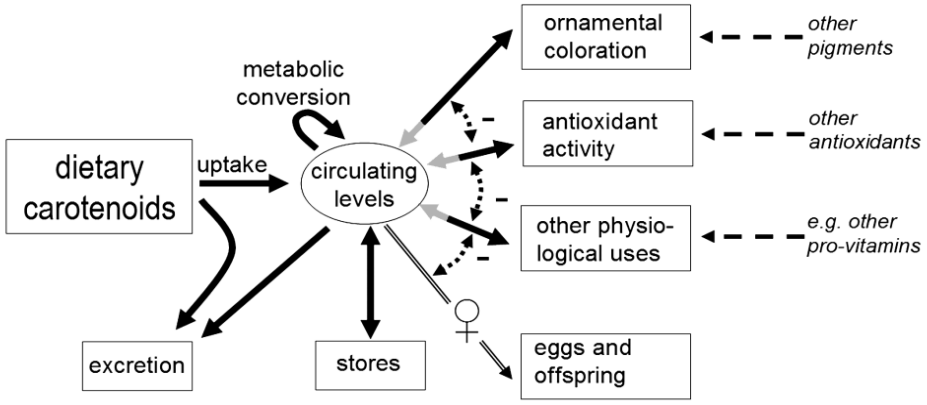

Figure 1 - Interactions that carotenoids can have in animals that display carotenoid-based ornamentation. Gray arrows highlight partial bidirectionality; dotted arrows highlight carotenoid trade-offs; dashed arrows highlight the shared functions between carotenoids and other components. The hollow arrow highlights the reallocation of carotenoids to eggs, a process that is exclusive to females. Image from Svensson and Wong (2011).

1.4 Sex steroids and ornamentation

The expression of ornamental traits can be highly regulated by hormones (Owens and Short, 1995), specifically sex steroid hormones like oestradiol and testosterone (Casagrande *et al.*, 2011; Pham *et al.*, 2014; Romero-Diaz *et al.*, 2022a). Hormones are chemical messengers, i.e., substances that influence the function of other cells (Nelson, 2005; Wingfield, 1994). Upon receiving external stimulation, endocrine cells release hormones that travel through blood interacting with other cells, triggering a response (Adkins-Regan, 2005; Schreiber, 2023; Wingfield, 1994). Sex steroid hormones, mainly produced in the gonads, include androgens, with testosterone being the most common; oestrogens with oestradiol being the most common; and progestogens (Schreiber, 2023; Wingfield, 1994). These hormones are responsible for the regulation of reproduction and secondary sexual characters (Norris and Lopez, 2010; Wingfield, 1994). Although

classified as female and male hormones, oestradiol and testosterone, respectively, both are present in both sexes, even if in lower concentrations, and therefore, both can influence ornamentation in both sexes (Norris and Lopez, 2010). Testosterone is generally associated with the enhancement of ornamental colours (Adkins-Regan, 1999; Ardia *et al.*, 2010; Beltrao *et al.*, 2021b; Casagrande *et al.*, 2011), while oestradiol tends to reduce their expression (Casagrande *et al.*, 2011; Romero-Diaz *et al.*, 2022a).

1.4.1 Testosterone and ornamentation

Although testosterone is considered a male hormone, since it is mainly produced in the testicles from the conversion of cholesterol by Leydig cells, it is also be synthetized in females by the adrenal glands, the ovaries, and the brain in smaller amounts (Hau and Goymann, 2015; Staub and De Beer, 1997; Wingfield, 1994). Testosterone seems to highly influence the development of ornamentation, especially the ornaments that are condition-dependent (Ardia *et al.*, 2010; Casagrande *et al.*, 2011; Newhouse and Vernasco, 2020), which can be due to androgens regulating pigment content in the tissue (McGraw and Ardia, 2007; Mougeot *et al.*, 2007) or stimulating blood flow in the tissue (Casagrande *et al.*, 2011). The role of testosterone in influencing bill colour has been documented not only in males, but also in females. For instance, the work developed in Laucht *et al.* (2010) showed that in male house sparrows (*Passer domesticus*) baseline testosterone levels were positively correlated with the blackening of the bill. In females, Pham *et al.* (2014) showed that in the American goldfinch (*Spinus tristis*), those with higher baseline levels of circulating testosterone are the ones with higher bill colour saturation, highlighting an association between testosterone and display of bill colour. The correlation between testosterone and ornamentation has also been supported by experimental manipulation of testosterone levels, using both short- and long-term exposures to the hormone while monitoring ornamental changes in different tissues. For instance, testosterone implanted moorhens (*Gallinula chloropus*) of both sexes presented thicker, bigger and enhanced colouration in frontal shields, (Eens *et al.*, 2000). In the male red grouse (*Lagopus lagopus scoticus*), increased testosterone levels due to implantation caused rapid increases in both comb size and redness (Martínez-Padilla *et al.*, 2010). When it comes to bill colour specifically, in the red-backed fairy-wren (*Malurus melanocephalus*), female bill colour became darker due to long-term exposure to exogenous testosterone (Lindsay *et al.*, 2016). In the zebra finch, (*Taeniopygia guttata*), where the orange-red bill colour is based on carotenoid pigmentation (McGraw and Schuetz, 2004; McGraw and Ardia, 2003), long-term exposure to testosterone resulted in elevated bill redness, in both sexes (McGraw, 2006; McGraw *et al.*, 2006a), and short-

term exposures, using injections, showed that testosterone propionate increased bill red chroma and hue in males, in only three days (Ardia *et al.*, 2010).

However, it is important to consider that often times, testosterone is a "double edge sword" and more factors should be taken into account. A study conducted by Martínez-Padilla *et al.* (2014) showed that the investment in colourful traits was enhanced in the presence of high testosterone levels, but these effects depended on the level of intrasexual competition, suggesting that the population context influences the relationship between testosterone and ornament expression (Martínez-Padilla *et al.*, 2014). On the other hand, the investment in ornamentation mediated by testosterone, could have a negative impact on the individuals' physiological health due to the carotenoid trade-offs between ornamentation and fitness (Zera and Harshman, 2001), meaning that only individuals in optimal condition can deal with the costs associated with maintaining both ornamentation and physiological health. One possible explanation for this, lies on the Immunocompetence hypothesis (Folstad and Karter, 1992), that proposes that testosterone has a suppressive effect on the immune system of animals, weakening immune responses to adverse situations. According to this theory, higher levels of testosterone can result in a less effective response from the immune system (Folstad and Karter, 1992). This has been supported by some authors (Foo *et al.*, 2017; Newhouse and Vernasco, 2020), although it is not universal for every species (Roberts *et al.*, 2004). Furthermore, the Oxidation handicap theory suggests that elevated testosterone levels increase oxidative stress, due to an imbalance between the production of free radicals and the body's ability to neutralize them. (Alonso-alvarez *et al.*, 2008; Newhouse and Vernasco, 2020). As it was stated before, carotenoids have different functions and are responsible not only for ornamentation but also for maintaining the immune system working when they are needed. Therefore, when a bird is not in optimal health the connection between carotenoid availability, testosterone levels, and the associated trade-offs in maintaining efficient physiological responses can result in decreased ornamentation, even if carotenoid concentration is high in the bloodstream, due to the reallocation of carotenoids, just like it was described in Alonso-alvarez *et al.* (2008).

1.4.2 Oestradiol and ornamentation

Like testosterone, oestradiol, the most abundant oestrogen in vertebrates (Norris and Carr, 2013; Norris and Lopez, 2010), also influences bird ornamentation. Oestradiol is mainly produced in the ovaries, in a process that begins with cholesterol, going through

the conversion of androstenedione, a weak androgen, into estrone and testosterone by aromatase enzymes and culminating in the formation of oestradiol (Schlinger and Arnold, 1992). However, there is much less research on the ornamental impacts of this hormone when compared to testosterone.

Since testosterone can be converted into oestradiol, and oestradiol can also be produced in males, in testicles and by the adrenal gland (Schreiber, 2023; Wingfield, 1994), it is suggested that oestradiol may influence ornamentation in both sexes. Oestradiol has been associated with originating dull coloration (Kimball and Ligon, 1999). For instance, females of many bird species that have undergone ovariectomy (i.e. removal of the ovaries) or have reached the end of reproductive ability, and have therefore lower levels of oestradiol, tend to develop coloration similar to that of males (e.g. mosaic canary) (Perez-Beato, 2008). This has also been supported by experimental manipulation of oestradiol levels, on both sexes. For example, a study on both sexes of the diamond dove (*Geopelia cuneata*) implanted with oestradiol showed that, although oestradiol did not influence the size of the eye ring, it seemed to reduced eye ring coloration (Casagrande et al. 2011). Furthermore, in female common waxbills (*Estrilda* astrild) implanted with oestradiol, the long-term exposure resulted in decreased colour saturation and hue (Romero-Diaz *et al.*, 2022a). Since the colouration of the common waxbills' bill is carotenoid-based (McGraw and Schuetz, 2004), these results suggest that female reproductive physiology may limit the allocation of resources to carotenoid-based colour expression (Romero-Diaz *et al.*, 2022a). Furthermore, exposure to oestradiol also enhanced sexual dichromatism in this species, making the colour differences between males and females more distinct when viewed through a model of avian vision (Romero-Diaz *et al.*, 2022a). Two possible explanations for these results were suggested in Romero-Diaz *et al.* (2022a). First, oestradiol may directly interact with hormone-responsive regulatory elements of genes responsible for carotenoid-based coloration, potentially affecting processes such as carotenoid uptake, transport, deposition, or degradation (Romero-Diaz *et al.*, 2022a). On the other hand, high levels of oestradiol may inhibit gonadotropin-releasing hormones in the hypothalamus through a negative feedback mechanism, which in turn decreases the release of luteinizing hormone (LH) from the anterior pituitary gland. As LH is vital for the expression of vibrant coloration in many species, lower concentrations of LH could result in less vivid bill colouration (Kimball and Ligon, 1999; Romero-Diaz *et al.*, 2022a). This study highlights sex steroid hormones influence on bill colour in the common waxbill, specifically in females. However, no studies have been done with this species to test the influence of

oestrogen in males or the influence of testosterone in both sexes, which could give a better insight into the mechanisms behind bill ornamentation in common waxbill.

