Environmental and social modulation of hormones and behavior in a free living songbird, the black redstart

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München, den 2. Dezember 2014

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Author contributions

In this thesis I present my doctoral work that was performed at the Max Planck Institute for Ornithology, Seewiesen and was carried out from December 2009 until December 2014. For the first three experimental studies (chapter 1-3), I performed most of the field work, data collection, data analysis and writing of the manuscripts under guidance of my supervisor Dr. Wolfgang Goymann, who also contributed to field work and writing. Dr. Beate Apfelbeck contributed to the field work (chapters 1 & 3). Dr. Julio Blas contributed to field work and helped with acquisition of permits necessary to perform field work in Andalucia, Spain (chapter 2). Chapter 4 was performed in collaboration with my work group. I contributed to field work and brain analyses, Dr. Beate Apfelbeck wrote the main body of the manuscript together with Dr. Wolfgang Goymann. Kim G. Mortega, Dr. Sarah Kiefer, and Prof. Dr. Silke Kipper contributed to field work and song analyses. Dr. Michiel Vellema contributed to the brain analyses. All coauthors contributed to the writing of the manuscript.

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Summary

Most organisms live in a seasonal environment and adjust their adult life cycle within different life-history stages. Endocrine secretions are important modulators of life history stages in most vertebrates. The steroid hormone testosterone affects several traits of animals including physiology, morphology and behavior. Seasonal elevation of testosterone often correlates with aggressive and mating behaviors and may interfere with parental care. In addition, in socially monogamous species, male-male interactions trigger testosterone increases. This implies that testosterone influences aggressive behavior, but aggressive behavior can also feed-back on testosterone levels. Testosterone has been related to territorial and mating behavior in a wide variety of taxa. However these relationships are not always straightforward. For example, black redstarts (*Phoenicurus ochruros*) are socially monogamous birds and territorial, but not only during breeding. They defend territories also outside a breeding context. Interestingly, they do not increase testosterone during male-male challenges. The objective of my PhD study was to investigate the role of testosterone and its relationship with various behaviors during different life-history stages, as well as looking for potential environmental factors affecting the modulation of testosterone in male black redstarts. I correlated testosterone with aggressive behavior during different life-history stages and contexts. In addition I investigated the relationship between testosterone, parental care, and extra-pair behavior. The results show that although testosterone levels were higher at the beginning of the breeding season, aggressive behavior was stable among stages. Further, testosterone concentrations did not increase during male-male challenges regardless of context. Also, testosterone did not correlate with parental care behavior, but there was a connection between low levels of testosterone during the parental phase and paternity loss. In combination with prior work, the data suggest that testosterone plays only a minor role in the control of territorial behavior and it does not interfere with parental care, but it may be related with mating behavior in this species. I propose that the control of territorial and parental care behaviors in species that are territorial throughout their annual life cycle can be decoupled from testosterone. In summary, my studies increases the growing evidence that current theory regarding hormone-behavior relationship needs to be refined, and that there is still much to learn about how hormones and behavior relate to each other.

General introduction

The main focus of my thesis was to investigate the relationship of hormones and behavior in a songbird, the black redstart (Phoenicurus ochruros). To introduce the topic I start by referring to how hormone-behavior relationships have been studied from a historical perspective and how it progressed from studies in captivity to studies with wild animals living in their natural habitat. Then I explain how hormones function and how they can affect behavior through action in the brain. I then focus on how behavior can affect hormone secretion, to finally state the aim of my work.

Behavioral endocrinology is the field that studies the interaction between hormones and behavior. The beginning of the discipline goes back to 1849 to what is considered the first true experiment in classical endocrinology done by Berthold (1849). He observed that castrated cockerels were smaller and failed to engage in aggressive and mating behaviors compared to normal roosters. However, after re-implantation or transplantation of testes, he could rescue the behavioral and morphological characteristics of males. With this experiment Berthold concluded that there is a substance that travels through the blood that is independent of the nervous system, but important for the normal development and behavior of roosters. Later it was discovered that testes are the main gland producing the sexual hormone testosterone. Follow-up castration experiments, together with the application of exogenous hormones, demonstrated a causal relationship between sexual and aggressive behaviors (Beach and Holz, 1946; Beach and Inman, 1965). Because of its’ pleiotropic effects, the steroid hormone testosterone influences numerous physiological, morphological and behavioral traits. Testosterone is responsible for spermatogenesis, the expression of secondary sexual traits and muscular hypertrophy (Adkins-Regan, 2005; Nelson, 2005). In addition, it has been described that testosterone influences courtship, mating, singing and aggressive behavior in various species (see Wingfield et al., 2001 for review).

The effect of hormones on morphology, physiology and behavior has been extensively studied in laboratory animals (Wingfield, 2005). However, with the development of less invasive techniques and sensitive immunoassays, the study of hormones and behavior has been extended to free living animals, mainly using birds as model species (Fusani, 2008; Konishi et al., 1989; Wingfield, 2005). One of the first studies to report the relation between environmental changes, endocrine system and behavior was conducted in 1925. Rowan (1925) described that changes in environmental cues, such as day length, affect gonadal development and migration in birds. This study led to a growing field of research on environmental control of breeding (Konishi et al., 1989). More recent studies propose that adult bird species adjust their life cycle to successive life-history stages in response to predictable changes of environmental cues (e.g. day length; Jacobs and Wingfield, 2000; Wingfield, 2008). The majority of bird species undergo at least three life-history stages: breeding, molting and non-breeding. It gets more complex when they breed in more severe or changing environments (Jacobs and Wingfield, 2000; Wingfield, 2008). For example, many species migrate to spend the winter in regions with a milder climate. This adds two more life-history stages, vernal and autumn migration. Environmental cues together with neuroendocrine mechanisms regulate each life-history stage (Wingfield, 2008). The neuroendocrine mechanisms of the breeding stage are the most frequently studied and best-known so far.

Environmental or social cues activate the hypothalamic–pituitary–gonadal axis or “HPG axis” to time the breeding stage. Upon receiving cues from the environment, the hypothalamus secretes gonadotropin releasing hormone (GnRH) into the pituitary portal system. In the anterior pituitary, GnRH then triggers the release of luteinizing hormone (LH). LH enters the blood circulation and stimulates testosterone production in the Leydig cells of the testes. High levels of testosterone in turn inhibit the production of LH and GnRH, thus acting as a negative feedback of its own production. Therefore, the stimulation of the HPG axis elevates the production of plasma testosterone to prepare males for reproduction. In most free-ranging male vertebrates, plasma testosterone varies seasonally. The highest levels of testosterone are reached at the beginning of the breeding stage, when males compete for territories and mates, suggesting that testosterone affect territorial and mating behaviors (Dawson, 1983; Horton et al., 2010).

The actions of testosterone are traditionally subdivided into "organizational" effects, referring to developmental actions which are permanent and "activational" effects, which are transitory and reversible throughout life. Similar to other steroids testosterone acts through nuclear receptors specific to each hormone. When testosterone reaches a cell, it enters through the cell membrane and binds to intracellular nuclear androgen receptors (Durdiakova et al., 2011). This leads to the formation of a hormone–receptor complex, which then binds to hormone response elements in the promotor region of genes to modulate gene expression and protein synthesis (Bennett et al., 2010; Durdiakova et al., 2011). Alternatively, as described recently, it can affect behaviors through rapid non-genomic effects via membrane receptors at the outside of the cell (Balthazart et al., 2004).

The brain is a key steroid target as it contains groups of cells sensitive to specific hormones (Adkins-Regan, 2005; Nelson, 2005). For example, the preoptic area and the hypothalamus in the brain of birds have been related to social and sexual behaviors and both regions are sensitive to androgens and estrogen. These brain areas also express aromatase, an enzyme that converts testosterone into estrogen (Balthazart et al., 2004; Canoine et al., 2007; Schlinger and Callard, 1990). Also, the so-called song control system in the brain of songbirds, which is involved in song learning and production (Nottebohm et al., 1976) is sensitive to steroid hormones. It is composed of several interconnected nuclei, including the HVC that expresses androgen and estrogen receptors (Gahr and Metzdorf, 1997).

The level of expression of hormone receptors in the avian brain also undergoes seasonal cyclical variations in a region-specific manner (Belle et al., 2003; Breuner and Orchinik, 2001; Canoine et al., 2007; Fusani et al., 2000; Gahr and Metzdorf, 1997). Therefore, the action of hormones on behavior can be altered not only by seasonal variation in hormone concentration, but also by variation in the sensitivity of the respective brain cells. However, little is known about the seasonality in hormone concentrations in combination with seasonal variation of hormone sensitivity of the brain in free living species (Breuner and Orchinik, 2001; Canoine et al., 2007; Lattin and Romero, 2013; Soma et al., 1999; Wacker et al., 2010). Such knowledge would be useful to understand the underlying mechanisms that control the seasonal expression of hormones receptors, through which environmental stimuli can modulate hormone action in the avian brain.

Not only hormones affects behavior but also the reverse can be true. Classical experiments in birds have shown that behavior affects hormone secretion and hormone-dependent behavior of the same or another individual (Hinde and Steel, 1976; Lehrman and Wortis, 1960). Follow-up studies have shown that social interactions can modulate circulating hormone levels in males (Harding, 1981). Large and essential contributions to this area have been made by John Wingfield and his studies in wild song and white-crowned sparrows. For example, in 1985 Wingfield showed that during aggressive interactions over territories or mates, plasma levels of testosterone increase (Wingfield, 1985). This study was the basis for the proposal of the challenge hypothesis. The challenge hypothesis (Wingfield et al., 1990) was the first hypothesis attempting to explain the huge variation of plasma testosterone concentrations among wild animals. It established that in socially monogamous bird species, testosterone is high at the beginning of the breeding season while lower levels are maintained during other times of the breeding life-history stage. However, when males are challenged by conspecific males they show a rise in testosterone. Thus, aggressive challenges between males can increase levels of plasma testosterone, promoting the aggressive response and the persistence of the behavior (Wingfield, 1994). In this way males can avoid to maintain high levels of testosterone through the breeding season because it may interfere with parental behavior and have other negative effects.

The classical way to test the challenge hypothesis is through a simulated territorial intrusion (STI, see e.g. Goymann et al., 2007). By placing a stuffed or an alive decoy in combination with a playback of a male song into the territory of a focal male, one can simulate the intrusion of a competitor. The STI is a useful method to assess the aggressive and territorial behavior of individuals. In addition, if you can capture the individual following the STI, it is possible to determine the concentration of various hormones and compare the hormone levels of males that have been subjected to an STI with control males. The challenge hypothesis predicts that males challenged with an STI should have higher levels of testosterone than control males (e.g. Goymann et al., 2007).

After the proposal of the challenge hypothesis, several studies demonstrated its applicability to different vertebrate taxa (e.g. Hirschenhauser and Oliveira, 2006). However, the support mainly came from correlational data that assumed that seasonal differences in testosterone concentrations would correspond to changes in hormone levels during situational encounters. Only few studies gathered hormone date from situational male-male encounters such as STIs (for a detailed discussion see Goymann et al., 2007). Although the acceptance of the challenge hypothesis is quite high, many species do not increase testosterone during male-male challenges, e.g. less than one third of all bird studies support the challenge hypothesis (Addis et al., 2010; Apfelbeck and Goymann, 2011; Deviche et al., 2012; DeVries et al., 2012; Landys et al., 2010; Scriba and Goymann, 2010; reviewed in Goymann, 2009). The challenge hypothesis has been an important framework for behavioral endocrinology, but variation in testosterone concentrations due to male-male challenges does not account for much of the variability and complexity of hormone responsiveness that has been observed. Thus, although different hypotheses have been suggested (Goymann et al., 2007; Landys et al., 2007; Lynn et al., 2002; Wingfield and Hunt, 2002), there is no clear explanation that would account for the lack of social modulation in different species.

According to the challenge hypothesis, testosterone should promote mating behaviors (including extra-pair behavior) but should interfere with parental care. The relationship between testosterone, extra-pair behavior and parental care has been mainly investigated using testosterone implants. In general hormone manipulation experiments has been shown to reduces or inhibit parental care (Hegner and Wingfield, 1987; Ketterson et al., 1991; Lynn et al., 2009; Moreno et al., 1999; Schoech et al., 1998; Silverin, 1980; Van Roo, 2004) and increase extra-pair behavior (Ketterson et al., 1996; Raouf et al., 1997, but see Foerster and Kempenaers, 2004). However, implants typically lead to pharmacological levels of a hormone (Edler et al., 2011; Fusani, 2008; Quispe et al., submitted) and it is not clear whether physiological levels of the hormone would have similar effects. Thus, it may be important to see whether the natural variation in hormone levels relates to parental care and extra-pair paternity. So far, only few studies have focused on individual variation of behavior and life-history traits in relation to variation of hormone concentrations, but such studies may be important to understand the physiological basis of such relationships.

Aim of the thesis

The aim of my thesis is to gain a better understanding of the relationship between hormones and behavior in the black redstart (*Phoenicurus ochruros*), a socially monogamous song bird.

Specifically, I study (i) the relationship between testosterone and territorial behavior. Do male black redstarts socially modulate testosterone? Does testosterone influence their territorial behavior? (ii) The relationship between testosterone and other behaviors and life-history traits. Is there a relationship between natural circulating levels of testosterone with parental care and paternity loss? (iii) The effects of testosterone on behaviors outside the breeding season. Is there a correlation between testosterone, brain androgen sensitivity, territorial behavior and song in males across seasons?

The black redstart is a small, insectivorous, and highly territorial songbird of central Europe (Glutz von Blotzheim and Bauer, 1988). Breeding pairs are typically socially monogamous and both parents provide care for their offspring. Males defend territories during breeding and during autumn (Apfelbeck and Goymann, 2011; Weggler, 2000), before they depart to spend the winter in the Mediterranean (Landmann, 1996). Further, males may also be territorial in the wintering grounds (Cuadrado, 1995). Previous studies in this species have indicated that testosterone levels show large variation among males. In addition, migratory populations of black redstarts respond very aggressively towards STIs without showing a corresponding increase in plasma testosterone levels, following one or several STIs (Apfelbeck et al., 2011; Apfelbeck and Goymann, 2011).

Figure 1. Adult male black redstart in response to a simulated territorial intrusion (STI).

In **chapter 1** we investigated whether a more natural simulation of social instability rather than an STI would increase testosterone levels in male black redstarts. Previous studies have shown that black redstarts do not increase testosterone after an STI. However, an STI could not represent real threat or may be too short to produce an increase in testosterone in this species. We removed males from their territories to create social instability and assess social modulation of testosterone. We assumed that replacement and neighboring males would compete for the free territory. We measured territorial behavior in replacements, neighbors and in males from a control area where no removals were done by doing STIs. In addition, we measured baseline testosterone and GnRH-induced testosterone levels in all captured males. By injecting GnRH it is possible to test whether a male was not able to further increase testosterone during social instability because its testosterone secretion may have been already at its maximum (see Apfelbeck and Goymann, 2011). We expected that replacements and neighbors would behave more aggressively and would have higher levels of testosterone than control males.

In **chapter 2** we asked whether the number of life-history stages influences the environmental modulation of hormones by comparing aggressive and social modulation of testosterone in two populations of black redstart, a migratory- and a sedentary -one. Migratory black redstarts express 5 life-history stages, whereas sedentary birds only express 3 stages. According to the finite-state-machine model, animals that express fewer life-history stages should be more flexible in timing and more responsive to environmental modulation of hormone concentrations. We measured the aggressive response towards an STI together with plasma levels of testosterone (baseline and GnRH-induced levels) in both populations at the beginning of the breeding season (March in sedentary and April in the migratory population). In addition, we measured testosterone levels (baseline and GnRH-induced levels) in a population during migration at the same time when the sedentary population started to breed (March). By injecting GnRH it was possible to assess the testicular status. Thus, in addition, we could assess testosterone secretion according to the physiological state of the testes of males during migration compared to breeding males (Goymann et al., 2007; Moore et al., 2002). We expected that sedentary males would behave less aggressive and would increase testosterone after an STI.