1.5 The common waxbill (*Estrilda astrild*)

The common waxbill (*Estrilda astrild*) (Figure 2) is a small granivorous estrildid finch native to sub-Saharan Africa (Harris and Davis, 1993). This is an extremely social species, moving in flocks, roosting communally, and feeding on various small seed-producing grasses and small seeds of sedges (Harris and Davis, 1993; Reino and Silva, 1998). The breeding season occurs between March and September, peeking in May, and during this period, the common waxbill forms monogamous pairs (Beltrão *et al.*, 2021c). Previous studies suggested that common waxbills do not breed successfully in captive conditions (Catry *et al.*, 2010; Harris and Davis, 1993; Romero-Diaz *et al.*, 2022a). It is thought that the common waxbill was first introduced in Portugal in the 1960s, due to escapes from captivity, and it has since spread throughout the country as a result of other introductions (Silva *et al.*, 2002). The common waxbill is a mutually ornamented species, where both adult males and females (Figure 2) present a vibrant red bill, a red mask around the eye area, red plumage on the breast, and different shades of brown plumage throughout the rest of the body (Harris and Davis, 1993). Red bills are very common in gregarious bird species (Dey *et al.*, 2015), including females of gregarious estrildids (Gomes *et al.*, 2016). While on average males exhibit a higher extent and saturation of red in both their plumage and bill, the differences in ornamentation between sexes are not always easily discernible (Cardoso *et al.*, 2014a; Cardoso *et al.*, 2014b). In common waxbills, bill colour does not signal aggression or social dominance (Funghi *et al.*, 2018) but influences social preferences similarly in both sexes (Cardoso *et al.*, 2014a). The red coloration of the common waxbill's bills is derived from carotenoid pigments, specifically astaxanthin, α-doradexanthin, adonirubin and canthaxanthin, which are metabolized from yellow dietary carotenoids and then deposited into the integument (Brush, 1990; McGraw and Schuetz, 2004; McGraw, 2004). In contrast to plumage, which maintains mainly a set colour after the moulting season, the colour of the avian bill can change over the course of only a few days or weeks (Karubian *et al.*, 2011; Rosenthal *et al.*, 2012). Previous work has shown that bill colour in common waxbills is a plastic trait, with females being more

Figure 2 - The common waxbill (*Estrilda astrild*)

flexible to change, probably because they need to balance investment in ornamentation with maintaining physiological condition for reproduction (Funghi *et al.*, 2018). Freitas *et al.* (2021) demonstrated that female bill ornamentation tends to be particularly sensitive to diet and environmental conditions, specifically cold-mediated stress. Moreover, sexual dichromatism can disappear if both males and females are maintained under ideal environmental conditions (Funghi *et al.*, 2018). Furthermore, as bill colour of female common waxbill decreases when oestradiol levels are high (Romero-Diaz *et al.*, 2022a), makes the common waxbill the perfect candidate to study howinvestment in ornamentation changes in response to environmental and socioecological conditions, as well as to hormonal changes (Funghi *et al.*, 2018; Romero-Diaz *et al.*, 2022a).

1.6 Objectives

Previous research has shown that long-term exposure to oestradiol decreases bill colour ornamentation in female common waxbills (Romero-Diaz *et al.*, 2022a). However, no research has been done with this species to test the influence of short-term oestradiol exposure nor the influence of testosterone in both sexes, which could give a better insight into the mechanisms behind bill colour ornamentation in the common waxbill. Therefore, in my thesis I aim to test two goals:

1: Assess the effects of sex-steroids short-term manipulations on bill colour ornamentation, by administration of oestradiol and testosterone injections on both sexes.

2: Test the effects of long-term exposure to testosterone on bill colour ornamentation, on both sexes.

My prediction for the first goal, based on previous research with oestradiol implants in common waxbills (Romero-Diaz *et al.*, 2022a), is that oestradiol injections will decrease bill colour saturation in females, while testosterone injections will increase bill colour saturation in both sexes.

My prediction for the second objective is that long-term testosterone treatment will mirror those obtained with androgen manipulation on relatedspecies (Ardia *et al.*, 2010; Casagrande *et al.*, 2011; De Ridder *et al.*, 2002; Enbody *et al.*, 2018; McGraw, 2006), with both males and females displaying increased bill colour saturation.

The results of this experiment will help complement the existing theories about the evolutionary forces that shape bird ornamentation. In the common waxbill, it is

expected that both sex-steroids influence female bill colour, supporting the theory that sexual dichromatism in the common waxbill is primarily driven by females.

2. Materials and Methods

2.1 Housing of the birds

For the short-term testosterone and oestradiol treatments, a total of 20 common waxbills (10 males and 10 females) were obtained from certified breeders in September 2019 and housed in five metal cages (88.5 cm × 30 cm × 40 cm) at a research aviary in CIBIO (Vairão, Portugal) (Figure 3). For the long-term testosterone treatment, a total of 24 common waxbills (12 males and 12 females) were obtained from certified breeders in May 2021 and housed in the same conditions. In both treatments, birds were ringed with unique colours and numbers, for individual identification, and were housed in randomly assigned mixed-sex groups of four birds per cage (2 males and 2 females) (Figure 3B) in order to avoid the stress of isolation since common waxbills are highly gregarious (Clement, 1999). The cages were visually but not acoustically isolated from each other, and the gridded walls of the cages allowed natural ventilation and natural light illumination that was complemented with artificial light in a cycle adjusted to the natural photoperiod (Figure 3A). Previous research using the common waxbill suggests that adults of this species do not reproduce in captive conditions, which enables the triggering of the reproductive physiology without the interference of actual breeding occurring (Romero-Diaz *et al.*, 2022a).

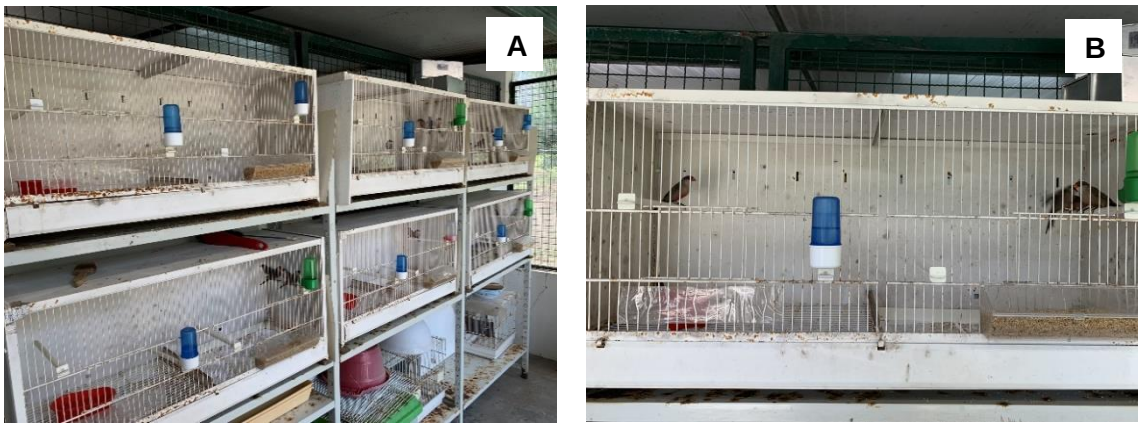

Figure 3 - Aviary **(A)** and individual cage with 2 males and 2 females **(B)**.

The cages were equipped with two water dispensers that provided ad libitum water to the birds, and one long feeder that provided *ad libitum* food (a commercial seed mixture, Tropical Finches Prestige, Versele-Laga) that allowed multiple birds to feed simultaneously. Furthermore, fine sand mixed with crushed oyster shells (Grit with Coral

Prestige, Versele-Laga) was placed at the bottom of the cages, to provide the birds with a vitamin supplement. The birds were monitored regularly to ensure their well-being.

2.2 Bill colour measurements

Bill color was measured using reflectance spectrophotometry (Figure 4). Scattered light reflectance was measured between 300 and 700 nm, the bird-visible spectrum (Romero-Diaz *et al.*, 2022b) relative to a WS-1-SL white Spectralon reflectance standard in a standardized set-up (Romero-Diaz *et al.*, 2022b), using an Ocean Optics USB4000 spectrophotometer coupled to a PX-2 pulsed xenon light source and a UV/Vis reflectance probe (Ocean Optics, Dunedin, FL, USA) (Figure 4A). Measurements were taken with the reflectance probe perpendicular to the bill surface, and each time, 4 measurements of the bill were taken, two on each side of the upper mandible, with the reflectance probe gently touching the bill surface (Figure 4B) and enveloping the area with a black opaque cloth to prevent contamination from daylight. Using the *getspec()* function in the "pavo" package v. 2.9.0 (White *et al.*, 2023), the collected reflectance data was uploaded to the R software v.4.4 (R Core Team, 2024), and with the *procspec()* function, the spectral data was smoothed with a span of 0.1 in order to reduce noise, and the negative values were zeroed to correct any possible measurement errors.

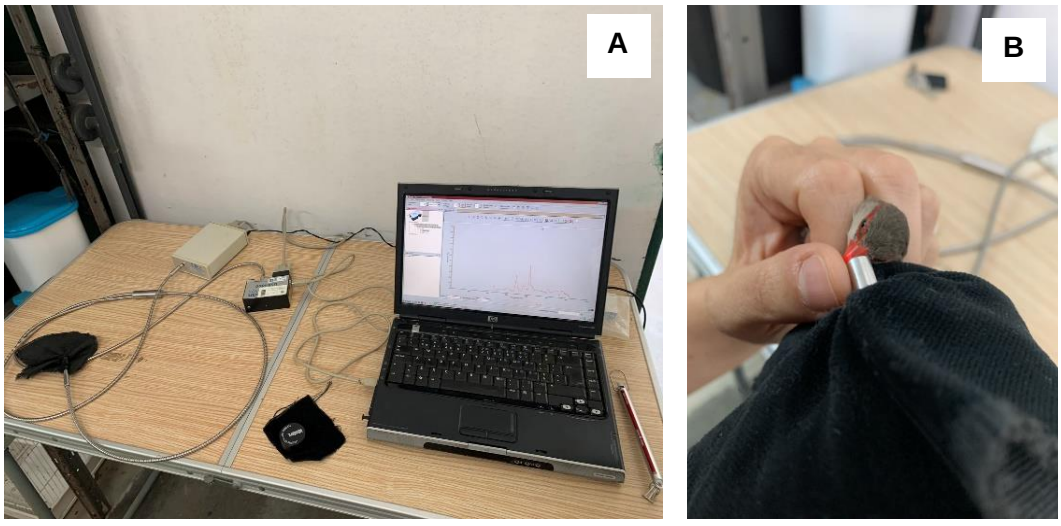

Figure 4 - Ocean Optics USB4000 spectrophotometer set-up **(A)** and bill colour measurement **(B)**.

Since the vision of estrildid finches is ultraviolet sensitive (UVS) (Montgomerie, 2006), using the *vismodel()* function of the "pavo" package, a visual model based on an average avian UVS visual system, and the achromatic sensitivities of a representative passerine was employed to the spectral data. Mirroring the approach used in (Romero-

Diaz *et al.*, 2022b), this visual model used quantal catches (Qi) to simulate the initial perception of light by photoreceptor cells, without considering Fechner's law (that states that visual receptors respond proportionally to the logarithm of light intensity (Houstoun and Shearer, 1930)) to solely focus on the response of the visual system of the species, a standard daylight illuminant (D65) to accurately model how birds perceive color under typical daylight conditions, and von Kries color correction was applied to ensure the model represents how birds adapt to light changes (Romero-Diaz *et al.*, 2022a,b; Vorobyev *et al.*, 2001).

With this visual model, the receiver-independent color metric *r* was extracted the *colspace()* function of the "pavo" package to create a representation of color perception in the tetrahedral color space based on the visual model. This metric represents color saturation (*r*, the distance from the achromatic center in tetrahedral color space, where a higher *r* value represents a more saturated color) (Romero-Diaz *et al.*, 2022a,b; Stoddard and Prum, 2008).

The main focus of this work was the color saturation (*r*) because it is directly related to carotenoid pigment concentration and is the colour metric that most accurately represents sexual dichromatism in the bill of the common waxbill (Cardoso *et al.*, 2014b; Romero-Diaz *et al.*, 2022b).

2.3 Short-term testosterone and oestradiol treatments

2.3.1 Hormonal treatment

For the short-term treatment, experiments took place over 5 weeks, from the 6th October to the 5th November 2020. There were three rounds of experiments, so that each bird received three treatments (Control, Testosterone, and Oestradiol) with an interval of 15 days between each treatment. Each treatment consisted of an intramuscular injection of a compound, on the right side of the pectoral muscle. Treatments were distributed in a balanced manner across all experimental birds, such that the different treatments were distributed evenly over time. One male died before the end of the experiments, and therefore the sample size was 19 birds (10 females and 9 males).

The control treatment consisted of an injection of 20 µl saline solution, and the hormonal treatments consisted of an injection of testosterone or oestradiol diluted in 20 µl saline solution. The dosage of each compound was based on previous studies of birds of the same size, especially the zebra finch (Adkins-Regan and Ascenzi, 1987; Ardia *et*

al., 2010; von Engelhardt *et al.*, 2004)**,** using as reference the mean body mass of the birds used for this experiment (9 g, measured before experiments): treatment with testosterone (Testosterone, 86500 Sigma-Aldrich, Darmstadt, Germany) and treatment with estradiol (β-Estradiol, E2758 Sigma-Aldrich) used 7.5 $g.l^{-1}$.

On the days of injection, the bird was removed from the cage at 10 am, injected with the corresponding treatment and placed again in its cage. After 3 hours the bird was taken again from the cage, bill color was measured, and the bird was once again returned to its cage. This was repeated 6, 24 and 48 hours after the injection.

2.3.2 Statistical analysis

In this treatment, to see if testosterone and oestradiol had an effect on bill colour saturation (*r*), the statistical analysis was done separately for males and females. Both the male and female data were organized in sub-datasets for each of the time points, allowing for the comparison of each bird with itself across the different treatments at each time point, individually. Histograms and QQ plots were analyzed to assure that the data followed an approximately normal distribution.

Linear Mixed Models (LMMs) were performed for each of the sub-datasets using the *lmer()* function in the R package “lme4” v. 1.1-35.4 (Bates *et al.*, 2015), where the dependent variable was the colour metric *r*, the treatment was a fixed factor and bird identity was a random factor in order to test if bill colour saturation in the control treatment differed from the oestradiol and testosterone treatments.

Using the *check_distribution()* and *check_model()* functions on R, the predicted distributions of the residuals and response from these model were analyzed and confirmed that the residuals followed an approximately normal distribution.

2.4 Long-term testosterone treatment

2.4.1 Hormonal treatment

Experiments took place in two different periods, with an interval of 15 days between them to allow the birds to recover. Each period lasted 8 weeks, the first one from 1st of February to the 28th of March 2024 and the second one from 11th of April to the 6th of June 2024. On the first period, 3 of the cages received the control treatment. and the other 3 received the Testosterone treatment. On the second period the treatments were reversed. Two females died before the end of the experiments but were

replaced with new unimplanted females to maintain the social context, and therefore the sample size was reduced to 22 birds (12 males and 10 females).

On the 1st of February 2024, the birds subjected to the testosterone treatment were implanted with a subcutaneous silastic tube (internal diameter = 0.76 mm, outer diameter = 1.65 mm, length = 10 mm; mono-lumen tubing, Helix Medical) filled to capacity with Testosterone propionate (product number 86541, Sigma-Aldrich) and the birds subjected to the control treatment were implanted with empty tubes of the same size. The size of the testosterone implants was based on previous studies with birds of similar size, especially the zebra finch, (Cynx *et al.*, 2005; Lopez-Rull and Gil, 2009; McGraw, 2006; Roberts *et al.*, 2007), to keep the amount of testosterone within the natural physiological bounds. The implants were sealed with non-toxic silicone and immersed for 24 h into phosphate-buffered saline prior to implantation, to prevent the release of a supraphysiological bolus of hormone from being released.

Right before implantation, the birds were locally anaesthetized with lidocaine gel (Lidonostrum Gele 2%, Sidefarma, Portugal) applied manually, and the implant was placed beneath the skin on the dorsal side after making a small incision in the right flank. The cut was then sealed with super-glue.

After implantation, the birds were held for a few seconds to allow the super-glue to dry before being released into their respective cages where their behaviour was monitored to ensure their well-being. The implants are expected to start releasing their content within a few hours after implantation and to continue releasing testosterone for approximately two months (Romero-Diaz *et al.*, 2022a).

After 2 months, on the 28th of March, the implants were retrieved by making a small incision and pulling out the implant using tweezers. Nevertheless, it was not possible to find 8 of the implants, which means they either fell of the bird or moved out of place, but the ones that were retrieved were empty. Then, on the 11th of April the treatments were switched and the whole process was repeated so that every bird was subjected to each of the treatments.

In this treatment, the birds were weighed every four weeks using a handheld analog scale to determine if the testosterone treatment influenced the weight of the birds.

On the days of implantation, each bird was removed from the cage at 10 am, implanted with the corresponding treatment and placed again in its cage. Bill color was measured 7 days after the implantation and then, every 7 days throughout 8 weeks for each period, at the same time of day.

2.4.2 Statistical analysis

Here, to see if testosterone influenced bill colour, the statistical analysis was also done separately for males and females. Firstly, using the saturation (*r*) values measured throughout the 8 weeks of the treatment, the mean of those values was calculated individually for each bird, in both the Testosterone and the Control treatment period. Then, using the *t.test()* function on the R software v.4.4 (R Core Team, 2024) those means were used to perform paired t tests, since the goal was to compare each individual in the testosterone treatment with itself in the control treatment, taking into account individual variability within the birds.

The implants were expected to start releasing testosterone within a few hours of implantation, but in case the bill colour took some time to change, these testes were repeated using only the data from the last 4 weeks of each treatment (Control and Testosterone).

Finally, in order to see if testosterone influenced the weight of the birds, the same paired t tests were performed using the means of the weights of each bird in each treatment, to compare each individual in the testosterone treatment with itself in the control treatment, taking into account individual variability within the birds.