Figure 2. The left image shows a breeding habitat of the migratory population of black redstart in Bavaria, Germany. The right image shows the habitat of the sedentary population in Andalucia, Spain

In **chapter 3** we assessed if natural variation in hormone levels (baseline and GnRH induced testosterone levels and corticosterone) correlated with parental care and paternity in male black redstarts. Testosterone manipulation studies suggested that the hormone promotes extra-pair behavior, but interferes with parental care. However, little is known if this relationship persists given natural variation in testosterone. In addition, few studies have investigated the relationship between physiological parameters (such as hormone concentrations), parental care and genetic paternity. Here we investigated this relationship in male black redstarts. We measured how often males and females fed their chicks within one hour during three consecutive days and assessed if males had extra-pair chicks in their nests. We measured baseline and GnRH-induced levels of testosterone as well as corticosterone during mating and during the parental care phase. Because testosterone levels are highly variable, the maximal production provides an opportunity to assess a “standard” testosterone parameter. Thus, the potential of an individual to increase testosterone release ($R_{potential}$ sensu Goymann et al. 2007) could account for individual variation and may be more relevant than baseline levels of testosterone in modulating behaviors (McGlothlin et al., 2007). We expected to find a negative correlation between testosterone and parental care and a negative relationship between paternity loss and testosterone.

In **Chapter 4** we assessed the relation between aggressive behavior, song and plasma levels of testosterone in combination with androgen and estrogen receptors as well as aromatase expression in the brain. We measured the testosterone profile of males from March to October. In addition, we related the aggressive behavior and spontaneous song rate and structure to the brain anatomy during breeding and non-breeding. We expected differences in aggression, song rate and structure between stages driven by differences in testosterone levels. We also classified which brain areas expressed androgen and estrogen receptor as well as aromatase mRNA in relation to season.

Chapter 1

Experimental induction of social instability during early breeding does not alter testosterone levels in male black redstarts, a socially monogamous songbird

Camila P. Villavicencio, Beate Apfelbeck, Wolfgang Goymann

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Experimental induction of social instability during early breeding does not alter testosterone levels in male black redstarts, a socially monogamous songbird

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ABSTRACT

Testosterone plays an important role in territorial behavior of many male vertebrates and the Challenge Hypothesis has been suggested to explain differences in testosterone concentrations between males. For socially monogamous birds, the challenge hypothesis predicts that testosterone should increase during male–male interactions. To test this, simulated territorial intrusion (STI) experiments have been conducted, but only about a third of all bird species investigated so far show the expected increase in testosterone. Previous studies have shown that male black redstarts (*Phoenicurus ochruros*) do not increase testosterone during STIs or short-term male–male challenges. The aim of this study was to evaluate whether black redstarts modulate testosterone in an experimentally induced longer-term unstable social situation. We created social instability by removing males from their territories and compared the behavior and testosterone concentrations of replacement males and neighbors with those of control areas. Testosterone levels did not differ among replacement males, neighbors and control males. Injections with GnRH resulted in elevation of testosterone in all groups, suggesting that all males were capable of increasing testosterone. We found no difference in the behavioral response to STIs between control and replacement males. Furthermore, there was no difference in testosterone levels between replacement males that had expanded their territory and new-coming males. In combination with prior work these data suggest that testosterone is not modulated by male–male interactions in black redstarts and that testosterone plays only a minor role in territorial behavior. We suggest that territorial behavior in species that are territorial throughout most of their annual life-cycle may be decoupled from testosterone.

Introduction

Testosterone has long been known to regulate vertebrate morphological, physiological and behavioral traits. In most seasonal bird species testosterone levels are elevated during the breeding season (reviewed in Wingfield and Farner, 1993; Wingfield and Silverin, 2002, 2009). An increase in testosterone during breeding is responsible for spermatogenesis, the development of some secondary sexual characters and other physiological and morphological traits that are relevant for reproduction (reviewed in Adkins-Regan, 2005). Additional short-term peaks in testosterone are known to be associated with mating and competitive (territorial) behaviors (e.g. Goymann et al., 2007a; Hirschenhauser et al., 2003; Wingfield et al., 1991, 1987, 2001). A causal relationship between testosterone and sexual behavior has been demonstrated, for example, by castration experiments. Castrated birds show a decline in sexual behaviors, which can be fully reestablished by implanting testosterone (e.g. Beach and Inman, 1965). Likewise, castrated male birds show a decrease in aggressive behaviors (Deviche, 1979; Tsutsui and Ishii, 1981) and testosterone administration can increase or extend aggression (e.g. Barfield, 1971; Wingfield, 1984). Similarly, blocking androgen receptors and conversion of androgens to estrogens can reduce territorial behaviors in some species (reviewed in Apfelbeck et al., 2013b). However, there is growing evidence that testosterone does not universally affect aggression between males (e.g. Apfelbeck et al., 2013b; Lynn, 2008).This raises the question regarding how common the influence of testosterone on agonistic behavior in birds actually is.

Conversely, social interactions such as competition for territories or mates can feed-back on hormone secretion, increasing plasma testosterone levels after male–male conflicts (Harding, 1981). The propensity to increase testosterone in response to social instability is known as the "challenge hypothesis" (Wingfield et al., 1990). This hypothesis states that when males of socially monogamous species are challenged by conspecific males they show a rise in testosterone levels, while they maintain lower breeding levels of testosterone during other times. By doing so they would avoid the potential interference of high levels of testosterone with parental care. Indeed, social interactions produce a rapid

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increase in plasma testosterone levels in a variety of taxa (reviewed in Hirschenhauser and Oliveira, 2006).

A common test of the challenge hypothesis in birds is simulated territorial intrusion (STI) experiments, during which a decoy typically in combination with playback is placed into a male's territory, because presentation of just one of these stimuli may not be sufficient to elicit a rise in testosterone (Wingfield and Wada, 1989). Males exposed to an STI should experience a short-term elevation of plasma testosterone ($R_{male–male}$ sensu Goymann et al., 2007a). However, a comparative study found that only 8 out of 24 bird species that were subjected to an STI showed the expected increase in testosterone (Goymann et al., 2007a and Fig. 4b in Goymann, 2009). This and the above suggest that the relationship between testosterone and territorial behavior could be dissociated in many species. Indeed, this seems to be the case in the black redstart (*Phoenicurus ochruros*), a small, insectivorous, and highly territorial songbird of central Europe (e.g. Glutz von Blotzheim and Bauer, 1988). Breeding pairs are typically socially monogamous and both parents provide care for their offspring. Males defend territories during breeding and in autumn (Apfelbeck and Goymann, 2011; Weggler, 2000), before they depart to spend the winter around the Mediterranean Sea (Landmann, 1996). Further, males may also be territorial in the wintering grounds (Cuadrado, 1995). Prior work in this species has shown a typical seasonal pattern of plasma testosterone with high levels in spring and low levels outside the breeding period (Apfelbeck et al., 2013a). Black redstarts respond very aggressively towards STIs without showing a corresponding increase in plasma testosterone levels following one or several STIs (Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011). This is in contrast to the prediction of the challenge hypothesis. Although there are some ideas as to why some species modulate testosterone in response to male–male aggression and others do not, the reasons underlying such differences in social modulation of testosterone remain unclear (Goymann, 2009).

Even though an STI may effectively simulate a real male–male challenge, this does not mean that it mimics a real threat in all species or that its duration may be too short to induce a change in testosterone in some species. This possibility has been little explored and may be a potential drawback in the methodological setup of such experiments (e.g. Goymann et al., 2007a). The aim of our current study was to assess testosterone modulation due to male–male interactions ($R_{male–male}$ sensu Goymann et al., 2007a) in a more natural and prolonged situation. In Germany, black redstarts live at high densities in human settlements (villages and towns). By removing a territorial male we created a vacant territory that could be occupied by a new-coming male, or by neighbors who could expand or rearrange their territories to fill the empty spot. We explored whether such a longer-term social instable situation could induce an increase in testosterone concentrations, as compared to the lack of such an increase during short-term agonistic interactions or experimentally induced STIs in this species (Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011). After having removed the territory owner we subsequently caught the replacement males (i.e. males that took over the vacated territory) and the neighboring males (i.e. the neighbors of the males that had been removed from their territories) to compare plasma levels of testosterone of these males with males from control areas that were not manipulated.

In addition, by creating social instability in areas with a high density of territorial males we expected that replacement males that established and rearranged the boundaries with their neighbors would behave more aggressively towards an unknown intruder (STI) than birds in control areas where boundaries had been settled. A similar experiment had been conducted by Wingfield (1985) in which he found that the removal of territorial male song sparrows (*Melospiza melodia*) resulted in social instability and increased levels of testosterone in replacement and neighboring males. However, in contrast to black redstarts, song sparrows are known to increase testosterone also during STIs (Wingfield and Wada, 1989). The only other study that tested the hormonal effect of removing males from their territory did not find any evidence for testosterone modulation (white-bellied antbirds *Myrmeciza longipes*; Fedy et al., 2006). Given the lack of an androgen responsiveness to short male–male interactions in black redstarts (Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011) we were curious to know whether and how black redstarts would respond to longer-term social instability. To exclude the possibility that black redstarts did not elevate testosterone during social instability because they were producing testosterone already at a maximal rate (see Goymann, 2009 for discussion of this possibility) we took a post-capture sample of testosterone and then injected birds with a high dose of GnRH (gonadotropin-releasing hormone). After 30 min, we took another testosterone sample to see whether birds increased testosterone production in response to the GnRH challenge (see e.g. Apfelbeck and Goymann, 2011; Goymann and Wingfield, 2004; Jawor et al., 2006; Moore et al., 2002; Wingfield et al., 1991). If an individual did not show elevated levels of testosterone during social instability, but still showed an increase after the GnRH injection, we assumed that it would have been capable to raise testosterone in response to a social challenge.

Methods

Study species and site

We conducted this study on a migratory population of European black redstarts in Upper Bavaria, Germany, in villages in the vicinity of the Max-Planck-Institut für Ornithologie (47°N, 11°E, 500–600 m above sea level). Males of this population arrive at the breeding grounds in late March. Breeding activities continue until July, enabling most pairs to raise at least two sequential broods per year (Apfelbeck and Goymann, 2011; Draganoiu et al., 2005). Black redstarts show delayed plumage maturation with second-year males (i.e. males in their first breeding season) resembling females and attaining their black coloration with white wing patches only after their first postnuptial molt (Landmann, 1996). Because this phenotypic difference between second-year and older males could relate to differences in behavior or testosterone concentrations (Schwarzova et al., 2010) we highlight this age difference throughout the manuscript.

Experimental procedure

To create social instability we temporarily removed males from their territories and kept them in captivity for 7–10 days (under a natural photoperiod, with food and water *ad libitum*). All males were removed by first luring them to the capture area with a short playback of black redstart song and then catching them with mealworm-baited ground- and tree-traps during the mating period between April 14th and May 5th in 2010 and 2011. We removed 23 males (14 adults, 9 second-year males). There was no obvious difference in territory size and location (roughly estimated by playing back song in various parts of the territory) between territories of second-year and adult males. In each case we tried to remove males from central territories with a similar number of neighbors. The number of neighbors did not differ between territories of second-year or adult males. After removing the territory owner, the vacated territories were observed on a daily basis. We assumed that a territory had been taken over by a new-coming male, if the number and location of neighbors in the surrounding areas had not changed, but the vacated territory was occupied and defended by a male that had not been present when the previous territory owner was removed. We assumed that a neighboring male had expanded his territory, if no additional bird was present in the area, but one of the previous neighbors now defended his old and the vacated territory. All territories were occupied by new-coming or neighboring males within a short period of time (1–3 days, mean ± std: 2.13 ± 0.8 days). Specifically we observed replacement males on the first day ($N = 4$), on the second day ($N = 6$) and on the third day ($N = 6$) within the territory of the removed male. In 7 cases, we were not able to determine the exact day when the territory was taken over by a replacement male. In 9 cases a simulated territorial intrusion (STI) was performed within 1–3 days (2.11 ± 0.78 days) after the vacated territory had been taken over, in 7 further cases we conducted a STI within 4–5 days (4.28 ± 0.48 days) after the territory had been taken over. In the 7 cases where we were not able to determine the exact day when the territory was taken over the STIs were conducted 3–7 days (mean ± std: 4.4 ± 1.6 days) after removing the previous territorial male. STIs were performed by placing a stuffed decoy at the same location where the previous male had been caught and playing back black redstart song for 20 min. To avoid pseudo replication 3 different stuffed males in full adult plumage were used as decoys, and songs from 20 different adult males. Following Apfelbeck and Goymann (2011) the following behaviors were recorded: the latency to approach within 5 m of the decoy for the first time (response latency), total time spent within 5 m of the decoy, frequency of head nodding, number of songs and attacks. After 20 min of STI the traps were opened to catch and sample the replacement male. We caught 17 (4 adults, 13 second-year males) of the 23 males that had replaced the former territory owner. Those males were caught 1–5 days (3.06 ± 1.29) after arrival.

The same STI procedure that was used to measure behavior and testosterone levels of replacement males was applied to score the behavior and measure testosterone in 16 control males (10 adults, 6 second-year males). These males were tested and caught in different villages that were at least 5 km away from villages in which males had been removed: we performed an STI to assess the control males' behavior. Following the STI we caught the respective male to obtain a blood sample for testosterone measurements. All behaviors were scored by the same observer (CPV).

In addition to these two experimental groups, we caught 13 males (7 adults, 6 second-year males) from territories adjacent to the territories that had been taken over by replacement males. These neighbors already had a territory, but they may have been negotiating the new boundaries with the replacement males. Neighbors were lured to the traps with short playback of black redstart song to obtain a blood sample for testosterone determination. Following the rationale of Wingfield (1985) we did not conduct STIs in these neighbors, because we wanted to make sure that if they should have elevated levels of testosterone compared to control males, these elevated levels were due to being neighbor of a new-coming male and not due to the STI procedure immediately before capturing the focal neighbor.

Males that had been removed from their territory were released back onto their territory 7 – 10 days after capture. Three days after releasing the former territory owner we conducted another STI to assess whether the replacement male was able to keep the territory or whether the previously removed territory owner successfully reclaimed his territory.

Blood sampling

Previous studies on black redstarts have demonstrated that this species does not increase testosterone concentrations following single or repeated STIs (Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011). Therefore, we did not expect differences in hormone concentrations due to differences in capture methodology. Immediately after capture, we took a blood sample from the wing vein (~120 μl). Then, to see whether individuals were still able to increase testosterone production we injected 50 μl of chicken GnRH-I (Bachem H 3106; 1.25 μg dissolved in 50 μl isotonic saline) into the *pectoralis major* muscle. A much smaller dose of 500 ng GnRH (2.5 times less) has been demonstrated to elicit a maximum response of LH (lutenizing hormone) and subsequent maximum rise in testosterone in a much larger songbird, the song sparrow (Moore et al., 2002; Wingfield and Farner, 1993). In addition, the dosage of 1.25 μg GnRH strongly elevated testosterone within 30 min in a previous study of black redstarts (Apfelbeck and Goymann, 2011) suggesting that it is effective in maximizing testosterone production. Then, each bird was measured (body mass, right tarsus, wing and tail length, and length and height of the cloacal protuberance), and ringed with a numbered aluminum ring (Vogelwarte Radolfzell) and three plastic split rings with a unique color combination for individual identification. Each bird was kept in a holding bag until we took a second blood sample (~60 μl) 30 min after the GnRH injection. Blood samples were immediately centrifuged with a Compur Minicentrifuge (Bayer Diagnosis) to separate the plasma from the blood cells. Plasma volume was measured using a Hamilton syringe and the plasma was stored in 500 μl ethanol (Goymann et al., 2007b). After returning from the field, samples were stored at −80 °C. All experimental procedures were approved by the governmental authorities of Upper Bavaria.