3. Results

3.1 Short-term testosterone and oestradiol treatments

In males, short-term hormonal treatments revealed that bill colour saturation (*r*) significantly decreased within the first three hours following the oestradiol treatment (T3: t=-2.217; p=0.036, Table 1, Figure 5A), compared to the control treatment. Although bill colour saturation (*r*) fluctuated throughout the 48 hours, no other significant results were found in the short-term hormonal treatment either for males (Table 1, Figure 5A) or females (Table 1, Figure 5B).

Table 1 - Results of the linear mixed models (LMMs) describing the effects of short-term manipulations of Testosterone and Oestradiol on bill colour saturation (*r*), for males and females. Time: 3, 6, 24 and 48 hours after injection (T3, T6, T24 and T48, respectively). Positive t-values indicate an increase in saturation relatively to control; negative t-values indicate a decrease in saturation relatively to control. Significant values in bold.

Sex	Time	Treatment	Estimate	Std. Error	df	*t* value	*p* value
Females	T3	Oestradiol	-0.003	0.012	18	-0.241	0.812
		Testosterone	0.012	0.012	18	0.983	0.339
	T6	Oestradiol	0.001	0.017	18	0.035	0.972
		Testosterone	-0.003	0.017	18	-0.190	0.852
	T24	Oestradiol	-0.015	0.015	18	-0.998	0.331
		Testosterone	-0.002	0.015	18	-0.123	0.903
	T48	Oestradiol	0.016	0.016	18	1.032	0.316
		Testosterone	-0.009	0.016	18	-0.547	0.591
Males	T3	Oestradiol	-0.027	0.012	24	-2.217	**0.036**
		Testosterone	-0.020	0.012	24	-1.688	0.104
	T6	Oestradiol	-0.026	0.019	24	-1.362	0.186
		Testosterone	-0.017	0.019	24	-0.915	0.369
	T24	Oestradiol	0.011	0.008	16	1.280	0.219
		Testosterone	-0.013	0.008	16	-1.621	0.125
	T48	Oestradiol	0.015	0.011	16	1.365	0.191
		Testosterone	-0.001	0.011	16	-0.084	0.934

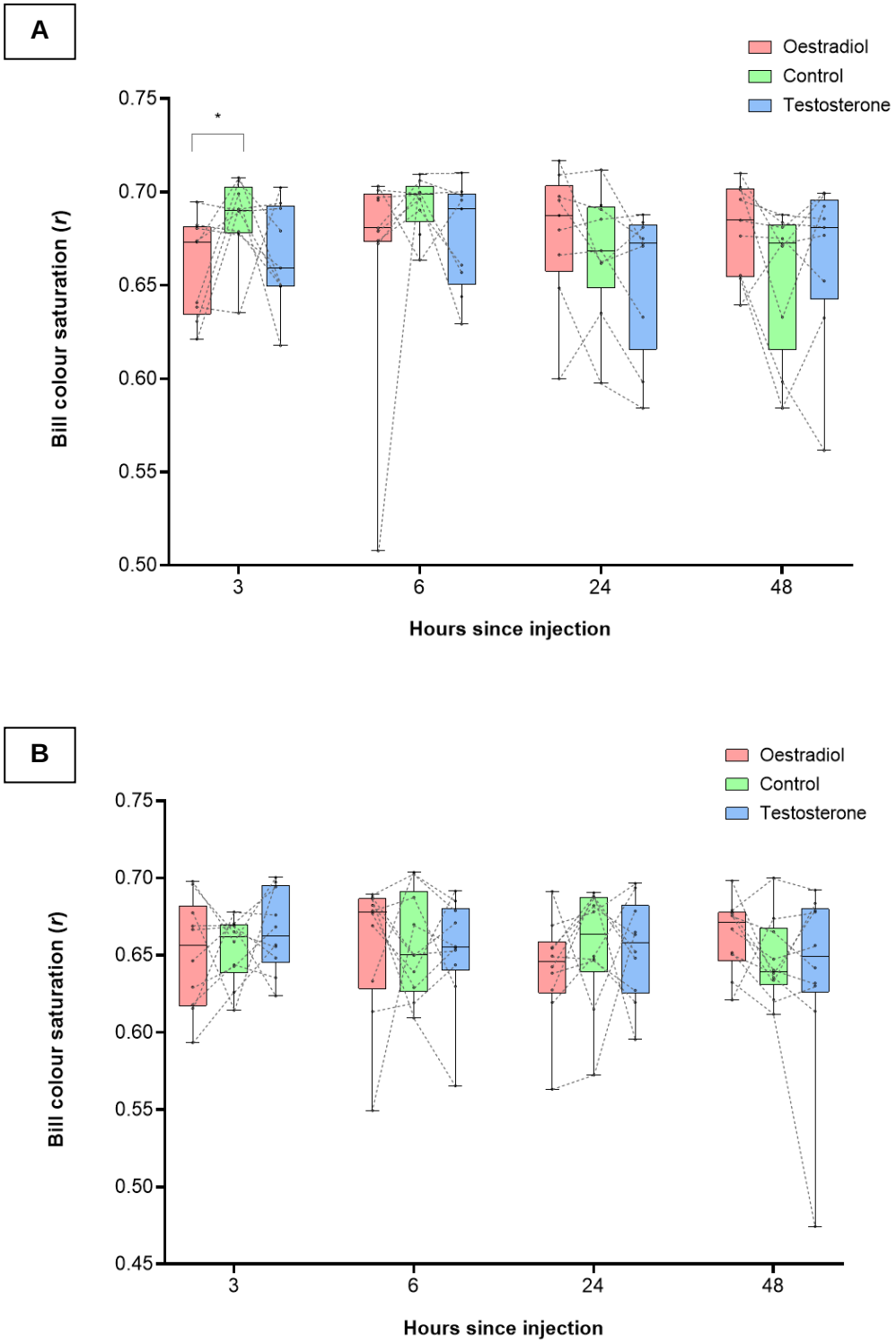

Figure 5 - Boxplots representing the effects of the sex steroids Oestradiol (pink boxplots) and Testosterone (blue boxplots) on male **(A)** and female **(B)** bill colour saturation (*r*), over time after injections. Green boxplots represent the control. (*) represents significant values. Dotted lines connect the same individual between treatments.

3.2 Long-term testosterone treatment

3.2.1 Testosterone effects on bill colour saturation (*r*)

In the long-term testosterone treatment, when assessing the fluctuations in bill colour saturation (*r*) for the entire 8 weeks that the implant was inserted, there were no significant changes in either males (t=1.802; N=12 birds; p=0.099; blue boxplots in Figure 6A) or females (t=0.041; N=10 birds; p=0.968; orange boxplots on Figure 6A). When assessing the fluctuations in bill colour saturation (*r*) considering the last 4 weeks that the implant was inserted, once again, no significant changes occurred in males (t=1.175; N=12 birds; p=0.265; blue boxplots in Figure 6B) or females (t=1.660; N=10 birds; p=0.1312; orange boxplots on Figure 6B).

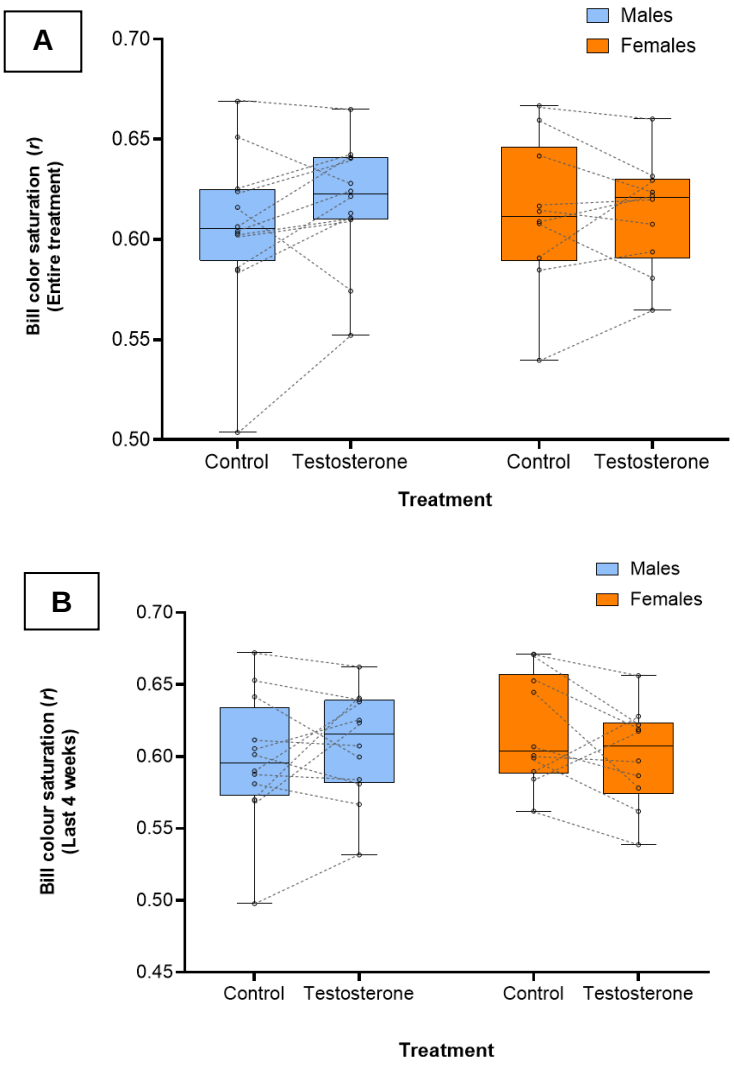

Figure 6 - Boxplots representing the effects of Testosterone on male (blue boxplots) and female (orange boxplots) bill colour saturation (*r*) throughout the entire treatment **(A)** and in the last four weeks **(B)**. Dotted lines connect the same individual between treatments.

3.2.2 Testosterone effects on common waxbill weight

The testosterone treatment did not significantly influence the weight of the common waxbills, either in males (t=-0.586; N=12 birds; p=0.569; blue boxplots in Figure 7), or in females (t=0.018; N=10 birds; p=0.986; orange boxplots in Figure 7).

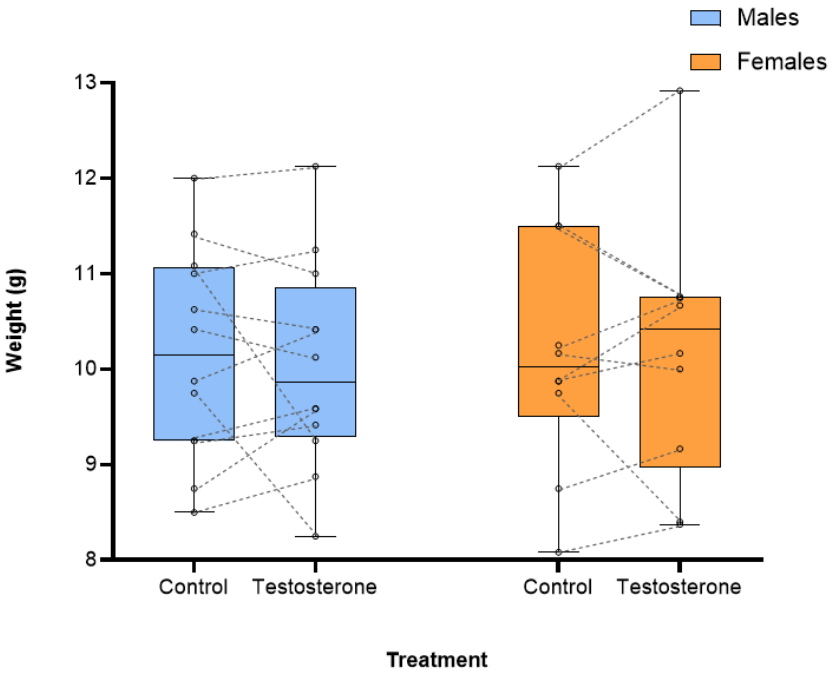

Figure 7 - Boxplots representing the effects of Testosterone in the common waxbill weight for males (blue boxplots) and females (orange boxplots). Dotted lines connect the same individual between treatments.

4. Discussion

In this study, the common waxbill was used as a model species to test the effects of long-term manipulation of testosterone and short-term manipulations of testosterone and oestradiol on bill colour ornamentation of the common waxbill (*Estrilda astrild*), specifically bill colour saturation (*r*). Bill colour saturation in this species is directly related to carotenoid pigment concentration in the bill (McGraw and Schuetz, 2004), and shows some degree of sexual dichromatism (Cardoso *et al.*, 2014). Previous work has shown that, in the common waxbill, bill red colour saturation is influenced by long-term treatments with oestradiol (Romero-Diaz *et al.*, 2022a), and also by environmental conditions (Cardoso *et al.*, 2014a; Freitas *et al.*, 2021; Funghi *et al.*, 2018).

Here, it was found that manipulating testosterone levels, whether in the short- or long-term treatments, did not lead to significant changes in bill colour saturation in either sex, contrary to the initial prediction that testosterone would enhance saturation in both males and females. Furthermore, long-term exposure to testosterone did not influence the weight of the common waxbills in either sex, suggesting that the treatment did not cause adverse health effects. Unlike the results observed in Romero-Diaz *et al.* (2022a) using long-term exposure to oestradiol, here short-term exposure to oestradiol did not affect bill colour saturation in females, but appeared to decrease bill colour saturation in males three hours after the injection. These results are further discussed below.

4.1 Testosterone effects on bill colour saturation (*r*)

Past research on various avian species has often demonstrated the effects of testosterone as a mechanism that influences colour ornamentation, especially carotenoid-based colour, enhancing the expression of ornaments, in both males and females (Ardia *et al.*, 2010; Casagrande *et al.*, 2011; Eens *et al.*, 2000; Perez-Beato, 2008; Serra *et al.*, 2024). However, in the common waxbill no significant changes in bill colour were found with testosterone manipulations. In both the long-term and short-term testosterone treatments, female bill colour saturation (*r*) did not differ significantly from the control treatment, suggesting that testosterone does not affect female bill colour, and it was very similar to male bill colour saturation, which can be explained with the fact that the common waxbill is not a sexually dichromatic species (Cardoso *et al.*, 2014a).

In males, no significant differences in bill colour saturation were found in the short-term testosterone treatment, but when treated with long-term testosterone there was a statistically non-significant suggestion for a small increase saturation. The lack of a significant effect could suggest that males are already expressing bill colour at near maximum capacity, which could minimize the impact of additional testosterone provided by the implant. To test this hypothesis, future research should consider analysing how male bill colour is affected by decreased levels of testosterone, for instance, by using anti-androgens or performing gonadectomy. Moreover, although the testosterone dosage used in the implants was based on studies with similar species and of similar size, the testosterone dosage could be insufficient to trigger changes in ornamentation. In this study, blood sampling was avoided to minimize disturbance to the individuals, but future work should include hormonal assays to measure baseline testosterone levels in common waxbills. This procedure could potentially enable a better and accurate determination of the testosterone dosage to use.

Another explanation for the absence of testosterone effects on bill colour saturation might lie on negative feedback in the hypothalamic-pituitary-gonadal (HPG) axis in response to high levels of testosterone. When testosterone levels rise, the HPG axis activates a negative feedback mechanism to prevent excessive hormone production. In this case, the hypothalamus detects the increased testosterone levels and reduces the secretion of gonadotropin-releasing hormone (GnRH). As a result, the pituitary gland decreases its release of luteinizing hormone (LH), an hormone responsible for stimulating the gonads to produce testosterone (Rosvall *et al.*, 2016). This decrease in LH could affect ornamentation in two ways: Previous research on LH indicates that it is necessary for the development of vibrant colours, and has been shown to be a key hormone in plumage of other passerine birds (Kimball and Ligon, 1999; Thapliyal and Tewary, 1963), therefore, its decrease could affect ornamentation. On the other hand, LH stimulates the gonads to produce testosterone through a chain of reactions (Rosvall *et al.*, 2016). This mechanism might have resulted in a reduction of endogenous testosterone, which balanced with the testosterone provided by the implant might have made testosterone-mediated changes in ornamentation stay undetected. Future studies should consider monitoring testosterone levels throughout the experiment, to better understand this negative feedback on the common waxbill.

Yet another possible explanation for the lack of testosterone-mediated changes in bill colour saturation in the common waxbill is the fact that testosterone induces oxidative stress and can weaken the immune system, even though no decline in physical condition was detected associated with the testosterone treatment. Only the healthiest

individuals can afford to invest resources in both ornamentation and managing adverse effects (Alonso-alvarez *et al.*, 2008; Folstad and Karter, 1992; Newhouse and Vernasco, 2020). This might help explain our results: testosterone may have disrupted the physiological health of the individuals, which might have originated carotenoid trade-offs between the bill and other tissues. This could have made changes in bill colour undetectable.

Results studying the common waxbill's bill colour, where testosterone administration did not affect ornamental colour, contrast with those on most other species studied so far. The Zebra finch (*Taeniopygia guttata*), another estrildid finch with a vibrant red bill, serves as a good comparison. In this species, testosterone has been shown to enhance bioavailability of carotenoids in zebra finches (McGraw, 2006), and to enhance bill colour both in short-term (Ardia *et al.*, 2010) and in long-term (McGraw, 2006) experiments.

There are key differences between the zebra finch and the common waxbill that perhaps could explain why testosterone influences bill ornamentation in one species but not the other. On one hand, zebra finches exhibit sexual dimorphism (Price and Burley, 1993), with males having conspicuously red bills while females have paler and more orange bills (Burley *et al.*, 1992). Zebra finch bill colour is mediated by testosterone (Ardia *et al.*, 2010; McGraw, 2006), and in males it signals aggressiveness and dominance (Green *et al.*, 2022). Also, zebra finch males appear to be more aggressive than females (Bolund *et al.*, 2007). Contrasting with the zebra finch, male and female common waxbills do not differ appreciably in aggressiveness or social dominance (Beltrao *et al.*, 2021a), there is minimal sexual dimorphism, with the sexes not being distinguishable based on their red colour ornamentation and males having on average only slightly more saturated red bills than females (Cardoso *et al.*, 2014a). Although testosterone increases aggression and dominance in common waxbills (Beltrao *et al.*, 2021b), bill colour does not signal aggressiveness or social dominance (Funghi *et al.*, 2018). Therefore, being a species without large sex differences in ornamentation or in social dominance, it makes sense that bill colour in common waxbills is not strongly determined by testosterone levels, since testosterone levels differ between the sexes and are related to aggression or social hierarchy.