Hormone analysis

Testosterone concentration was determined by radioimmunoassay following the procedures described in Goymann et al. (2007b). Extraction recovery (mean ± sd) was 88 ± 5%. Samples were measured in duplicate and distributed randomly between two assays. The testosterone concentration was calculated with Immunofit 3.0 (Beckmann Inc., Fullerton, CA, USA). The lower detection limits of the assays were 3.5 pg/ml and 4.5 pg/ml and all samples were above the detection limit. The intra-assay coefficients of variation were 8.7% and 13%, respectively; the intra-extraction coefficients of variation of chicken pools were 0.02% and 5.6%, respectively. The inter-assay coefficient of variation was 14.8% and the inter-extraction coefficient of variation was 11.1%.

Statistical analysis

Data were analyzed using R (2.13.0; R Development Core Team). To investigate the influence of social instability (controls vs. replacements vs. neighbors), age class (second-year vs. adult males) and GnRH injection on log-transformed testosterone concentrations we used a linear mixed model (Lme).We included the interaction between social instability treatment and GnRH-injection to establish whether the degree of social instability led to a difference in GnRH-induced testosterone concentrations. Because post-capture and GnRH-induced testosterone levels were included in one model, bird ID was included as a random effect to account for repeated measures.

For the analysis of the behavior we assessed whether the latency until the first approach within 5 m of the decoy (in seconds), the time spent within 5 m of the decoy (in seconds), the frequency of head nodding and the number of songs differed between control and replacement males. All behavioral variables were analyzed with the Wilcoxon rank sum test for independent samples (this test is equivalent to the Mann–Whitney U-test and should not be confused with the Wilcoxon signed-rank test for paired data) in R, because the data did not meet the assumptions for parametric testing. The frequency of head nodding was corrected for the time we were able to see the focal male.

A general linear model was used to assess whether the post-capture testosterone concentrations of replacements males that had established themselves in the vacant territory differed from those of males that had expanded their own territory, including age as a fixed factor. Because data from GnRH-induced testosterone levels and the increase of testosterone from post-capture to GnRH-induced levels did not meet the requirements for linear models we performed a Wilcoxon rank sum test for independent samples to assess differences between replacement males that expanded their own territory and those that established a new territory.

Furthermore, we conducted another Wilcoxon rank sum test to analyze whether post-capture and GnRH-induced testosterone concentrations of removed males were related to their ability to regain their previous territory or not. Significance was accepted at $\alpha < 0.05$ (two tailed).

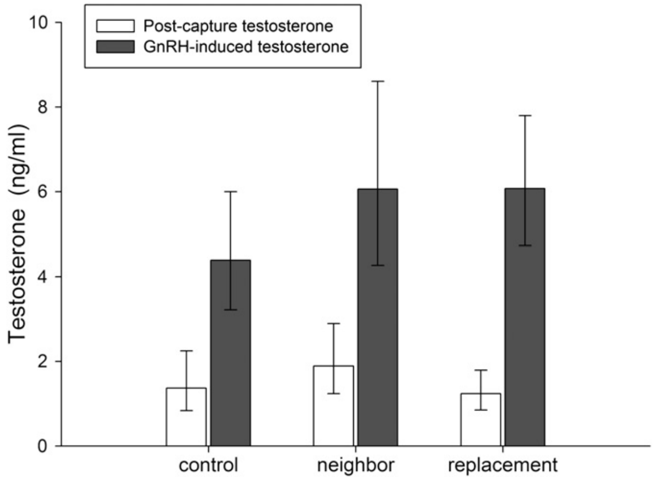

Fig. 1. Back transformed means (±95% confidence intervals) of post-capture and GnRH-induced testosterone levels did not differ ($p = 0.33$) between replacements ($n = 17$), neighbors ($n = 13$) and control males ($n = 16$). Testosterone levels significantly increased ($p < 0.01$) to a similar degree in all three groups after the GnRH challenge. Open bars represent post-capture testosterone levels; grey-shaded bars represent GnRH induced testosterone levels.

Results

Overall testosterone concentrations did not differ between replacement, neighboring and control males ($F_{2,43} = 1.13$, $p = 0.33$). The GnRH injection led to a significant increase of testosterone in all males ($F_{2,42} = 172.3$, $p < 0.0001$; Fig. 1), but the three groups did not differ with respect to the magnitude of the GnRH-induced increase in testosterone (interaction between social instability and GnRH injection: $F_{2,42} = 1.58$, $p = 0.22$; Fig. 1).

Control and replacement males did not differ in any of the behaviors measured: approach latency to within 5 m of the dummy ($W = 178$, $p = 0.71$), time within five meters of the dummy ($W = 238$, $p = 0.21$), frequency of head nodding ($W = 212$, $p = 0.59$), and song frequency ($W = 143.5$, $p = 0.18$; Fig. 2).

Replacement males occupied all "vacant territories" within 3 days after the territory owner had been removed. Out of all 23 replacements 15 were second-year males (65.2%). Replacement males were either new birds that had not been seen in the area before (1 adult, 11 second-year males) or neighbors that expanded their territories (7 adult and 4 second-year males). From all 23 replacement males we caught 17 (4 adult and 13 second-year males): post-capture testosterone levels did not differ between males expanding their own territories and males that had established themselves newly in the vacant territories ($F_{1,17} = 0.07$, $p = 0.79$) and also did not depend on age ($F_{1,17} = 0.1$, $p = 0.76$, Table 1). GnRH-induced testosterone levels ($W = 30$, $p = 0.67$) or the increase in testosterone between post-capture and GnRH-injection ($W = 27$, $p = 0.47$) did not differ between males that expanded their previous territories and males that established themselves newly in the vacant territories (Table 1).

After release, 57.1% ($N = 12$) of the previously removed males could regain their territories, whereas 42.9% ($N = 9$) could not get their territories back (excluding 2 males that died in captivity). Of all 12 males that regained their territories 83.3% ($N = 10$) were adult males. Only three adult males were not able to regain their territories. Post-capture levels of testosterone and GnRH-induced levels of testosterone did not differ between males that successfully recovered their territories and males that did not (post-capture testosterone: $W = 28$, $p = 0.53$; GnRH-induced testosterone: $W = 23$, $p = 0.49$; Fig. 3).

Discussion

By removing male black redstarts from their territories we tested whether longer-term social instability leads to elevated levels of

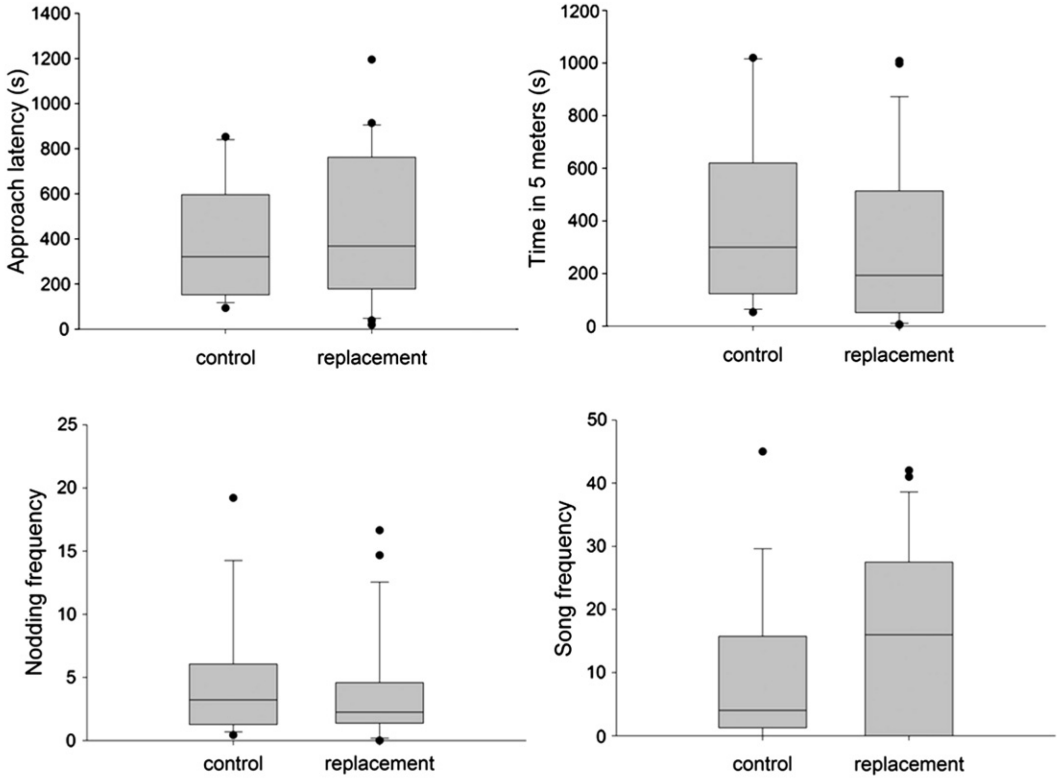

Fig. 2. Medians, 10th, 25th, 75th, 90th percentiles, and outliers of behaviors measured during simulated territorial intrusions (STI). There were no differences in approach latency, time spent within five meters of the dummy, head nodding and song frequency (all p's > 0.18) between replacement ($n = 25$) and control males ($n = 16$).

Table 1
Median with lower and upper quartiles of post-capture and GnRH-induced testosterone levels, and the increase from post-capture to GnRH induced testosterone concentrations (ng/ml) between adult and second-year males that expanded their own territory (expanders) and new-coming males that took over a "vacant territory".

	N	Post-capture testosterone Median [lower;upper quartile]	GnRH-induced testosterone Median [lower;upper quartile]	Testosterone increase Median [lower;upper quartile]
Adult expander	3	1.86 [0.37;5.68]	9.41 [7.82;11.01]	5.65 [5.33;5.97]
First-year expander	5	1.16 [0.87;1.21]	3.84 [3.35;6.28]	2.48 [1.88;5.64]
Adult newcomer	1	1.02	5.56	4.54
First-year newcomer	9	1.23 [0.88;2.17]	5.95 [4.29;8.27]	4.72 [3.24;7.00]

testosterone in neighbors of the previous territory owner and the respective males that adopted the empty territory. However, there was no difference in testosterone concentrations between replacement or neighboring males, and males caught in control areas. Also, all males responded with a similar level of territorial aggression and regardless of whether they experienced experimentally induced social instability or not. Furthermore, injection of GnRH resulted in a substantial increase of testosterone in all males, thus excluding the possibility that testosterone levels of male black redstarts were already maximal in the control context (see Goymann, 2009; Goymann et al., 2007a). Together, these results suggest that longer-term social instability does not lead to a change in testosterone concentrations or intensity of aggression in male black redstarts. These data are consistent with previous experiments in which we demonstrated that neither single or multiple simulated territorial intrusions, nor real short-term intrusions of rivaling males led to an increase in testosterone in this species (Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011; see also DeVries et al., 2012 for similar results in northern cardinals *Cardinalis cardinalis*).

Wingfield (1985) was the first to conduct an experiment regarding the effects of prolonged social instability on plasma testosterone concentrations. He found that replacement and neighboring males of previously removed song sparrows had elevated levels of testosterone when compared to control males. Furthermore, Wingfield (1985) demonstrated that song sparrow males show a pronounced increase in testosterone during simulated territorial intrusions. To our knowledge, testosterone concentrations were investigated in only one other removal study: Fedy et al. (2006) studied a tropical species, the white-bellied antbird (*Myrmeciza longipes*). Males of this species had low levels of plasma testosterone throughout the year and also did not increase testosterone after playing back song of a conspecific male, or after inducing longer-term social instability (Fedy et al., 2006). Thus, even though song sparrows and black redstarts are similar in that they are both from the temperate zone, have a socially monogamous mating system and provide biparental care, they seem to differ completely with regard to their androgen responsiveness to male–male competition ($R_{\text{male–male}}$ sensu Goymann et al., 2007a). Song sparrows show a high degree of androgen responsiveness in that males elevate levels of testosterone during times of artificial short-term (10–30 min STIs) or longer-term (removal experiment: 1–3 days after arrival of a replacement male) social instability (Wingfield, 1985). Similar to song sparrows, 12 out of 17 of the black redstart replacement males were caught within 1–3 days after the arrival of the respective replacement male. Five further males were caught a bit later, within 4–5 days after their arrival in the vacant territory. However, in contrast to song sparrows we found no evidence of social testosterone modulation in response to artificial short term social instability (20 min STI; Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011) and to longer-term social instability (this study). Our results are consistent even if we remove the 5 males caught only 4–5 days after the removal or the original territory owner. Even though we cannot exclude the possibility that we may have missed the critical period for social instability, the androgen responsiveness of black redstarts differs from that of song sparrows caught within 1–3 days after arrival of a replacement male. Moreover, given the lack of a short-term (from 10 min up to 1 h) androgen response to single or multiple STIs and to real short-term intrusions in this species, we consider the possibility that we missed the critical period unlikely.

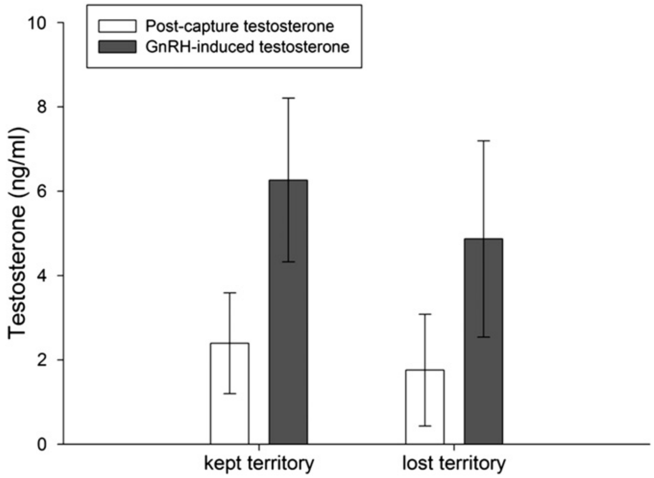

Fig. 3. Means (±95% confidence intervals) of post-capture and GnRH-induced testosterone levels did not differ ($p = 0.53$ and $p = 0.49$ respectively) between males that successfully recovered their territories after release ($n = 12$) and males that did not ($n = 9$). Males were sampled immediately after capture and before they were held in captivity for 7–10 days. Open bars represent post-capture testosterone levels; grey-shaded bars represent GnRH induced testosterone levels.

Removal experiments are typically used to assess habitat saturation and to determine the existence of floater males or the availability of females (e.g. Marra and Holmes, 1997). In our population of black redstarts, all vacated territories were occupied by a replacement male within 3 days after removing the previous territory owner, suggesting that there is a pool of males in search for territories. In our case, the majority of territories (15 out of 23) were taken over by second-year males that presumably did not have a territory or may have occupied a low quality territory before. In contrast, the majority of expanders were adult neighboring males (7 out of 11) that already had a territory, but were able to expand to include the vacated one and probably also took over the "widowed" female. Removal of males can render previously monogamous neighboring males to become polygynous (e.g. in the house wren, *Troglodytes aedon musculus;* Llambías, 2012). We have evidence that some of the neighboring males of removed black redstarts became polygynous. However, we did not find differences in testosterone concentrations between males that expanded their territories or new-coming males, suggesting that there is no strong association between these two kinds of replacement males, age and testosterone in black redstarts. Furthermore, post-capture and GnRH-induced levels of testosterone were also not associated with keeping (replacement male) or being able to regain (released previous owner) a territory.

So far, all studies of black redstarts suggest that males of this species do not conform to the predictions of the challenge hypothesis, which states that males of socially monogamous species with a high degree of paternal care should show a strong modulation of androgen levels during male–male interactions (Wingfield et al., 1990). Further, considering all other hypotheses that have been suggested to explain a lack of androgen modulation (summarized in Goymann, 2009) black redstarts do not conform to any of them. First, the short season hypothesis (Wingfield and Hunt, 2002; extended by Goymann, 2009) or the number of broods hypothesis (Landys et al., 2007) suggests that species with a short breeding season or a single brood per season may not modulate testosterone to facilitate a fast transition from the mating to the parental phase. However, neither black redstart populations with a short breeding season (typically single-brooded) nor those with a long breeding season (multiple-brooded) modulate testosterone during STIs (Apfelbeck and Goymann, 2011). Second, an extension of the essential paternal care hypothesis (Lynn et al., 2002) suggests that species with essential paternal care may not increase testosterone during territorial challenges, because they are insensitive to the effects of testosterone (Goymann et al., 2007a). The definition of whether paternal care is essential or not is controversial, but according to a popular description paternal care is essential when a decrease in male parental care leads to a significant decrease in offspring survival (Lynn, 2008; Lynn et al., 2002). For black redstarts, there is no evidence for such essentiality, as females are able to raise a clutch without help from a male (Landmann, 1996; C. Villavicencio et al. in prep.). Why then, is testosterone not modulated during social challenges in male black redstarts?

Previous experiments have demonstrated that black redstarts show the same level of territoriality not only during the breeding season when testosterone concentrations are high, but also during the non-breeding territorial phase in autumn when plasma testosterone concentrations are non-detectable (Apfelbeck and Goymann, 2011; Apfelbeck et al., 2013a). In some species brain androgen sensitivity or aromatase activity may be involved in regulating territorial behavior. For example, in some birds an increase in aromatase activity is related to an increase in aggressive behavior (Pintér et al., 2011; Schlinger and Callard, 1990; Silverin et al., 2004; Trainor et al., 2006). Furthermore, there is growing evidence that social interactions can modulate aromatase activity (Trainor et al., 2006, but see Charlier et al., 2011). In black redstarts, blocking the action of testosterone or its estrogenic metabolites does not decrease territorial aggression (Apfelbeck et al., 2013b), suggesting that androgen sensitivity or aromatase activity is not playing a major role in controlling aggressive behavior in this species. In summary, current evidence suggests that territorial aggression is likely to be independent of testosterone in black redstarts. A behavior that is more or less constantly expressed throughout the year should not necessarily be modulated by hormones (see Adkins-Regan, 2005). Rather, other hormones may be involved in the suppression of territoriality during short periods when black redstarts are not territorial, in particular during migration. If territorial aggression is not under androgenic control in black redstarts, then territorial behavior may also not feed-back on hormone levels, thus explaining the lack of a testosterone response of black redstarts to STIs (Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011) or social instability (this study). Similar to black redstarts, some other species that are territorial year-round, do not modulate testosterone concentrations (Landys et al., 2010, 2013). A clear exception is the song sparrow, which is also territorial for most of the year, but in which territorial aggression influences hormone concentrations and vice versa (e.g. Sperry et al., 2010; Wingfield, 1994; Wingfield and Soma, 2002). Possibly, the biodiversity of species is accompanied by a “biodiversity of mechanisms” (Wingfield and Silverin, 2009) which may limit our ability to find general patterns. Species-specific differences in the relation between testosterone and aggressive behavior during breeding and during non-breeding do not seem to follow simple patterns (see Landys et al., 2013). In some species testosterone is strongly related to territorial behavior (e.g. McGlothlin et al., 2007), but in others such as the black redstart variation in testosterone may mainly be associated with reproduction and cues from the female partner rather than territorial aggression (Apfelbeck et al., 2013a,b).

Conclusions

Black redstarts did not show an increase in testosterone levels during prolonged social instability and the levels of aggression did not differ between replacement and control males. Hence, in this species, there does not seem to be an association between experimentally induced longer-term social instability and testosterone. Because black redstarts may be territorial throughout most of the year (except during migration) we suggest that territorial aggression might be decoupled from testosterone in this and other species in which territoriality is decoupled from reproduction.

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Chapter 2

The number of life-history stages does not influence the androgen responsiveness to male-male interactions: Sedentary and migratory black redstarts (*Phoenicurus ochruros*) do not elevate testosterone in response to simulated territorial intrusions

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The number of life-history stages does not influence the androgen responsiveness to male–male interactions: Sedentary and migratory black redstarts (*Phoenicurus ochruros*) do not elevate testosterone in response to simulated territorial intrusions

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ABSTRACT

High plasma levels of testosterone at the beginning of the breeding season adjust male physiology for mating and promote territorial behavior in birds. Conversely intra-sexual competition may elicit a temporary increase in circulating testosterone. Male black redstarts (*Phoenicurus ochruros*) from migratory populations show the expected increase in baseline testosterone during early breeding, but circulating testosterone levels do not change in response to male–male interactions. Because sedentary populations express fewer life-history stages they may be more flexible in timing of life-history stages and more responsive to environmental modulation of hormone concentrations. Therefore, we tested whether the androgen responsiveness to male–male interactions differs between migratory (6 life-history stages) and sedentary black redstarts (3 life-history stages) during early breeding, predicting that in contrast to migratory birds, sedentary birds would modulate testosterone in response to simulated territorial intrusions (STI). In contrast to our prediction, sedentary males did not modulate post-capture testosterone levels in response to simulated territorial intrusions. Males of both populations increased testosterone after an injection of gonadotropin releasing hormone (GnRH), demonstrating that they were capable of increasing testosterone. Interestingly, in sedentary males the GnRH injection elicited a higher testosterone response in STI males than in control males. The two populations did not differ in their behavioral response to the STIs, except that sedentary males spent less time close to the decoy. In combination with previous data from black redstarts and other socially monogamous and biparental birds our current study adds to the growing evidence that current theory regarding hormone-behavior relationship needs to be refined.

1. Introduction

Most organisms live in seasonal environments and time successive life-history stages according to predictable changes in environmental cues (Jacobs and Wingfield, 2000; Wingfield, 2008). The finite-state-machine model (Jacobs and Wingfield, 2000; Wingfield, 2008) suggests that animals living in environments with large seasonal changes express more life-history stages than animals living in environments with a low degree of seasonal change. Because hormones are involved in the regulation of development, onset and termination of life-history stages, the numbers of stages and the degree of overlap may influence the neuroendocrine mechanisms and their control by environmental cues (Wingfield, 2008). In particular, the finite-state-machine theory predicts that species or populations expressing more life-history stages (e.g. migratory, compared to sedentary populations) should be less flexible in timing and thus, the succession of life-history stages may be under tighter hormonal control. Furthermore, if life-history stages are tightly controlled they may be less susceptible to environmental modulation (Goymann, 2009; Wingfield, 2008, 2005). In addition, it has been proposed that birds breeding at higher latitudes have higher peaks of plasma testosterone than lower latitude birds (reviewed in Garamszegi et al., 2008). However, these differences may be driven by differences in life history rather than latitude per se (Goymann and Landys, 2011;

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Goymann et al., 2004). Accordingly, specific life-history traits, such as short and synchronous breeding seasons may be more important determinants of hormone profiles than latitude.

Sex steroids are important regulators of the breeding life-history stage. Testosterone, for example, is involved in spermatogenesis, the development of secondary sexual characters (reviewed by Adkins-Regan, 2005), sexual (Ball and Balthazart, 2004; Beach and Inman, 1965), and territorial behavior within a reproductive context (e.g. Canoine and Gwinner, 2002; Wingfield et al., 1990, 2001; Wingfield, 1984). At the same time social interactions such as competition for territories or mates may feed-back on hormone secretion, potentially leading to an increase in testosterone during male–male conflicts (Harding, 1981). The challenge hypothesis predicts a short-term rise in circulating testosterone in response to male–male interactions, especially in socially monogamous species (Wingfield et al., 1990; termed $R_{\text{male–male}}$ by Goymann et al., 2007a). Indeed, male–male interactions produce a rapid increase in plasma testosterone levels in males of socially monogamous species of a variety of vertebrate taxa (reviewed by Hirschenhauser and Oliveira, 2006). However, in the majority of birds that have been experimentally tested so far, males fail to elevate testosterone during male–male challenges (Addis et al., 2010; Apfelbeck and Goymann, 2011; Deviche et al., 2012; DeVries et al., 2012; Landys et al., 2010; Scriba and Goymann, 2010; Villavicencio et al., 2013; and older studies reviewed by Goymann et al., 2007a; Goymann, 2009). The finite-state-machine theory offers one possible explanation for the lack of $R_{\text{male–male}}$ in some birds. If the tightness of hormonal control mechanisms increases with the number of life-history stages, then those avian species (or populations) expressing more life-history stages may also be less responsive to social modulation of hormones (i.e. to avoid interference with the control mechanism; Goymann, 2009). Here, we test this idea by comparing testosterone dynamics in males from two populations of one bird species that differ in the number of life-history stages.

Prior work on the black redstart (*Phoenicurus ochruros*), a small, insectivorous, and territorial songbird indicates that testosterone shows a strong seasonal pattern with high levels in spring, when territories are established, intermediate levels during the rest of the breeding season, and low levels in autumn (Apfelbeck et al., 2013). Males respond aggressively, but do not elevate plasma testosterone when challenged by a real or a simulated territorial intrusion, by a multiple simulated territorial intrusion, or when removing a territory neighbor. Black redstarts thus, do not support the predictions of the challenge hypothesis for socially monogamous birds with biparental care (Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011; Villavicencio et al., 2013). Previous work on this species has focused on migratory populations expressing six life-history stages: spring migration, breeding, molt, autumn territoriality, autumn migration and wintering. In southern Europe, sedentary populations of black redstarts exist (Bueno, 1992) and they express only three life-history stages: breeding, molt and non-breeding. If social modulation of testosterone is more likely to occur in birds displaying fewer life-history stages, then we hypothesized that sedentary redstarts should modulate testosterone in response to male–male interactions. To test this hypothesis we compared territorial behavior, post-capture and GnRH-induced testosterone concentrations in males from one sedentary and two migratory black redstart populations. The sedentary population in Spain had already started to breed, allowing us to establish comparisons with either (i) a migratory population sampled at the same time of the year, during active northward migration across the Mediterranean sea (i.e. same dates, but different life-history stages) or (ii) a migratory population in Germany sampled later in the year, during early breeding (i.e. different dates, but the same life-history stage as the sedentary population). We assessed both post-capture and GnRH-induced testosterone levels to exclude the possibility that birds were not capable of elevating testosterone during male–male interactions ($R_{\text{male–male}}$), because they may have been producing the hormone at their maximal rate even before the social challenge (Goymann, 2009). Further, the GnRH-treatment allowed us to test whether the populations differed in their maximum physiological capacity to produce testosterone (termed $R_{\text{potential}}$ by Goymann et al., 2007a; see also e.g. Moore et al., 2002).

2. Material and methods

We conducted this study on one sedentary and two migratory populations of black redstarts. The resident, non-migrant population was located in southern Spain, in the natural reserve Torcal de Antequera (Malaga, Andalusia; 36°N, 4°W, 1100–1200 m above sea level). Males were sampled during early breeding (between March 17th and April 1st in 2012) when they courted females and females started nesting (i.e. the mating stage). One migratory population was sampled during migration on Ponza, a small island in the Tyrrhenian Sea of Italy (40°55′ N, 12°58′ E, 0–100 m above sea level) that is frequently used by migrants as a stop-over site (samples were collected during active northward migration, between March 14th and March 27th in 2011). The second migratory population was sampled in Upper Bavaria, Germany in villages in the vicinity of the Max-Planck-Institut für Ornithologie (47°N, 11°E, 500–600 m above sea level). Males of this population arrive on the breeding grounds between mid-March and beginning of April. Once territories are established and weather conditions permit they start to breed at the end of April. We sampled males when they were actively defending territories and mating with females, which started to nest (i.e. the mating stage between April 20th and May 13th in 2010 and April 11th and May 6th in 2011).

Black redstarts show delayed plumage maturation with second-year males (i.e. males in their first breeding season) resembling females and attaining their black coloration with white wing patches only after their first postnuptial molt (Landmann, 1996). Because this phenotypic difference between second-year and older males could relate to differences in behavior or testosterone concentrations (Schwarzova et al., 2010) we considered age differences throughout the manuscript.

2.1. Experimental procedure

On the breeding grounds males were caught with two different protocols: (i) STI males were caught with mealworm-baited ground traps following a simulated territorial intrusion, during which we placed a stuffed decoy onto a male's territory and played-back black redstart songs for 20 min. (ii) Control males were caught with mealworm-baited traps either passively while searching for food, or within 10 min of the onset of playing back black redstart song (assuming that testosterone concentrations do not change within 10 min of playback presentation; Wingfield and Wada, 1989). To avoid pseudo-replication we used four different stuffed males in full adult plumage as decoys and songs from 20 different adult males. For the STI group territorial behavior was assessed during 20 min after placing the dummy and starting playbacks. We recorded the following behaviors: latency to first approach within 5 m of the decoy (response latency), total time spent within 5 m of the decoy, frequency of head nodding, number of songs and attacks. All behaviors were scored by the same observer (CPV), and the frequency was defined as total number of events per 20 min. After 20 min of STI, traps were activated to catch the territorial male. All males were caught with ground traps and tree traps bated with mealworms. We caught 13 control males with short playback (latency to be caught: 4.5 ± 1.7 min (mean ± 95%

CI)) and 14 STI males from the migratory population in Germany (latency to be caught: 9.9 ± 6.3 min after activating the traps). From the non-migratory population in Spain we caught 16 control males (passively without playback, latency to be caught: 70.7 ± 28.1 min) and 15 STI males (latency to be caught: 29.3 ± 19.4 min after activating the traps). On Ponza (migratory population sampled during migration), males ($N = 17$) were caught passively with mist nets while they were moving along the island during stop-over.

2.2. Capture and blood sampling

Immediately after a bird was caught (5 ± 0.6 min after capture; mean ± 95% CI, for males from Germany and Spain, and 12.1 ± 7.4 for males from Ponza), a blood sample was taken from the wing vein (approx. 120 μl) and collected into heparinized capillaries. Then we injected 50 μl of chicken GnRH-I (Bachem H 3106; 1.25 μg dissolved in 50 μl isotonic saline) into the breast muscle. Birds were kept in a holding bag until a second blood sample (approx. 60 μl) was taken 30 min after the GnRH injection to measure the potential to increase testosterone ($R_{\text{potential}}$). The dosage of 1.25 μg GnRH strongly elevated testosterone within 30 min in previous studies of black redstarts (Apfelbeck and Goymann, 2011; Villavicencio et al., 2013). From two breeding males of the sedentary population in Spain and the migratory population in Germany, and from one migrating male on Ponza we could not get a second blood sample. Each bird was subjected to the following measurements: body mass (to the nearest 0.1 g), right tarsus, wing and tail length (to the nearest 0.1 mm), and length and height of the cloacal protuberance (to the nearest 0.1 mm). The latter two measurements allowed us to estimate the volume of the cloaca (assuming a cylindrical shape: volume = $\pi r^2 h$). Before release, birds were ringed with a metal ring and three plastic rings with a unique color-combination for individual recognition (males captured during migration on Ponza were only ringed with an aluminum ring [ISPRA, Italia]). Blood samples were immediately centrifuged (7000 rpm, 10 min) with a Compur minicentrifuge (Bayer Diagnosis). The plasma was separated and the collected volume measured with a Hamilton syringe before adding 500 μl ethanol (Goymann et al., 2007b). After returning from the field, samples were stored at −20 °C in Spain and Ponza, and at −80 °C upon return to the laboratory in Germany and until laboratory analyses. All experimental procedures were approved by the governmental authorities of Upper Bavaria and the Consejería de Medio Ambiente de la Junta de Andalucía in Spain.