Finally, previous research on the common waxbill has uncovered an interplay between environmental factors and female ornamentation, suggesting that bill colour in females is more plastic and flexible than in males. This plasticity could reflect the evolutionary need for females to carefully balance their investment in ornamentation with

the imperative of maintaining reproductive health. Freitas *et al.* (2021) demonstrated that female ornamentation tends to be particularly sensitive to diet and environmental conditions, and Funghi *et al.* (2018) has shown that sexual dichromatism tends to diminish when ecological conditions are favourable, allowing females to express more colour ornamentation. These results suggest that in more favourable environments, the pressure on females to invest less on ornamentation is reduced. This way, in the common waxbill, bill colour and sexual dimorphism appear to be primarily regulated by females, leading to a convergence in appearance between the sexes. This greater plasticity of female than male investment in colour ornamentation may be an additional reason for the evolution of mechanisms that are less dependent on testosterone.

4.2 Short-term oestradiol effects on bill colour saturation (*r*)

In females, no significant results were found in the short-term oestradiol treatment, suggesting that rapid exposures to high levels of oestradiol are insufficient to induce changes in bill colour saturation. In Romero-Diaz *et al.* (2022a), long-term exposure to oestradiol decreased bill colour in common waxbill females, specifically hue and saturation, increasing sexual dichromatism. Although bill colour is a plastic trait capable of changing in short periods of time, the differences in these results may be explained by the fact that females already have high basal oestradiol levels, in addition to a possible delay in the adaptation time of the female common waxbill's bill to this hormonal change. This suggests that when oestradiol levels are high, but stable, the hormone may induce a response in carotenoid concentration in the bill, which is not achieved when the hormonal increase is sudden and short in time. One hypothesis proposed by Romero-Diaz *et al.* (2022a) was that oestradiol might interact with hormone-responsive genes that regulate carotenoid-based coloration, such as genes involved in carotenoid deposition, inducing to a negative feedback (Gazda *et al.*, 2020). The short time that oestradiol levels were high in waxbill's blood in the present study might not have been enough to trigger this genetic process.

In males, three hours after the oestradiol injection, bill colour saturation (*r*) decreased, but reversed to similar values to the control measurements after another three hours, and afterwards. Male bill colour response to oestradiol may thus be more acute than in females. Interestingly, while males do show an acute response to oestradiol, this change is rapidly corrected to maintain a consistent signal. This temporary decrease in male bill colour saturation could also be explained by the negative

feedback in the HPG axis, which probably did not affect females because they have higher baseline levels of oestradiol: increased oestradiol levels trigger the HPG axis that supresses hormone secretion, which reduces the secretion of GnRH and consequently decreases the release of LH, resulting in duller coloration (Kimball and Ligon, 1999; Romero-Diaz *et al.*, 2022a; Rosvall *et al.*, 2016).

5.Conclusion

Bill colour in the common waxbill has been shown to be influenced by long-term exposures to oestradiol and environmental factors in females (Freitas *et al.*, 2021; Funghi *et al.*, 2018; Romero-Diaz *et al.*, 2022a). Although testosterone has been shown to mediate the expression of carotenoid-based ornamentation in many species (Ardia *et al.*, 2010; Casagrande *et al.*, 2011; Eens *et al.*, 2000; Laucht *et al.*, 2010), bill colour of the common waxbill appears not to be greatly influenced by this hormone, in either sex. Furthermore, short-term exposure to oestradiol seems to temporarily decrease bill colour saturation in males, but it reverses after a few hours.

Bringing together these experiments with previous ones on common waxbills (*Estrilda astrild*), the conclusion seems to be that in this species, males express bill colour at near maximum capacity and sexual dimorphism is primarily regulated by females (Freitas *et al.*, 2021; Funghi *et al.*, 2018; Romero-Diaz *et al.*, 2022a). Their greater flexibility and heightened sensitivity to environmental and hormonal changes positions them as the key drivers in modulating bill colouration differences. This adaptive capacity likely serves as a critical strategy for balancing the demands of ornamentation with the overarching goal of reproductive success.

References

Adkins-Regan, E. (1999). Testosterone increases singing and aggression but not male-typical sexual partner preference in early estrogen treated female zebra finches. *Hormones and Behavior*, *35*(1), 63-70.

Adkins-Regan, E. (2005). *Hormones and animal social behavior*. Princeton University Press.

Adkins-Regan, E., and Ascenzi, M. (1987). Social and sexual behaviour of male and female zebra finches treated with oestradiol during the nestling period. *Animal behaviour*, *35*(4), 1100-1112.

Alonso-alvarez, C., Pérez-rodríguez, L., Mateo, R., Chastel, O., and Viñuela, J. (2008). The oxidation handicap hypothesis and the carotenoid allocation trade-off. *Journal of evolutionary biology*, *21*(6), 1789-1797.

Amundsen, T. (2000a). Female ornaments: genetically correlated or sexually selected. *Animal signals: Signalling and Signal Design in Animal Communication*, 133-154.

Amundsen, T. (2000b). Why are female birds ornamented? *Trends in Ecology & Evolution*, *15*(4), 149-155.

Andersson, M. (1994). *Sexual selection* (Vol. 72). Princeton University Press.

Ardia, D. R., Broughton, D. R., & Gleicher, M. J. (2010). Short-term exposure to testosterone propionate leads to rapid bill color and dominance changes in zebra finches. *Hormones and Behavior*, *58*(3), 526-532.

Arnqvist, G., and Rowe, L. (2005). *Sexual conflict* (Vol. 31). Princeton university press.

Assersohn, K., Brekke, P., & Hemmings, N. (2021). Physiological factors influencing female fertility in birds. *Royal Society Open Science*, *8*(7), 202274.

Bates D, Ma¨chler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw. 2015; 67(1). Available from: http://www.jstatsoft.org/v67/i01/

Beltrao, P., Marques, C. I., Cardoso, G. C., & Gomes, A. C. R. (2021a). Plumage colour saturation predicts long-term, cross-seasonal social dominance in a mutually ornamented bird. *Animal behaviour*, *182*, 239-250.

Beltrao, P., Silva, P. A., Soares, M. C., Cardoso, G. C., & Trigo, S. (2021b). Testosterone treatment produces sex-dependent effects in social dominance. *Animal behaviour*, *179*, 307-315.

Beltrão, P., Gomes, A. C. R., Marques, C. I., Guerra, S., Batalha, H. R., & Cardoso, G. C. (2021c). European breeding phenology of the invasive common waxbill, a sub-Saharan opportunistic breeder. *acta ethologica*, *24*(3), 197-203.

Bendich, A. (1989). Carotenoids and the immune response. *The Journal of nutrition*, *119*(1), 112-115.

Berglund, A., Bisazza, A., & Pilastro, A. (1996). Armaments and ornaments: an evolutionary explanation of traits of dual utility. *Biological Journal of the Linnean Society*, *58*(4), 385-399.

Blount, J. D., and McGraw, K. J. (2008). Signal functions of carotenoid colouration. *Carotenoids: Volume 4: Natural Functions*, 213-236.

Blount, J. D., Surai, P. F., Nager, R. G., Houston, D. C., Møller, A. P., Trewby, M. L., & Kennedy, M. W. (2002). Carotenoids and egg quality in the lesser black-backed gull Larus fuscus: a supplemental feeding study of maternal effects. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *269*(1486), 29-36.

Bolund, E., Schielzeth, H., & Forstmeier, W. (2007). Intrasexual competition in zebra finches, the role of beak colour and body size. *Animal behaviour*, *74*(4), 715-724.

Britton, G. (1983). *The biochemistry of natural pigments*. Cambridge University Press.

Brush, A. H. (1990). Metabolism of carotenoid pigments in birds. *The FASEB Journal*, *4*(12), 2969-2977.

Burley, N. T., Price, D. K., & Zann, R. A. (1992). Bill color, reproduction and condition effects in wild and domesticated zebra finches. *The Auk*, *109*(1), 13-23.

Cardoso, G., Leitão, A., Funghi, C., Batalha, H., Lopes, R., & Mota, P. (2014a). Similar preferences for ornamentation in opposite-and same-sex choice experiments. *Journal of evolutionary biology*, *27*(12), 2798-2806.

Cardoso, G. C., Batalha, H. R., Reis, S., & Lopes, R. J. (2014b). Increasing sexual ornamentation during a biological invasion. *Behavioral Ecology*, *25*(4), 916-923.

Casagrande, S., Dijkstra, C., Tagliavini, J., Goerlich, V. C., & Groothuis, T. G. (2011). Differential effects of testosterone, dihydrotestosterone and estradiol on carotenoid deposition in an avian sexually selected signal. *Journal of Comparative Physiology A*, *197*, 1-13.

Catry, P., Costa, H., Elias, G., & Matias, R. (2010). *Aves de Portugal: Ornitologia do território continental*. Assirio & Alwin.

Chew, B. P., and Park, J. S. (2004). Carotenoid action on the immune response. *The Journal of nutrition*, *134*(1), 257S-261S.

Clement, P. (1999). *Finches and sparrows: an identification guide.* Princeton University Press.

Clutton-Brock, T. (2009). Sexual selection in females. *Animal behaviour*, *77*(1), 3-11.

Cynx, J., Bean, N. J., & Rossman, I. (2005). Testosterone implants alter the frequency range of zebra finch songs. *Hormones and Behavior*, *47*(4), 446-451.

Dale, J. (2006). Intraspecific variation in coloration. *Bird coloration*, *2*, 36-86.

Dale, J., Dey, C. J., Delhey, K., Kempenaers, B., & Valcu, M. (2015). The effects of life history and sexual selection on male and female plumage colouration. *Nature*, *527*(7578), 367-370.

Darwin, C. (1871). *The descent of man, and selection in relation to sex* (Vol. 1). John Murray.

De Ridder, E., Pinxten, R., Mees, V., & Eens, M. (2002). Short-and long-term effects of male-like concentrations of testosterone on female European starlings (Sturnus vulgaris). *The Auk*, *119*(2), 487-497.