2.3. Hormone analysis

Testosterone concentration was determined by radioimmunoassay following the procedures described in Goymann et al. (2007b). Extraction recovery (mean ± sd) was 87 ± 4.5%. Samples were measured in duplicate and distributed among 4 assays. The testosterone concentration was calculated with Immunofit 3.0 (Beckmann Inc., Fullerton, CA, USA). The lower detection limits of the assays ranged from 3.5 to 4.5 pg/ml and all samples were above the detection limit. The intra-assay coefficients of variation of standard samples added to the assays were 8.7%, 13%, 9.1%, and 13.2%, respectively. The intra-extraction coefficients of variation of extracted chicken plasma pools were 0.02%, 5.6%, 6.1%, and 0.9%, respectively. The inter-assay coefficient of variation of standard samples was 24.3% (a relatively high value caused by one low assay control standard sample in the Ponza assay) and the inter-extraction coefficient of variation of extracted chicken plasma pools was 15.9%. The latter is much more informative than the inter-assay coefficient of variation, because it does not only take into account the variation between assays, but in addition also the variation due to the extraction.

2.4. Statistical analysis

Data were analyzed using R 2.13.0 (R Development Core Team) package "lme4" (Bates et al., 2013). Biometric differences between study populations were assessed through linear models to compare the body mass, the lengths of the tarsus, wing and tail, and the cloaca volume. *T*-tests were used to compare the behavioral responses to STIs (i.e. response latency, total time spent within 5 m of the decoy, frequencies of head nodding and songs) between the migratory and sedentary populations during breeding (i.e. sedentary in Spain vs. migratory in Germany). Post-capture levels of testosterone were log-transformed before analysis, and a linear model was fitted to assess the influence of behavioral treatment (control vs. STI), age (second-year vs. adult), population (sedentary in Spain vs. migratory in Germany), catch latency (time lapse between opening the traps and catching the bird) and the interaction between treatment, age and catch latency with population. A similar model was used to investigate the influence of the same parameters on log-transformed GnRH-induced testosterone levels.

To compare testosterone concentrations of migratory males on migration with those of the sedentary population during early breeding we used two linear models. For this comparison we used only the testosterone data of breeding sedentary males that were caught in the control setup (see above; non-STI birds) and compared them to passively caught males on migration. First we tested whether post-capture levels of log-transformed testosterone could be explained by population/stage (during migration on Ponza vs. sedentary breeders in Spain), age (second-year vs. adult males) and the interaction between both variables. In a second linear model we tested whether the same variables explained the variability in log-transformed GnRH-induced testosterone levels. In addition, we assessed if the increase in testosterone after a GnRH-challenge (subtracting post-capture testosterone from GnRH-induced testosterone) was influenced by population/stage (during migration on Ponza vs. sedentary breeders in Spain) using a linear model.

3. Results

Tarsus and wing lengths did not differ between populations (Table 1). However, sedentary males had significantly higher body mass and significantly longer tail than individuals from the two migratory populations (Table 1). Breeding migrants were significantly heavier than those on migration (Table 1). Breeding migrants had significantly larger cloacas compared to males on migration or sedentary males (Table 1).

During breeding, males from the sedentary and the migratory population did not differ in their latency to respond to the STI ($t = -1.02$, df = 25.7, $p = 0.32$), in their frequencies of head nodding ($t = 1.42$, df = 20, $p = 0.17$), or in how often they sang ($t = 0.71$, df = 28.7, $p = 0.48$; see Fig. 1). However, breeding sedentary males spent significantly less time within 5 m of the decoy than breeding migrants ($t = 2.19$, df = 25.1, $p = 0.038$; Fig. 1). In addition, only one out of 14 sedentary males (7%) attacked the dummy, while in the migratory population four males out of 12 (33%) attacked the dummy.

To estimate whether breeding sedentary males and breeding migrants differed in their androgen responsiveness to male–male interactions ($R_{\text{male–male}}$) we compared testosterone concentrations of males caught in a control vs. a STI situation. Overall, post-capture testosterone concentrations did not differ between control males and STI males ($F_{1,52} = 0.02$, $p = 0.89$), suggesting that males of

Table 1
Biometric differences (mean ± confident intervals) between males from the three studied populations. Statistically significant differences are shown in bold, different superscript letters indicate significant differences from the Tukey post hoc comparisons.

	Migratory (Germany) during breeding (mean ± CI)	Migratory (Ponza) during migration (mean ± CI)	Sedentary (Spain) during breeding (mean ± CI)	*F*-value	*P*-value
Body mass	16.4 ± 0.3^{a}	15.0 ± 0.6^{b}	17.4 ± 0.4^{c}	**24.31**	**<0.001**
Tarsus length	23.5 ± 0.2	23.6 ± 0.3	23.7 ± 0.2	0.48	0.62
Wing length	86.8 ± 0.8	85.6 ± 1.1	85.8 ± 0.7	2.18	0.12
Tail length	63.1 ± 1.0	No data	65.4 ± 0.8	**12.37**	**<0.001**
Cloaca volume	312.4 ± 49.8^{a}	78.3 ± 9.1^{b}	106.5 ± 13.7^{b}	**58.12**	**<0.001**

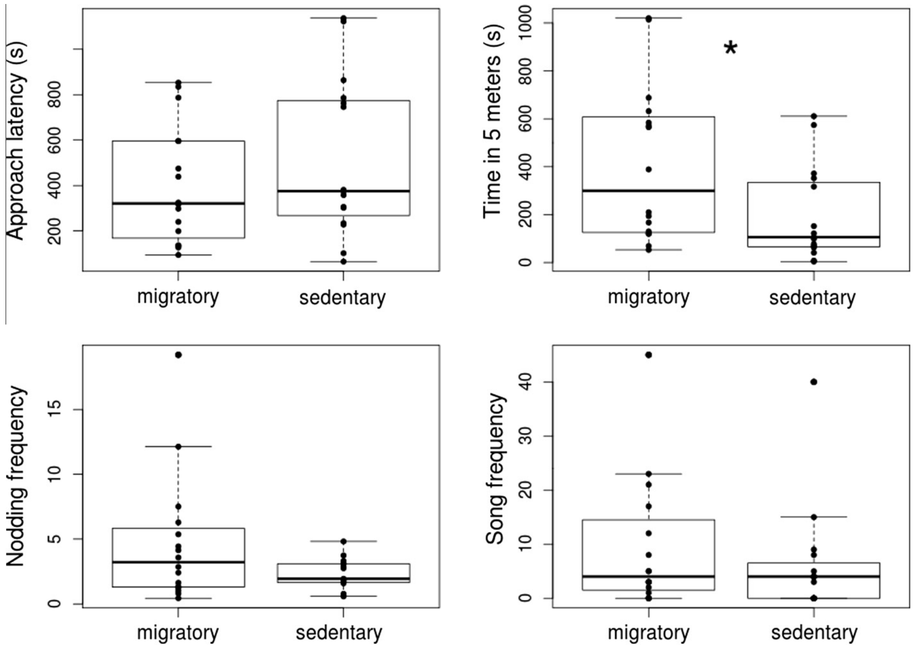

Fig. 1. Agonistic behaviors during simulated territorial intrusions (STIs) in migratory and sedentary male black redstarts during the early breeding stage. Box plots indicate medians, 10th, 25th, 75th, and 90th percentiles, dots indicate individual values. Approach latency (time to first approach to decoy), head nodding and song frequency (events per 20 min) did not differ significantly between sedentary and migrating males (all *p*'s > 0.17), but sedentary males spent significantly less time within 5 m of the decoy than migrants ($p = 0.038$).

both populations did not modulate $R_{\text{male–male}}$. Age ($F_{1,52} = 0.24$, $p = 0.62$), population ($F_{1,52} = 2.38$, $p = 0.13$; Fig. 2) and catch latency ($F_{1,52} = 2.28$, $p = 0.14$) did not significantly affect post-capture testosterone. Also, there was no significant interaction between population and age ($F_{1,52} = 3.26$, $p = 0.08$), or between population and catch latency ($F_{1,52} = 2.05$, $p = 0.16$). But we found a significant interaction between treatment and population ($F_{1,52} = 4.87$, $p = 0.03$; Fig. 2), indicating that the slope between STI and control birds differs between the two populations. However, when we further explored this relationship by investigating each population separately, we found that testosterone levels did not differ significantly between control and STI birds in the migratory ($F_{1,24} = 2.74$, $p = 0.12$) or in the sedentary population ($F_{1,28} = 0.96$, $p = 0.34$, Fig. 2). Therefore, the significant interaction describes the phenomenon that STI birds expressed a slightly lower median testosterone concentration than controls in the migratory, but not in the sedentary population (Fig. 2); none of the populations increased testosterone after STIs.

In general, GnRH-induced testosterone levels ($R_{\text{potential}}$) did not differ between STI and control males ($F_{1,48} = 2.17$, $p = 0.15$), but there was a significant effect of population, indicating that $R_{\text{potential}}$ was lower in the sedentary than in the migratory population ($F_{1,48} = 4.72$, $p = 0.04$). Also, the interaction between population and treatment was significant ($F_{1,48} = 9.6$, $p = 0.003$; Fig. 2), suggesting that similar to post-capture testosterone levels there was a population-specific difference between STI and control males. Therefore we investigated each population separately: $R_{\text{potential}}$ did not differ significantly between control and STI birds in the migratory population ($F_{1,22} = 1.15$, $p = 0.29$) but in sedentary males, $R_{\text{potential}}$ was significantly higher in STI birds than in control birds ($F_{1,26} = 8.75$, $p = 0.006$, Fig. 2). Furthermore, age affected $R_{\text{potential}}$ with older males expressing significantly higher levels of testosterone than second-year males ($F_{1,48} = 6.79$, $p = 0.01$). Catch latency did not have a significant effect ($F_{1,48} = 1.94$, $p = 0.17$) and also the interactions between population and age ($F_{1,48} = 0.21$, $p = 0.65$), and between population and catch latency ($F_{1,48} = 2.83$, $p = 0.1$) were non-significant.

Finally, we compared testosterone concentrations of migratory males on migration with those of breeding sedentary males caught at the same time of the year (i.e. passive birds captured on Ponza vs. control males in Spain). Post-capture testosterone concentrations differed significantly between both groups ($F_{1,29} = 57.04$, $p < 0.001$; Fig. 3): breeding sedentary males had significantly higher levels of testosterone than migrants on migration caught at the same time of the year. Age ($F_{1,29} = 2.54$, $p = 0.12$) and the interaction between age and population ($F_{2,29} = 3.03$, $p = 0.09$) did not have significant effects in this comparison. Also $R_{\text{potential}}$ differed significantly between both groups ($F_{1,27} = 49.2$, $p < 0.001$;

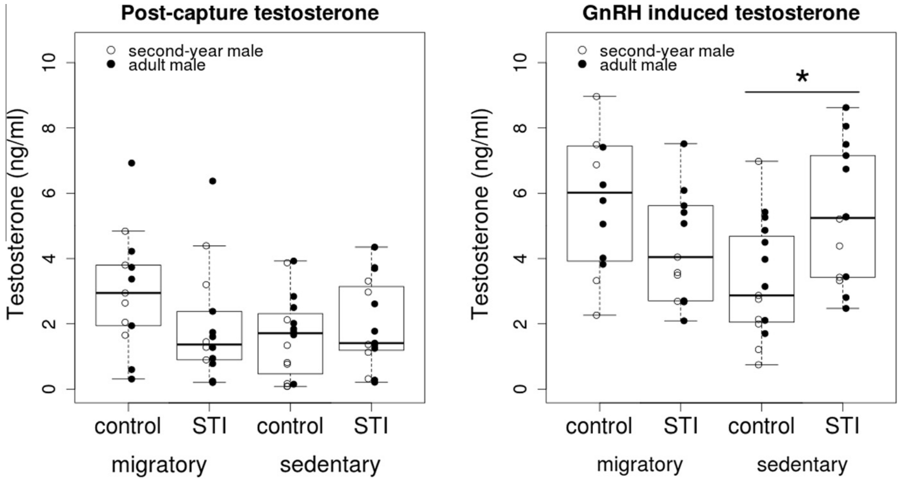

Fig. 2. Post-capture levels of testosterone (left) and GnRH-induced levels of testosterone ($R_{\text{potential}}$) (right) in migratory and sedentary male black redstarts during early breeding in relation to male–male challenges (control vs. simulated territorial intrusion STI) and age (open dots represent second-year males and black dots represent older males in adult plumage). Box plots indicate medians, 10th, 25th, 75th, 90th percentiles.

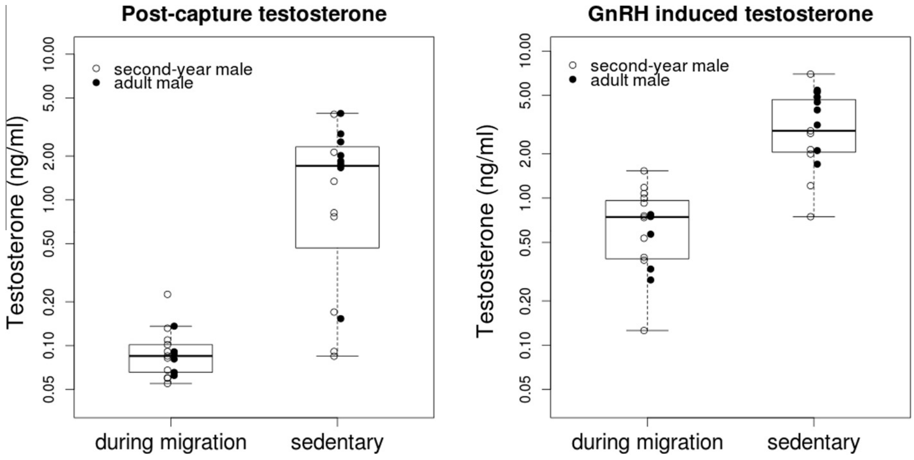

Fig. 3. Medians, 10th, 25th, 75th, 90th percentiles of (left) post-capture testosterone levels and (right) GnRH-induced testosterone levels ($R_{\text{potential}}$) of males caught passively at the same time of the year (March; please note the logarithmic *y*-axis). Both measures of testosterone were significantly higher in males of the sedentary population than in males during migration ($p < 0.001$). All individuals increased testosterone levels after the GnRH challenge and sedentary males showed a significantly higher increase in testosterone than migrating males ($p = 0.003$). Open dots represent second-year males and black dots represent older males in adult plumage.

Fig. 3), with breeding sedentary males in Spain expressing significantly higher levels than individuals on migration. Age ($F_{1,27} = 0.33$, $p = 0.57$) and the interaction between age and population ($F_{1,27} = 3.02$, $p = 0.09$) did not have significant effects. Individuals on migration (from Ponza) and sedentary breeding birds (from Spain) increased testosterone levels after a GnRH challenge and this increase was significantly higher in the sedentary population than in migrants on migration ($F_{1,29} = 10.75$, $p = 0.003$).

4. Discussion

Breeding males of a sedentary population of black redstarts responded similarly to a simulated territorial intrusion as breeding males from a migratory population. However, sedentary males spent less time within 5 m of the decoy than migrants. In contrast to our prediction derived from the finite-state-machine theory and the challenge hypothesis, breeding sedentary males were similar to breeding migrants and did not modulate testosterone in response to simulated territorial intrusions. Thus, expressing fewer life-history stages did not alter $R_{\text{male–male}}$, the androgen responsiveness to male–male interactions, in black redstarts. This result differs from similar data from song sparrows (*Melospiza melodia*) and white-crowned sparrows (*Zonotrichia leucophrys*): STIs resulted in a significant increase in testosterone in migratory white-crowned sparrows, while sedentary song sparrows only showed a trend to increase testosterone (Wingfield and Hahn, 1994). Because male black redstarts from both populations increased testosterone after injection of GnRH ($R_{\text{potential}}$), we conclude that they would have had the capacity to increase testosterone in response to a male–male challenge ($R_{\text{male–male}}$). These results confirm our previous studies of migratory black redstarts (Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011; Villavicencio et al., 2013) and a study in northern cardinals (*Cardinalis cardinalis*; DeVries et al., 2012), and question the generality of the challenge hypothesis, which would predict a strong $R_{\text{male–male}}$ in this class of birds (i.e. socially monogamous and biparental species).

Males from both black redstart populations vigorously responded to a simulated territorial intruder. However, sedentary breeders seemed to respond less strongly to the STIs, because they spent less time within 5 m of the dummy and a smaller proportion of them attacked the dummy. Similarly to black redstarts, sedentary European stonechats (*Saxicola torquata*) responded less aggressively towards a simulated territorial intrusion than males of a migratory population (Marasco et al., 2011). This could have several reasons: First, territorial conflicts may be less frequent in the sedentary population, because the birds may stay in the same location throughout the year (even though some or all birds may make small altitudinal movements during winter). Second, it could be that differences in territory size, in number of social interactions or in vegetation cover could affect the way males respond to an intruder (see Marasco et al., 2011 for a discussion of these factors in stonechats). Third, the dummies (stuffed birds) and the playback recordings that we used were all from the migratory population of black redstarts in Upper Bavaria. Thus, even though sedentary black redstarts clearly responded to these models and playbacks, we cannot exclude the possibility that their response may have been attenuated due to subtle differences in the appearance of the dummy or the playback.

Interestingly, sedentary males exposed to STIs showed a significantly higher $R_{\text{potential}}$ (testosterone increase after GnRH-injection) than control males. We have no compelling explanation for this observation. Did the agonistic interaction prime the hypothalamus–pituitary–gonadal-axis (HPG) so that the STI birds showed faster dynamics of testosterone release in response to GnRH compared to control birds? Or did the STI procedure select for males that had a higher $R_{\text{potential}}$? The latter is unlikely, though, because we caught all males from all territories in which we conducted STIs and in which males responded to these STIs. Thus, we were unlikely to be selecting for particularly aggressive males, which could have been the case if we would have caught only a subset of all males that had been tested. However, on more than one occasion an STI was performed and no bird responded, suggesting that maybe not all males were territorial at that moment. Although there was some variance in the latency to be caught between STI birds, this difference was not significant between populations, and was not related to post-capture testosterone or GnRH-induced testosterone levels. Differences in $R_{\text{potential}}$ of control and STI males between the sedentary and the migratory population might be due to differential sensitivity in the hypothalamus or testis (Ubuka et al., 2013). However, we do not have a compelling explanation for this result, and further experiments would be needed to determine whether this was a random effect or whether male–male interactions might prime the HPG-axis in the sedentary population.

According to the finite-state-machine theory sedentary populations should be more susceptible to environmental modulation of testosterone (Goymann, 2009; Wingfield, 2008, 2005), because they may be more flexible in the neuroendocrine regulation of behavior (fewer life-history stages). While this was not the case in response to male–male interactions ($R_{\text{male–male}}$), the modulation of GnRH-induced testosterone ($R_{\text{potential}}$) in STI birds may indeed indicate a higher degree of flexibility.

Males on migration had higher levels of testosterone than males during molt (see e.g. Apfelbeck et al., 2013), but lower testosterone levels than breeding males from the sedentary population at the same time of the year. Furthermore, males on migration responded to an injection of GnRH with an increase of testosterone, suggesting that their gonads had already started to develop. This is interesting both from a perspective of overlapping phases of two separate life-history stages (migration and breeding) and with regard to how different populations of black redstarts respond to changes in photoperiod. Long-distance migrants such as garden warblers (*Sylvia borin*) exhibit greater testicular mass during migration compared with the regressed (winter) state (Bauchinger et al., 2005), and testosterone levels are significantly correlated with testicular mass on migration (Bauchinger et al., 2007), suggesting that garden warblers start to initiate the breeding life-history stage while still on migration. There is little information with regard to this overlap in life-history stages in short-distance migrants, but our data on testosterone suggest a similar phenomenon in short-distance migratory black redstarts.

Obviously, sedentary males begin to develop their gonads earlier than migratory males. Because sedentary males breed at the same latitude in which migratory males winter, this suggests that sedentary and migratory populations interpret similar photoperiodic information in a different way. This has been experimentally demonstrated in stonechats, in which members of a short-distance and a long-distance migratory population that were held in common garden conditions used photoperiodic information differently (Helm et al., 2009). Thus, data from black redstarts are consistent with the idea that specific annual schedules result from a different organization of the annual cycle in interaction with photoperiod (Helm, 2009; Helm et al., 2009, 2006).

5. Conclusions

In conclusion, our study demonstrated that the number of life-history stages does not seem to explain the lack of androgen responsiveness to male–male interactions ($R_{\text{male–male}}$) in sedentary and migratory black redstarts. Previous work has demonstrated that black redstarts and most other socially monogamous birds in which males provide parental care lack $R_{\text{male–male}}$ (Addis et al., 2010; Apfelbeck and Goymann, 2011; Deviche et al., 2012; DeVries et al., 2012; Landys et al., 2010; Scriba and Goymann, 2010; Villavicencio et al., 2013; and older studies reviewed by Goymann et al., 2007a; Goymann, 2009). These studies thus question the generality of the challenge hypothesis. Neither single-brooded nor multiple-brooded populations of black redstarts modulate testosterone in response to real or simulated territorial intrusions (Apfelbeck and Goymann, 2011). Furthermore, members of a multiple–brooded population do not modulate testosterone in response to repeated simulated territorial intrusions (Apfelbeck et al., 2011) or when territorial males are removed (Villavicencio et al., 2013). Thus, $R_{\text{male–male}}$ is basically absent in male black redstarts. The current study adds to the growing evidence that current theory relating testosterone and territorial behavior needs refinement by demonstrating that a population that expresses fewer life-history stages does not modulate testosterone in response to STIs. Individual males of many bird species show huge differences in testosterone concentrations; in black redstarts, testosterone concentrations range from less than 0.2 ng/ml to more than 10 ng/ml during the early breeding season (Apfelbeck et al., 2013). The challenge hypothesis was an ingenious attempt to explain that this variation is due to differences in the degree of male–male challenge and social stability. The data from black redstarts and the other species cited above suggest that we may need to search for alternative explanations. The observation that sedentary black redstarts show a stronger $R_{\text{potential}}$ when they had been exposed to a STI before the GnRH-injection suggests that male–male interactions may alter HPG-responsiveness (Dijkstra et al., 2012). But our inability to explain this phenomenon underlines the realization that we still need to learn much about how hormones and behavior interact.

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Chapter 3

Parental care, loss of paternity and circulating levels of testosterone and corticosterone in a socially monogamous song bird

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Parental care, loss of paternity and circulating levels of testosterone and corticosterone in a socially monogamous song bird

Camila P Villavicencio[1*], Beate Apfelbeck[1,2] and Wolfgang Goymann[1]

Abstract

Introduction: In biparental birds testosterone levels of males are typically high during the mating phase and decrease during the parental phase. Testosterone implants may enhance mating behaviors, increase the likelihood of males to engage in extra-pair mating behavior and may reduce paternal care. Thus, sex steroids such as testosterone influence reproductive behaviors. Little is known, however, as to whether the more subtle differences in physiological concentrations of testosterone that occur between individuals are related to differences in paternal care, extra-pair behavior, and genetic paternity between those males. Here, we investigate these relationships in the male black redstart (*Phoenicurus ochruros*), a socially monogamous songbird with a low breeding synchrony. We used nestling provisioning as a proxy for parental care behavior and genetic paternity loss as a proxy for the efficiency of mate-guarding.

Results: There was no relationship between nestling provisioning and paternity loss of males. Baseline and gonadotropin releasing hormone (GnRH)-induced levels of testosterone, but not baseline corticosterone, were significantly higher during the mating than during the provisioning phase. Males fed more often when temperatures decreased and fed less when they sang more, but we found no correlation between parental behavior and baseline or GnRH-induced testosterone, and baseline corticosterone – both measured during either the mating or the parental phase. However, males that experienced loss of paternity had lower levels of testosterone during the provisioning phase than males that did not lose paternity. Further, males that lost paternity also expressed higher baseline levels of corticosterone.

Conclusions: Physiological differences in testosterone or baseline corticosterone were not related to differences in parental care, suggesting that the variation of testosterone within a physiological range may not relate to the degree of paternal care in this species. However, the profile of both hormones may indicate quality traits that influence the likelihood of the respective male to lose paternity.

Keywords: Steroid hormones, Black redstart, Aves, Mate-guarding, Extra-pair paternity

Introduction

Steroid hormones regulate traits central to growth, metabolism and reproduction, and thus may influence a wide range of life-history traits (e.g. [1]). For instance, in many seasonally reproducing species testosterone levels are high at the beginning of the breeding season, thus potentially enhancing mating and aggressive behaviors [2-5]. The majority of bird species are socially monogamous and biparental, i.e. males and females form pairs and both partners provision their offspring [6]. However, during the last two decades research using genetic tools revealed that females and males frequently engage in extra-pair behavior leading to extra-pair fertilization [7]. Therefore, males taking care of their young may diminish their possibilities to gain extra-pair paternity, in particular in species that breed asynchronously. Because sex steroids influence reproductive behaviors, an adequate seasonal modulation of testosterone can be important for optimal breeding performance [8] for several reasons. First, high testosterone levels during the parental phase can interfere with paternal

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behavior [9]; in particular, experimentally elevated levels of testosterone have been shown to reduce paternal care [10-19]. Second, high levels of testosterone during the mating season may increase the likelihood of males to show extra-pair behavior [1,20,21]. This has been corroborated by a comparative study indicating that males of bird species with higher rates of extra-pair paternity expressed higher peak levels of testosterone [22]. Third, testosterone may enhance copulatory behavior [23] and mate guarding, in which males attempt to prevent their female partners from engaging in extra-pair fertilizations [10,24].

Hormones other than testosterone have also been suggested to play a role in the regulation of reproductive traits. For example, levels of baseline corticosterone (a hormone involved in energy metabolism and the stress response [25]) have been shown to relate to clutch mass, number of nestlings and their growth rate [26], or to parental behavior and reproductive success [27-31]. However the nature of this relationship can change across stage or context [29,32] rendering it difficult to come up with clear predictions.

Although experimental studies have established that there is an apparent close association between steroid hormones and reproductive behaviors, little is known about the natural variation of reproductive traits in relation to physiological variation of hormone levels [33,34]. Testosterone manipulation studies often work with supraphysiological doses [35] and do not take into account the huge variation of testosterone levels among males. To better understand the role of hormones in the variation of life history traits, it is thus necessary to focus on the individual variation of hormones within their natural range [36]. So far, only few studies have investigated the individual variation between testosterone (or corticosterone) levels and parental behavior [36]. In addition to baseline concentrations of plasma hormones, another – so far little explored – hormonal parameter that can be used to assess the link with individual variation of traits are gonadotropin releasing hormone (GnRH) induced levels of testosterone, which – depending on the testicular status – can be an indicator of maximum production of testosterone [37-40]. In dark-eyed juncos (*Junco hyemalis*), the increase of testosterone after a GnRH challenge correlated positively with aggressive behavior, and negatively with parental care, suggesting that natural variation in testosterone (and not only pharmacological manipulations) can mediate the trade-off between mating and parental effort in this species [8]. The junco study implies that the potential of an individual to increase testosterone release (e.g. the individual variation in GnRH-induced increase of testosterone) could account for individual variation and may be more relevant than baseline levels of testosterone in modulating mating and parental behaviors.

Studies that combine measures of testosterone, parental care and genetic paternity within the same population are still rare [41], but could be important in clarifying the role of this sex steroid in the mediation of traits related to paternal care and extra-pair behavior [1]. Here, we assess the relationship between natural variation in baseline and GnRH-induced levels of testosterone, and baseline corticosterone with parental care and extra-pair paternity in male black redstarts (*Phoenicurus ochruros*), which are socially monogamous songbirds. From the male's point of view extra-pair behavior has two main aspects: first, gaining additional offspring with one or more extra-pair females and second, mate guarding during which a male prevents his female partner to engage in extra-pair behavior with other males. Because we could not monitor the whole population of black redstarts we could not reliably assess paternity gain. We thus focused on paternity loss as a result of ineffective mate guarding. Therefore, we investigated whether natural variation in levels of testosterone and corticosterone were related to paternal care and paternity loss. Opportunities for extra-pair fertilizations are typically higher when the parental period overlaps with the opportunity of males to gain additional mates [7], i.e. when breeding attempts of females are not synchronized and when females have more than one brood per breeding season. Female black redstarts are not synchronous with regard to successfully initiating a first clutch and they can raise up to three broods per season with clutch size ranging from 2 to 6 eggs [42,43]. In our population, the initiation of first clutches range from end of April until beginning of June, probably depending on female condition, quality of the territory, nest site and weather conditions (C. Villavicencio, W. Goymann and B. Apfelbeck, unpublished observations). The rate of extra-pair paternity in black redstarts has not yet been quantified, but this is essential to assess a possible relationship between mating and parental behavior and testosterone. Previous studies in this species have indicated that testosterone levels show large variation among males [44], but this variation was not related to territorial aggression [45,46] and thus could be related to mating behavior and/or paternal care [47].

In the current study, we first aimed to assess the rate of extra-pair paternity in black redstarts. Second, we investigated whether paternal care was related to baseline or GnRH-induced levels of testosterone and baseline concentrations of corticosterone measured during the mating and parental periods. In addition, we assessed other parameters affecting parental care such as singing behavior, which is mainly related to territory defense and mate attraction [48]. Third, we asked whether paternal care was related to loss of paternity to assess whether the level of care could be a response to certainty of genetic paternity. Finally, we asked whether baseline and GnRH-induced levels of testosterone or baseline corticosterone concentrations measured during the mating and parental periods were related to the loss of paternity in this species.

Results

Extra-pair paternity

The paternity analyses revealed that 30.2% of nests (N = 16 out of 53) in the study population contained extra-pair offspring. Overall, 28.8% of the nestlings (N = 64 out of 222) were not sired by the social father. Taking into account all nests, the proportion of extra-pair offspring in one nest was 27.1 ± 11.4% (mean ± 95% confidence interval).

Paternal care

The feeding rates of females and males were not significantly correlated (linear mixed model: $\chi^2 = 1.85$, $P = 0.174$; Pearson's correlation, $r = -0.03$, $P = 0.83$). We first investigated which factors were related to the relative degree of paternal care, i.e. the proportion of male parental care: The mean effect size of post-capture testosterone on paternal behavior was close to zero and also the credible interval for the effect size included zero (Table 1). Thus, based on a Bayesian framework, post-capture levels of testosterone were not related to paternal behavior (Table 1, Figure 1). Also GnRH-induced testosterone concentrations were not predictive for paternal behavior, as the effect size was very low and the credible interval included zero (Table 2, Figure 1). The only factors that were related to the relative degree of paternal care, in both models, were ambient temperature and song frequency: the lower the ambient temperature, the higher the degree of paternal care, and the more a male sang the less it contributed to parental care (Tables 1 and 2). When we used the absolute feeding rate of males rather than the proportion only song frequency had a significant negative impact on paternal care (Tables 1 and 2).

Table 1 Male nestling provisioning in relation to post-capture testosterone, breeding and environmental parameters

	Relative male provisioning rate	**Absolute male provisioning rate**
	Estimate [Credible intervals (2.5% – 97.5%)]	**Estimate [Credible intervals (2.5% – 97.5%)]**
Intercept (Stage feeding)	0.08 [–0.83 – 1.05]	9.14 [3.08 – 15.7]
Stage mating	0.43 [–0.05 – 0.9]	0.69 [–2.14 – 3.42]
Post-capture testosterone	0.0001 [–0.00016 – 0.0004]	0.0003 [–0.002 – 0.002]
Brood	0.15 [–0.21 – 0.51]	–0.19 [–2.14 – 3.42]
Rain	–0.002 [–0.008 – 0.005]	–0.02 [–0.06 – 0.04]
Temperature	**–0.054 [–0.096 – –0.01]**	–0.17 [–0.47 – 0.1]
Cloud cover	0.003 [–0.003 – 0.008]	0.003 [–0.04 – 0.04]
Songs	**–0.05 [–0.08 – –0.03]**	**–0.14 [–0.23 – –0.06]**
Testosterone: stage	–0.0002 [–0.0006 – 0.0001]	–0.0002 [–0.002 – 0.002]

The left column shows the relative male provisioning rate (proportion) and the right column the absolute male provisioning rate, each with the corresponding Bayesian estimate and its' credible intervals. Estimates of cofactors refer to differences from the intercept estimate, which represents the feeding stage. If 0 (zero) is not included in the credible intervals there is an effect of this parameter on the dependent variable. 'Significant' effects with credible intervals not including zero are shown in bold.