Dey, C. J., Valcu, M., Kempenaers, B., & Dale, J. (2015). Carotenoid-based bill coloration functions as a social, not sexual, signal in songbirds. *Journal of evolutionary biology*, *28*(1), 250-258.

Dunn, P. O., Garvin, J. C., Whittingham, L. A., Freeman-Gallant, C. R., & Hasselquist, D. (2010). Carotenoid and melanin-based ornaments signal similar aspects of male quality in two populations of the common yellowthroat. *Functional Ecology*, *24*(1), 149-158.

Eens, M., Van Duyse, E., Berghman, L., & Pinxten, R. (2000). Shield Characteristics Are Testosterone-Dependent in Both Male and Female Moorhens. *Hormones and Behavior*, *37*(2), 126-134.

Emlen, D. J. (2008). The evolution of animal weapons. *Annual review of ecology, evolution, and systematics*, *39*, 387-413.

Enbody, E. D., Boersma, J., Schwabl, H., & Karubian, J. (2018). Female ornamentation is associated with elevated aggression and testosterone in a tropical songbird. *Behavioral Ecology*, *29*(5), 1056-1066.

Folstad, I., and Karter, A. J. (1992). Parasites, bright males, and the immunocompetence handicap. *The American Naturalist*, *139*(3), 603-622.

Foo, Y. Z., Nakagawa, S., Rhodes, G., & Simmons, L. W. (2017). The effects of sex hormones on immune function: a meta-analysis. *Biological Reviews*, *92*(1), 551-571.

Fox, D. L. (1976). *Animal biochromes and structural colours: physical, chemical, distributional & physiological features of coloured bodies in the animal world*. Univ of California Press.

Freitas, R., Marques, C., Cardoso, G. C., & Trigo, S. (2021). Ecological effects on female bill colour explain plastic sexual dichromatism in a mutually-ornamented bird. *Scientific Reports*, *11*(1), 14970.

Funghi, C., Trigo, S., Gomes, A. C. R., Soares, M. C., & Cardoso, G. C. (2018). Release from ecological constraint erases sex difference in social ornamentation. *Behavioral ecology and sociobiology*, *72*, 1-12.

Gazda, M. A., Araújo, P. M., Lopes, R. J., Toomey, M. B., Andrade, P., Afonso, S., Marques, C., Nunes, L., Pereira, P., Trigo, S., Hill, G. E., Corbo, J. C., & Carneiro, M. (2020). A genetic mechanism for sexual dichromatism in birds. *Science*, *368*(6496), 1270-1274.

Ghiselin, M. T., Leonard, J., & Córdoba-Aguilar, A. (2010). The distinction between primary and secondary sexual characters. *The evolution of primary sexual characters in animals*, 9-14.

Gomes, A. C. R., Sorenson, M. D., & Cardoso, G. C. (2016). Speciation is associated with changing ornamentation rather than stronger sexual selection. *Evolution*, *70*(12), 2823-2838.

Green, P. A., George, E. M., Rosvall, K. A., Johnsen, S., & Nowicki, S. (2022). Testosterone, signal coloration, and signal color perception in male zebra finch contests. *Ethology*, *128*(2), 131-142.

Harris, A., and Davis, J. (1993). *Finches and sparrows: an identification guide*. In: London: Christopher Helm.

Hau, M., & Goymann, W. (2015). Endocrine mechanisms, behavioral phenotypes and plasticity: known relationships and open questions. *Frontiers in Zoology*, *12*(Suppl 1), S7.

Hill, G. E. (2006). 4. Female Mate Choice for Ornamental Coloration. In E. H. Geoffrey (Ed.), *Bird coloration* (pp. 137-200). Harvard University Press.

Hill, G. E., and McGraw, K. J. (2006). *Bird coloration* (Vol. 1). Harvard University Press.

Hill, G. E., Hood, W. R., & Huggins, K. (2009). A multifactorial test of the effects of carotenoid access, food intake and parasite load on the production of ornamental feathers and bill coloration in American goldfinches. *Journal of Experimental Biology*, *212*(8), 1225-1233.

Hill, G. E., and Yasukawa, K. (2014). The evolution of ornaments and armaments. *Animal behavior: how and why animals do the things they do*, *2*, 145-172.

Hill, G. E. (2015). Sexiness, individual condition, and species identity: the information signaled by ornaments and assessed by choosing females. *Evolutionary Biology*, *42*(3), 251-259.

Hipfner, J. M., Dale, J., & McGraw, K. J. (2010). Yolk carotenoids and stable isotopes reveal links among environment, foraging behavior and seabird breeding success. *Oecologia*, *163*, 351-360.

Hõrak, P., Ots, I., Vellau, H., Spottiswoode, C., & Pape Møller, A. (2001). Carotenoid-based plumage coloration reflects hemoparasite infection and local survival in breeding great tits. *Oecologia*, *126*, 166-173.

Houstoun, R., and Shearer, J. F. (1930). Fechner's law. *Nature*, *125*(3163), 891-892.

Jawor, J. M., Gray, N., Beall, S. M., & Breitwisch, R. (2004). Multiple ornaments correlate with aspects of condition and behaviour in female northern cardinals, Cardinalis cardinalis. *Animal behaviour*, *67*(5), 875-882.

Karadas, F., Pappas, A. C., Surai, P. F., & Speake, B. K. (2005). Embryonic development within carotenoid-enriched eggs influences the post-hatch carotenoid status of the chicken. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *141*(2), 244-251.

Karubian, J., Lindsay, W. R., Schwabl, H., & Webster, M. S. (2011). Bill coloration, a flexible signal in a tropical passerine bird, is regulated by social environment and androgens. *Animal behaviour*, *81*(4), 795-800.

Kimball, R. T., and Ligon, J. D. (1999). Evolution of Avian Plumage Dichromatism from a Proximate Perspective. *The American Naturalist*, *154*(2), 182-193.

Kraaijeveld, K., Kraaijeveld-Smit, F. J. L., & Komdeur, J. (2007). The evolution of mutual ornamentation. *Animal behaviour*, *74*(4), 657-677.

Lande, R. (1980). Sexual dimorphism, sexual selection, and adaptation in polygenic characters. *Evolution*, 292-305.

Landmann, A., and Kollinsky, C. (1995). Age and plumage related territory differences in male black redstarts: the (non)-adaptive significance of delayed plumage maturation. *Ethology ecology & evolution*, *7*(2), 147-167.

Laucht, S., and Dale, J. (2012). Development of badges of status in captive male house sparrows (Passer domesticus) in relation to the relative ornamentation of flock-mates. *Ethology*, *118*(7), 644-653.

Laucht, S., Kempenaers, B., & Dale, J. (2010). Bill color, not badge size, indicates testosterone-related information in house sparrows. *Behavioral ecology and sociobiology*, *64*, 1461-1471.

Leonard, J., and Córdoba-Aguilar, A. (2010). *The evolution of primary sexual characters in animals*. Oxford University Press.

Lindsay, W. R., Barron, D. G., Webster, M. S., & Schwabl, H. (2016). Testosterone activates sexual dimorphism including male-typical carotenoid but not melanin plumage pigmentation in a female bird. *Journal of Experimental Biology*, *219*(19), 3091-3099.

Lopez-Rull, I., and Gil, D. (2009). Elevated testosterone levels affect female breeding success and yolk androgen deposition in a passerine bird. *Behavioural Processes*, *82*(3), 312-318.

Lucas, A. M. (1972). *Avian anatomy integument* (No.362). Avian Anatomy Project, Poultry Research Branch, Animal Science Research Division, Agricultural Research Service, US Department of Agrculture.

Lyon, B. E., and Montgomerie, R. (2012). Sexual selection is a form of social selection. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *367*(1600), 2266-2273.

Martínez-Padilla, J., Pérez-Rodríguez, L., Mougeot, F., Ludwig, S., & Redpath, S. M. (2014). Intra-sexual competition alters the relationship between testosterone and ornament expression in a wild territorial bird. *Hormones and Behavior*, *65*(5), 435-444.

Martínez-Padilla, J., Mougeot, F., Webster, L. M. I., Pérez-Rodríguez, L., & Piertney, S. B. (2010). Testing the interactive effects of testosterone and parasites on carotenoid-based ornamentation in a wild bird. *Journal of evolutionary biology*, *23*(5), 902-913.

McGraw, K. J., and Ardia, D. R. (2003). Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. *The American Naturalist*, *162*(6), 704-712.

McGraw, K. J. (2004). Colorful songbirds metabolize carotenoids at the integument. *Journal of Avian Biology*, *35*(6), 471-476.

McGraw, K., and Schuetz, J. (2004). The evolution of carotenoid coloration in estrildid finches: a biochemical analysis. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *139*(1), 45-51.

McGraw, K. J. (2006). Sex steroid dependence of carotenoid-based coloration in female zebra finches. *Physiology & Behavior*, *88*(4), 347-352.

McGraw, K. J., Correa, S. M., & Adkins-Regan, E. (2006a). Testosterone upregulates lipoprotein status to control sexual attractiveness in a colorful songbird. *Behavioral ecology and sociobiology*, *60*, 117-122.

McGraw, K., Nolan, P., & Crino, O. (2006b). Carotenoid accumulation strategies for becoming a colourful House Finch: analyses of plasma and liver pigments in wild moulting birds. *Functional Ecology*, *20*(4), 678-688.

McGraw, K. J., and Ardia, D. R. (2007). Do carotenoids buffer testosterone-induced immunosuppression? An experimental test in a colourful songbird. *Biology Letters*, *3*(4), 375-378.