In the next analysis we included all birds for which we had data on the relatedness between the social father and nestlings to investigate if the degree of paternal care was related to paternity loss. Because ambient temperature and song seemed to influence paternal care (see above) we included these two variables in this follow-up model. There was no effect of paternity loss on relative paternal care, neither in first nor in second broods (Table 3, Figure 2). Similar to the previous models, ambient temperature and song frequency were negatively related to relative paternal care, while the proportion of male care increased with the number of nestlings (Table 3). When investigating absolute male feeding rates the results were similar: males fed less when they sang more and they fed more depending on the number and age of nestlings (Table 3).

Testosterone and paternity loss

In a third step, we were interested whether paternity loss as a measure of the degree of mate-guarding efficiency was related to testosterone concentrations. The Bayesian effect sizes and credible intervals indicated that testosterone concentrations were higher after a GnRH-challenge (Table 4, Figure 3) and higher during the mating stage than during the parenting stage (Table 4, Figure 3). Furthermore, the effect sizes and credible intervals for the interaction between paternity loss and breeding stage indicated that, during the nestling provisioning phase, males that had experienced a loss in genetic paternity expressed lower levels of testosterone than males that did not lose paternity (Figure 3 inset). This was not the case during the mating stage. There also was an overall effect of paternity loss on testosterone concentrations, but this was mainly driven by the lower levels of testosterone in males that lost genetic paternity during the parental stage. Brood sequence was not related to testosterone concentrations (Table 4).

Corticosterone, parental care and paternity loss

Finally, we assessed factors that were related to baseline corticosterone concentrations. Corticosterone levels of males did not differ between breeding stages (Table 5, Figure 4), relative degree of male parental care (Table 5,

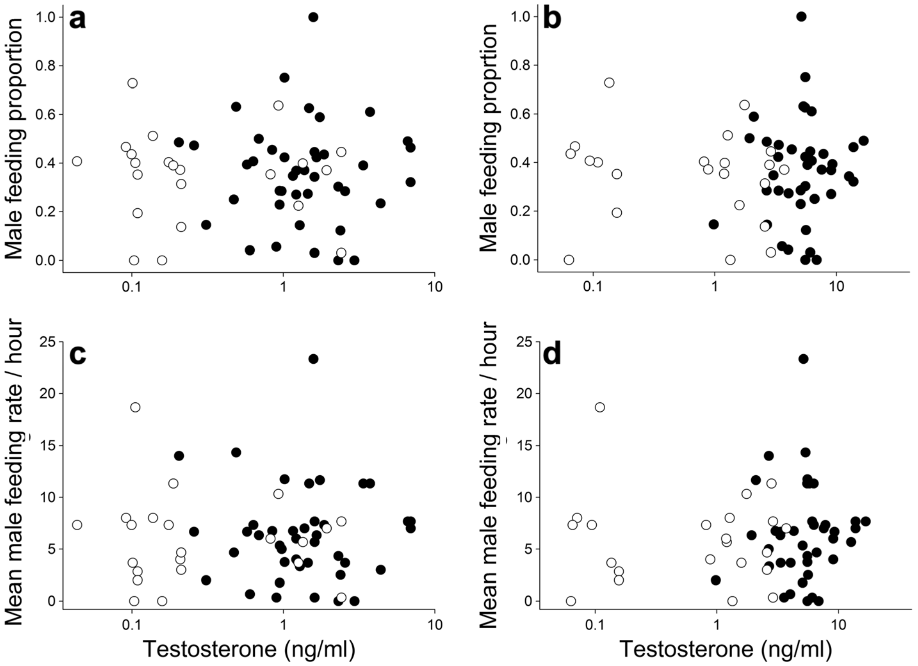

Figure 1 Parental care and testosterone. Relative and absolute male parental care was not related to **(a,c)** post capture testosterone and **(b,d)** GnRH-induced testosterone concentrations (log scale). Black dots represent males sampled during mating; open dots represent males sampled when they were feeding nestlings.

Table 2 Male nestling provisioning in relation to GnRH-induced testosterone, breeding and environmental parameters

	Relative male provisioning rate	Absolute male provisioning rate
	Estimate [Credible intervals (2.5% – 97.5%)]	**Estimate [Credible intervals (2.5% – 97.5%)]**
Intercept (Stage feeding)	−0.51 [−1.96 – 0.93]	9.17 [2.31 – 15.71]
Stage	−0.07 [−4.99 – 4.98]	0.89 [−2.63 – 4.37]
GnRH-induced testosterone	0.11 [−0.06 – 0.28]	0.0003 [−0.001 – 0.002]
Brood	0.19 [−0.19 – 0.57]	0.08 [−2.16 – 2.4]
Rain	−0.002 [−0.009 – 0.004]	−0.01 [−0.06 – 0.04]
Temperature	**−0.06 [−0.1 – −0.02]**	−0.21 [−0.49 – 0.09]
Clouds cover	0.003 [−0.003 – 0.008]	0.002 [−0.04 – 0.04]
Songs	**−0.06 [−0.08 – −0.03]**	**−0.14 [−0.23 – −0.05]**
Testosterone: stage	0.00004 [−0.59 – 0.58]	−0.0003 [−0.002 – 0.001]

The left column shows the relative male provisioning rate (proportion) and the right column the absolute male provisioning rate, each with the corresponding Bayesian estimate and its' credible intervals. Estimates of cofactors refer to differences from the intercept estimate, which represents the feeding stage. If 0 (zero) is not included in the credible intervals there is an effect of this parameter on the dependent variable. 'Significant' effects with credible intervals not including zero are shown in bold.

Figure 4) or brood sequence. When investigating absolute male nestling provisioning rates, the results were the same: no relationship between corticosterone and male nestling provisioning rates (see Table 5). There was also no relation between corticosterone and testosterone

Table 3 Male nestling provisioning in relation to paternity loss, environmental parameters and song behavior

	Relative male provisioning rate	Absolute male provisioning rate
	Estimate [Credible intervals (2.5% – 97.5%)]	**Estimate [Credible intervals (2.5% – 97.5%)]**
Intercept (No paternity loss)	−1.45 [−3.06 – 0.17]	−2.78 [−11.49 – 6.27]
Paternity loss	0.22 [−0.51 – 0.98]	0.07 [−3.14 – 3.25]
Temperature	**−0.06 [−0.1 – −0.01]**	−0.16 [−0.48 – 0.15]
Brood	−0.07 [−0.45 – 0.32]	−1.28 [−3.68 – 1.16]
Age of nestlings	0.05 [−0.01 – 0.11]	**0.68 [0.28 – 1.09]**
Number of nestlings	**0.37 [0.1 – 0.65]**	**1.66 [0.38 – 2.97]**
Songs	**−0.05 [−0.07 – −0.02]**	**−0.11 [−0.21 – −0.02]**

The left column shows the relative male provisioning rate (proportion) and the right column the absolute male provisioning rate, each with the corresponding Bayesian estimate and its' credible intervals. Estimates of cofactors refer to differences from the intercept estimate, which represents no paternity loss. If 0 (zero) is not included within the credible intervals there is a 'significant' effect of this parameter on the dependent variable. 'Significant' effects with credible intervals not including zero are shown in bold.

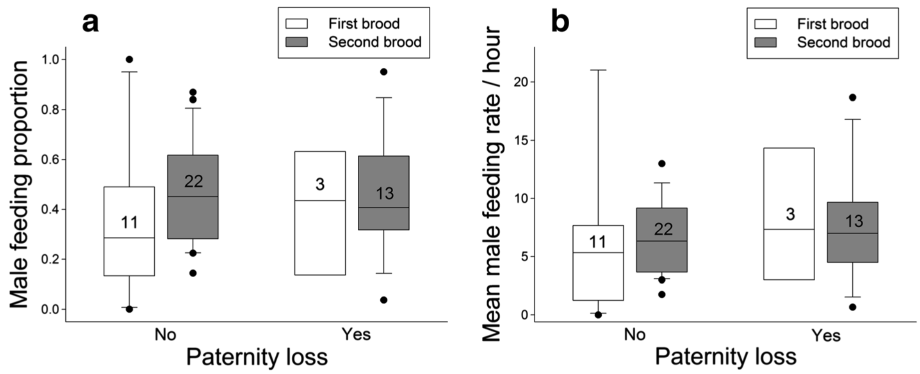

Figure 2 Parental care and paternity loss. Medians, 10th, 25th, 75th, 90th percentiles, and outliers of **(a)** relative and **(b)** absolute male parental care in relation to paternity loss. There was no difference of paternal care behavior regardless of whether a male lost paternity or not, neither in the first (open box) nor in the second brood (grey box).

concentrations, a relationship that was tested separately (Table 6). However, from the follow-up model where we included the subset of all birds for which we had data on the genetic relatedness between the social father and the nestlings to investigate if corticosterone was related to paternity loss, we inferred that males that had lost genetic paternity expressed significantly higher levels of corticosterone than males that did not lose paternity (Table 6, Figure 5).

Discussion

The results of this study suggest that male black redstarts face a relatively high degree of genetic paternity loss within socially monogamous birds [7,49]. The relative degree of paternal care in redstarts increased with lower ambient temperature and decreased with song output. Also the absolute feeding rate was negatively related to song output, but did not vary with ambient temperature. Neither the relative nor the absolute male feeding rate varied with any of the physiological measures, i.e. post-capture or GnRH-induced levels of testosterone, or baseline corticosterone. Also, relatedness with the nestlings did not influence the provisioning behavior of social fathers. However, males that lost paternity had lower levels of testosterone during the parental phase and had overall higher levels of corticosterone than males that did not lose paternity. These data suggest that the hormonal state of males may be related to mate guarding and describe the susceptibility of males to extra-pair behavior of the female.

Table 4 Testosterone concentrations in relation to paternity loss

	Estimate	Credible intervals (2.5%)	Credible intervals (97.5%)
Intercept (no GnRH injection)	6.15	5.42	6.85
GnRH injection	0.9	**0.56**	**1.25**
Paternity loss	−1.08	**−1.79**	**−0.38**
Stage	1.11	**0.5**	**1.73**
Brood	−0.08	−0.58	0.44
Male age	−0.36	−0.99	0.27
Paternity loss: stage	1.45	**0.6**	**2.29**
GnRH injection: stage	0.46	−0.06	0.96

The second column shows the estimates which indicate the direction of the relationship. The third and fourth columns shows the credible intervals (Bayesian), if zero is not included within the credible intervals there is a 'significant' effect of this parameter on the dependent variable. Estimates of cofactors refer to differences from the intercept estimate, which represents no GnRH injection. 'Significant' differences are shown in bold.

Parental care, testosterone and corticosterone

Our data suggest that the natural variation in testosterone concentrations does not influence male provisioning behavior of black redstarts, because the degree of parental care and testosterone – including the potential to produce testosterone (GnRH-induced levels) did not covary (see Figure 1). A suppressive effect of high levels of testosterone on paternal care has been found in testosterone manipulation studies. However, it is not clear whether the effects on paternal care may have been caused by pharmacological levels of the hormone, which may be apparent especially few days after the implantation [19,35]. To date, only few studies have related the natural physiological variance in testosterone concentrations to paternal care: similar to our study, paternal behavior was not related to post-capture testosterone in European starlings [50], barn swallows [41], northern cardinals [51] and dark eyed juncos [8]. Similar to northern cardinals [51], but unlike dark eyed-juncos [8] the GnRH-induced increase in testosterone was also not related to paternal care in black redstarts. The absence of a relationship between parental care and GnRH-induced

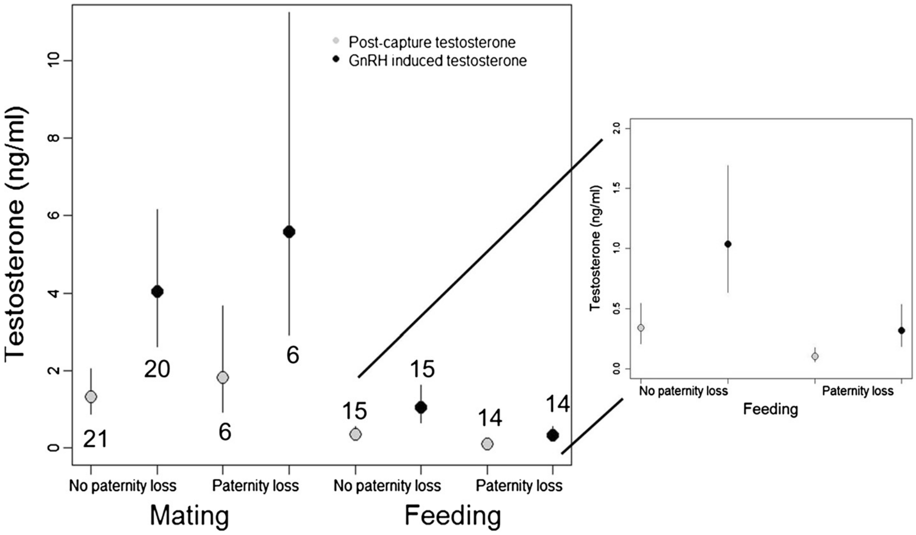

Figure 3 Testosterone and paternity loss. Back transformed means (± 95% credible intervals) of post-capture and GnRH-induced testosterone levels did not differ between males that lost or did not lose paternity, when sampled during mating. When sampled during the nestling provisioning phase males that lost paternity had lower baseline and GnRH-induced levels of testosterone than males that did not lose paternity (inset). Testosterone levels increased after the GnRH-challenge in all groups. Grey dots represent post-capture testosterone levels; black dots represent GnRH-induced testosterone levels.

testosterone levels in black redstarts and northern cardinals on the one hand, and the presence of such a relationship in dark eyed-juncos on the other hand, is difficult to compare: In northern cardinals and in our study male and female behavior was analyzed together and feeding behavior was assessed during three consecutive days; in contrast, in the dark-eyed junco study the female was removed prior to the observation during which only males provisioned the nestlings. It is unlikely that lack of statistical power prevented us from detecting a biologically meaningful correlation between testosterone and paternal care. First, our sample size for testosterone during the provisioning phase was similar to the dark-eyed junco study (dark-eved juncos: N = 25; black redstarts: N = 21) and during the mating phase our sample consisted of 41 birds. Second, an *a priori* power analysis for a simple correlation aiming for a medium effect size (r = 0.5) with a power of 0.80 requires a sample of 29 birds. Thus, for the mating stage our analysis had a power of approximately 0.92 and for the parental phase a power of 0.64, rendering it rather unlikely that we may have missed a medium effect of testosterone on parental behavior. Because we sampled birds during two breeding sub-stages (mating and provisioning stage) this leads to two conclusions. First, testosterone concentrations during the mating phase did not predict parental care behavior in black redstarts. In *Peromyscus* mice mating-induced levels of testosterone correlated with paternal care behavior [52]. It is unknown, whether mating induces an increase in testosterone in male black redstarts. But if so, the injection of GnRH should have a similar effect on males' testosterone concentrations. Because baseline and GnRH-induced levels of testosterone during the mating phase did not predict paternal care we consider it unlikely that testosterone is predictive of paternal care in this species. Second, the actual levels of testosterone expressed during the nestling provisioning phase were also not indicative of the degree of paternal care. Furthermore, even though testosterone levels were higher when black redstarts fed nestlings during the first brood than when they were feeding the second brood [45,53], males did not contribute differently to parental care between first and second

Table 5 Corticosterone levels in relation to the relative proportion of male nestling provisioning

	Relative male provisioning rate	Absolute male provisioning rate
	Estimate [Credible intervals (2.5% – 97.5%)]	Estimate [Credible intervals (2.5% – 97.5%)]
Intercept (Stage feeding)	8.53 [7.96 – 9.12]	8.69 [8.03 – 9.35]
Stage	0.07 [−0.28 – 0.41]	0.1 [−0.29 – 0.46]
Male provisioning rate	0.93 [−0.5 – 2.33]	0.01 [−0.07 – 0.08]
Brood	−0.22 [−0.75 – 0.28]	−0.04 [−0.48 – 0.41]

The left column shows the relative male provisioning rate (proportion) and the right column the absolute male provisioning rate, each with the corresponding Bayesian estimate and its' credible intervals. Estimates of cofactors refer to differences from the intercept estimate, which represents the feeding stage. If zero is not included in the credible intervals there is a 'significant' effect of this parameter on the dependent variable. We found no 'significant' effects.