Moller, A., Biard, C., Blount, J., Houston, D., Ninni, P., Saino, N., & Surai, P. (2000). Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? *Poultry and Avian Biology Reviews*, *11*(3), 137-160.

Moller, A., and Pomiankowski, A. (1993). Why have birds got multiple sexual ornaments? *Behavioral ecology and sociobiology*, *32*, 167-176.

Montgomerie, R. (2006). Analyzing colors. In: Hill, GE and McGraw, KJ (eds). *Bird coloration, volume 1*: *Mechanisms and Measurements* (pp. 90-147). Cambridge: Harvard University Press, Cambridge, 90-147.

Mougeot, F., Pérez-Rodríguez, L., Martínez-Padilla, J., Leckie, F., & Redpath, S. (2007). Parasites, testosterone and honest carotenoid-based signalling of health. *Functional Ecology*, 886-898.

Navara, K. J., and Hill, G. E. (2003). Dietary carotenoid pigments and immune function in a songbird with extensive carotenoid-based plumage coloration. *Behavioral Ecology*, *14*(6), 909-916.

Nelson, R. J. (2005). *An introduction to behavioral endocrinology*. Sinauer Associates.

Newhouse, D. J., and Vernasco, B. J. (2020). Developing a transcriptomic framework for testing testosterone-mediated handicap hypotheses. *General and Comparative Endocrinology*, *298*, 113577.

Norris, D., and Carr, J. (2013). Comparative aspects of vertebrate reproduction. *Vertebrate Endocrinology. Springer US*, 375-441.

Norris, D. O., and Lopez, K. H. (2010). *Hormones and reproduction of vertebrates* (Vol. 1). Academic Press.

Owens, I. P. F., and Short, R. V. (1995). Hormonal basis of sexual dimorphism in birds: implications for new theories of sexual selection. *Trends in Ecology & Evolution*,

Perez-Beato, O. (2008). *Fundamentals of color genetics in canaries: reproduction and control*. Rosedog Press.

Pérez-Rodríguez, L. (2008). Carotenoid-based ornamentation as a dynamic but consistent individual trait. *Behavioral ecology and sociobiology*, *62*, 995-1005.

Pérez-Rodríguez, L. (2009). Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. *BioEssays*, *31*(10), 1116-1126.

Pérez-Rodríguez, L., Martínez-Padilla, J., & Mougeot, F. (2013). Carotenoid-based ornaments as signals of health status in birds: evidences from two galliform species, the red-legged partridge (*Alectoris rufa*) and the red grouse (*Lagopus lagopus scoticus*). *Carotenoids: food sources, production and health benefits.* Nova Science Publishers*,* pp. 173-198.

Pham, T. T., Queller, P., Tarvin, K. A., & Murphy, T. G. (2014). Honesty of a dynamic female aggressive status signal: baseline testosterone relates to bill color in female American goldfinches. *Journal of Avian Biology*, *45*(1), 22-28.

Price, D. K., and Burley, N. T. (1993). Constraints on the evolution of attractive traits: genetic (co) variance of zebra finch bill colour. *Heredity*, *71*(4), 405-412.

R Core Team. (2024). Team R: A language and environment for statistical computing. R Foundation for Statistical Computing*, Vienna, Austria. URL* https://www*. R-project. org*.

Reino, L., and Silva, T. (1998). The distribution and expansion of the Common Waxbill (Estrilda astrild) in the Iberian Peninsula. *Biol Cons Fauna*, *102*, 163-167.

Roberts, M. L., Buchanan, K. L., & Evans, M. R. (2004). Testing the immunocompetence handicap hypothesis: a review of the evidence. *Animal behaviour*, *68*(2), 227-239.

Roberts, M. L., Buchanan, K. L., Hasselquist, D., & Evans, M. R. (2007). Effects of testosterone and corticosterone on immunocompetence in the zebra finch. *Hormones and Behavior*, *51*(1), 126-134.

Romero-Diaz, C., Silva, P. A., Cardoso, G. C., & Trigo, S. (2022b). Evaluating different metrics to study small color differences: the red bill and plumage of common waxbills as a case study. *Behavioral ecology and sociobiology*, *76*(9), 126.

Romero-Diaz, C., Silva, P. A., Soares, M. C., Cardoso, G. C., & Trigo, S. (2022a). Oestradiol reduces female bill colour in a mutually ornamented bird. *Proceedings of the Royal Society B*, *289*(1984), 20221677.

Rosenthal, M. F., Murphy, T. G., Darling, N., & Tarvin, K. A. (2012). Ornamental bill color rapidly signals changing condition. *Journal of Avian Biology*, *43*(6), 553-564.

Rosvall, K. A., Bergeon Burns, C. M., Jayaratna, S. P., Dossey, E. K., & Ketterson, E. D. (2016). Gonads and the evolution of hormonal phenotypes. *Integrative and Comparative Biology*, *56*(2), 225-234.

Saino, N., Ferrari, R., Romano, M., Martinelli, R., & Møller, A. P. (2003). Experimental manipulation of egg carotenoids affects immunity of barn swallow nestlings. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1532), 2485-2489.

Saks, L., Ots, I., & Hõrak, P. (2003). Carotenoid-based plumage coloration of male greenfinches reflects health and immunocompetence. *Oecologia*, *134*, 301-307.

Schlinger, B. A., and Arnold, A. P. (1992). Plasma sex steroids and tissue aromatization in hatchling zebra finches: implications for the sexual differentiation of singing behavior. *Endocrinology*, *130*(1), 289-299.

Schreiber, A. M. (2023). *General and Comparative Endocrinology: An Integrative Approach*. CRC Press.

Searcy William, A., and Nowicki, S. (2005). *The evolution of animal communication: Reliability and deception in signaling systems*. Princeton University Press.

Serra, L., Griggio, M., Casasole, G., Pirrello, S., Fusani, L., & Pilastro, A. (2024). Elevated testosterone levels during moult have contrasting effects on structural and carotenoid-based plumage colours in Eurasian Blue Tits. *Ibis*.

Seyfarth, R. M., and Cheney, D. L. (2012). The evolutionary origins of friendship. *Annual review of psychology*, *63*(1), 153-177.

Silva, T., Reino, L. M., & Borralho, R. (2002). A model for range expansion of an introduced species: the common waxbill Estrilda astrild in Portugal. *Diversity and Distributions*, *8*(6), 319-326.

Smith, J. M. (1982). Evolution and the Theory of Games. In *Did Darwin get it right? Essays on games, sex and evolution* (pp. 202-215). Springer.

Smith, J. M., and Harper, D. (2003). *Animal signals*. Oxford University Press.

Staub, N. L., and De Beer, M. (1997). The role of androgens in female vertebrates. *General and Comparative Endocrinology*, *108*(1), 1-24.

Stoddard, M. C., and Prum, R. O. (2008). Evolution of avian plumage color in a tetrahedral color space: a phylogenetic analysis of new world buntings. *The American Naturalist*, *171*(6), 755-776.

Svensson, and Wong. (2011). Carotenoid-based signals in behavioural ecology: a review. *Behaviour*, *148*(2), 131-189.

Thapliyal, J., and Tewary, P. (1963). Effect of estrogen and gonadotropic hormones on the plumage pigmentation in Lal munia (Estrilda amandava).

Tobias, J. A., Montgomerie, R., & Lyon, B. E. (2012). The evolution of female ornaments and weaponry: social selection, sexual selection and ecological competition. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *367*(1600), 2274-2293.

Tsuboi, M., Gonzalez-Voyer, A., Höglund, J., & Kolm, N. (2012). Ecology and mating competition influence sexual dimorphism in Tanganyikan cichlids. *Evolutionary Ecology*, *26*, 171-185.

Vergara, P., and Martínez-Padilla, J. (2012). Social context decouples the relationship between a sexual ornament and testosterone levels in a male wild bird. *Hormones and Behavior*, *62*(4), 407-412.

Vergara, P., Martínez-Padilla, J., Mougeot, F., Leckie, F., & Redpath, S. (2012a). Environmental heterogeneity influences the reliability of secondary sexual traits as condition indicators. *Journal of evolutionary biology*, *25*(1), 20-28.

Vergara, P., Mougeot, F., Martínez-Padilla, J., Leckie, F., & Redpath, S. M. (2012b). The condition dependence of a secondary sexual trait is stronger under high parasite infection level. *Behavioral Ecology*, *23*(3), 502-511.

von Engelhardt, N., Dijkstra, C., Daan, S., & Groothuis, T. G. G. (2004). Effects of 17-β-estradiol treatment of female zebra finches on offspring sex ratio and survival. *Hormones and Behavior*, *45*(5), 306-313.

Vorobyev, M., Marshall, J., Osorio, D., Hempel de Ibarra, N., & Menzel, R. (2001). Colourful objects through animal eyes. *Color Research & Application, 26*(S1), S214-S217.

White, T., Maia, R., Gruson, H., Endler, J., Eliason, C., Bitton, P.-P., & White, M. T. (2023). Package 'pavo'.

Willson, M. F. (1979). Sexual selection in plants. *The American Naturalist*, *113*(6), 777-790.

Wingfield, J. (1994). Hormone-behavior interactions and mating systems in male and female birds. *The differences between the sexes*, *303*, 330.

Zahavi, A. (1975). Mate selection—A selection for a handicap. *Journal of theoretical biology*, *53*(1), 205-214.

Zera, A. J., and Harshman, L. G. (2001). The physiology of life history trade-offs in animals. *Annual review of Ecology and Systematics*, *32*(1), 95-126.