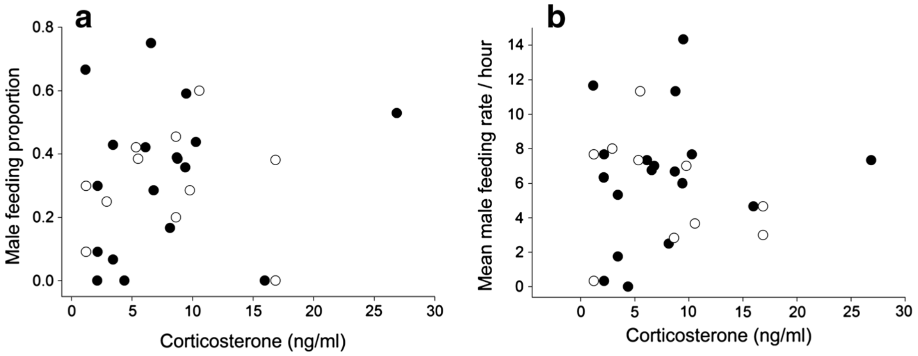

Figure 4 Parental care and corticosterone. (a) Relative and **(b)** absolute male parental care was not related to plasma levels of corticosterone. Black dots represent males sampled during mating; open dots represent males sampled when they were feeding nestlings.

broods (Figure 2). Although there is the possibility that males may differ with respect to the amount of food they provided during each nest visit (we could not quantify this for a sufficient number of birds), our data suggest that the natural variation in testosterone levels does not have a large impact on parental behavior in this species. Thus, it is questionable whether testosterone is involved in mediating a trade-off between mating and paternal behavior in black redstarts. Definitely, more studies on different species are needed to resolve the question whether physiological concentrations of testosterone are related to paternal care. In particular, we need to explore the relationship between the reaction norm of an individual's testosterone concentration and its potential relationship to parental behavior.

Also baseline corticosterone concentrations did not vary with paternal behavior, which is in contrast to some other studies in which baseline corticosterone predicted fitness and feeding behavior [27,29-31]. Because corticosterone is a metabolic hormone one may expect higher levels to be associated with a higher feeding frequency [30]. However, in black redstarts this was not the case. Corticosterone concentrations can be very dynamic and the relationship of corticosterone and parental care can change across stages [29,32]. In an effort to account for stage-related differences we measured corticosterone levels during two breeding stages. However, corticosterone levels did not differ between stages and did not correlate with parental behavior.

Table 6 Corticosterone levels in relation to testosterone and paternity loss

	Estimate	Credible intervals (2.5%)	Credible intervals (97.5%)
Intercept	8.84	8.41	9.26
Testosterone	−0.00005	−0.0002	0.00009
Intercept (no paternity loss)	7796	3986	11727
Paternity loss	4238	**200**	**8130**

They were assessed in separate model and the intercept is shown for each model. The second column shows the estimates which indicate the direction of the relationship. The third and fourth columns shows the credible intervals (Bayesian), if zero is not included in the credible intervals there is a 'significant' effect of this parameter on the dependent variable, which are shown in bold.

Interestingly, environmental factors rather than physiological parameters influenced male feeding behavior, as the relative proportion of the male feeding was higher at lower ambient temperatures. Previous studies have reported an effect of ambient temperature on parental care [54-57], but unlike our study, they did not find differences in the relative provisioning rate of females and males. In addition, males contributed proportionally more if there were more young in the nest, unlike for example hen harriers (*Circus cyaneus*) where female but not male provisioning rate varies positively with brood size [58]. However, provisioning rates typically increase with brood size (e.g. [54,55,59,60]). Also, feeding behavior was negatively related to singing behavior, i.e. males fed more when they sang less (like, for example in the reed bunting, *Emberiza schoeniclus*; [48]). Because a

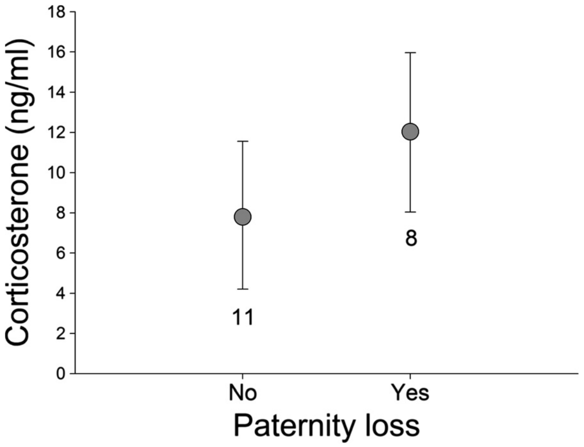

Figure 5 Corticosterone and paternity loss. Back transformed means (± 95% credible intervals) of corticosterone were significantly higher in males that lost paternity than in males that did not lose paternity.

male cannot sing and feed nestlings at the same time it is not surprising that males that spent more time singing contributed less to offspring care.

Paternity loss and parental care

Loss of paternity was relatively high (~30% of nests) in black redstarts compared to other birds species with a similar mating system [7,49]. Such high levels of paternity loss may result from a low degree of breeding synchrony and multiple-broodedness [7,49] in this species. However, paternal care did not differ between males that lost paternity and those that did not, suggesting that males did not adjust the degree of paternal care to paternity. This finding is in line with the observation that male birds in general do not discriminate between their own and extra-pair young [61].

Testosterone, corticosterone and paternity loss

Males that lost paternity expressed lower concentrations of testosterone than males that did not lose paternity when they were feeding nestlings (mainly of the second brood, see Figure 5). This suggests a possible link between testosterone and paternity loss, which may be related to the effectiveness of mate-guarding. Possibly, testosterone concentrations during the late breeding season indicate some trait related to male condition, whereby males with lower condition start to shut-down their reproductive system earlier than males in better condition. This explanation is supported by the data on GnRH-induced levels of testosterone during the nesting stage: males that lost paternity were not able to increase testosterone as much as males that did not lose paternity. Thus, the regression of the reproductive system of males that lost paternity was further advanced than that of males that did not lose paternity. Also the corticosterone data are consistent with this idea: males with higher levels of baseline corticosterone (possibly indicating a higher allostatic load *sensu* [62] experienced a higher degree of paternity loss (see also [63] and [29] for similar results). Alternatively, males that showed a higher investment in mating behavior maintained higher levels of testosterone for a longer period of time, and hence, were more capable of preventing their female from engaging in extra-pair fertilizations. Thus, the seasonal dynamics of an individuals' testosterone profile or individual hormonal reaction norms may be more important in relation to fitness-relevant traits than currently recognized. So far, the rate at which testosterone concentrations decline during the breeding season within individuals remains largely unknown. On a population level, the decline in testosterone has been reported to correlate with the duration of mating behaviors and the breeding season [64]. Surprisingly, there are only few other published studies that relate testosterone concentrations and extra-pair behavior (see also [1] for further discussion of this topic). Possibly, there is a publication bias, i.e. non-significant relationships between testosterone and paternity may not be published (e.g. [65] did not report testosterone because there was no relationship of testosterone with extra-pair paternity). To date, we are only aware of one study relating natural variation in testosterone levels and extra-pair paternity [66]. They found no correlation between testosterone levels and paternity loss in barn swallows. Another study measured cuckoldry risk or extra-pair opportunities in Seychelle warblers (*Acrocephalus sechellensis*) and did not find a relationship with testosterone [67]. Three studies have analyzed the effect of testosterone implants on extra-pair behavior. In two studies of dark-eyed juncos testosterone implants led to an increase in extra-pair paternity [21,68]. In contrast, testosterone implants reduced extra-pair paternity in blue tits (*Cyanistes caeruleus* [69]), which might have been due to negative feedback of testosterone leading to a shut-down of sperm production.

Conclusions

In summary, the data on black redstarts provide little evidence that the level of care could be a response to certainty of paternity. Importantly, there is also no indication that high levels of testosterone would interfere with parental care. Nevertheless, the maintenance of high levels of testosterone and low levels of corticosterone throughout the breeding season may be related to effective mate-guarding or good condition in male black redstarts, thus reducing the likelihood of losing paternity. Further, unlike in dark-eyed juncos [8] a GnRH-induced increase in testosterone did not appear to explain parental care behavior. However, both baseline and GnRH-induced testosterone levels during the provisioning phase seemed to relate to paternity loss. These data suggest that in order to find relationships of hormones with fitness-relevant traits we may need to better understand the seasonal dynamics and the hormonal reaction norms of individuals. Our data also add further support to the idea that testosterone in black redstarts (and possibly other birds that are territorial during most of the year) is mainly related to mating behavior, while it does not play a major role in territorial behavior ([53,70]). Studies relating parental care, paternity and testosterone (or other hormones) within the same individuals are still scarce, but are urgently needed to better understand individual variation in life-history traits and their physiological basis.

Material and methods

This study was conducted in a migratory population of black redstarts in Upper Bavaria, Germany, in villages in the vicinity of the Max-Planck-Institut für Ornithologie (47°N, 11°E, 500–600 m above sea level). Black redstarts of this population arrive on their breeding grounds from late March to the beginning of April. In the years in which we conducted the experiments, the first broods were raised from May 10th until June 8th in 2010, and from May 5th until June 15th in 2011. Second broods were raised between June 15th until July 29th in 2010 and from June 20th until July 30th in 2011. Black redstarts typically build their nests in houses or barns in human settlements. Females incubate the clutch of 2–6 eggs, but both parents feed nestlings and fledglings [42]. Black redstarts show delayed plumage maturation with second-year males (i.e. males in their first breeding season) resembling females and attaining their black coloration with white wing patches only after their first postnuptial molt [43].

Field procedures and blood sampling

Black redstarts were caught in mealworm-baited traps by first luring them to the traps with a short playback of black redstart song or by placing a stuffed decoy into their territory and playing back black redstart song for 20 minutes (simulated territorial intrusion; STI). Previous studies have demonstrated that black redstarts do not increase testosterone concentrations following single or repeated simulated territorial intrusions [45,46]. Therefore, we did not expect differences in testosterone concentrations depending on the catching method. Males were caught during the mating period between April 15th until June 10th (n = 35) and during the parental care period between May 25th until July 31st (n = 34) in 2010 and 2011, 8 of these males were sampled during both stages. Three males were caught while feeding the first brood (25th of May until June 11th), and 31 males were sampled during the second brood (June 23rd until July 31st). A subset of males (n = 25) was sampled also for plasma levels of corticosterone, but only in 2011: for corticosterone 17 males were caught during mating and 10 males were caught while feeding their nestlings, four of these males were sampled twice. The breeding stage of males (mating or parental) was determined using behavioral and nest observations. All males caught during April were assumed to be in the mating stage, because we did not find any nests in this period. The mating stage was further confirmed *a posteriori*, after finding and back-dating the nests of the respective pairs. Males were considered in the parental stage when they had an active nest in their territory and made frequent nest visits with food in their beaks.

Immediately after catching, a blood sample from the wing vein was obtained to determine baseline corticosterone (2.4 ± 0.2 min after capture; mean ± 95% CI) and post-capture testosterone concentrations (4.6 ± 0.4 min after capture). Following the first blood sample, 50 μl of chicken GnRH-I (Bachem H 3106; 1.25 μg dissolved in 50 μl isotonic saline) was injected into the *pectoralis major* muscle for the determination of GnRH-induced testosterone levels (see also [39]). After the injection, each bird was kept in a holding bag for 30 minutes until the second blood sample was taken. Each bird was measured (body mass, right tarsus, wing and tail lengths, and width and height of the cloacal protuberance) and banded with a unique numbered aluminum ring (Vogelwarte Radolfzell) and unique color bands for individual identification. Blood samples were immediately centrifuged with a Compur Minicentrifuge (Bayer Diagnostics) to separate the plasma from blood cells. Plasma volume was measured using a Hamilton syringe and stored in 500 μl ethanol [71]. The blood cells were dissolved in Queen's lysis buffer [72] for genetic paternity analyses and stored at room temperature. After returning from the field, plasma samples in ethanol were stored at −80°C. All experimental procedures were approved by the animal ethics committees of Upper Bavaria.

Parental behavior

Nestling provisioning rates were used as a proxy for parental behavior. Feeding behavior of both parents was measured by direct observation of the nest. The number of nest visits of both parents was counted during one hour on three consecutive days from days 6–13 after hatching. Song frequency was also assessed during that time. Although we attempted to measure the amount of food carried by the parents, we were not able to measure this for all the individuals and for each nest visit. However, we found quite some variability within individuals regarding the amount of food they carried even within just one observation (ranging from 1 to 4 beak sizes). All observations took place in the morning from 06:00 to 11:00 hours. We only conducted observations if at least one parent was color-banded. When one parent was unbanded we assumed that all feedings visits of unbanded birds were performed by the same individual. Black redstarts typically raise two (sometimes three) broods per season, with the first nestling period lasting between May 5th until June 18th, and the second between June 21st until July 30th 2010 and 2011. We observed parents during the nestling phase of 22 first and 41 second broods (see Table 7). Most nests were hard to access (or inaccessible) and hence regular nest

Table 7 Details of the feeding protocols

Observation dates	Year	Brood no.	No. of nests observed
19-May/29-May	2010	1st	8
15-Jun/29-Jul	2010	2nd	35
11-May/9-Jun	2011	1st	13
15-Jun/28-Jul	2011	2nd	18

Date, year and number of first and second broods observed to determine parental nestling provisioning rate.

inspections could not be done. The age of the nestlings was thus estimated by the fledging date, which is typically 14 days after hatching [43]. In addition, we collected weather data to assess if environmental parameters influence the feeding behavior of the parents. We used ambient temperature, cloud cover, and rainfall. The environmental weather parameters were obtained from the local weather center (Wetterstation Wielenbach) as means of the morning from 7:00–13:00, on the exact days we performed the nest observations.

Paternity

From the nestlings we obtained a small (ca. 5 μl) blood sample by puncturing the wing vein when they were at least one week old. In total, we collected DNA samples from 138 males, 68 females and 222 nestlings from 53 broods during the two consecutive years. For paternity analysis we used 13 microsatellite markers, combined in 4 mixes (Asμ15-ZEST, CcaTgu3, CcaTgu15, CcaTgu21, DkiB102-ZEST, TguEST09-005, TguEST09-021 [73], TG01-124, TG02-088 [74], ADCYAP1 [75], Tgu7 [76], Mcyμ4 [77], Gf06 [78], see Additional file 1: Table S1). DNA extraction was performed with NucleoSpin Blood QuickPure Kit (Machery-NagelGmbH & Co. KG, Düren, Germany). Multiplex PCR-reaction were performed with the Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany) and primer mixes containing three to five primer pairs (mix A – D, Additional file 1: Table S1) at primer mix specific temperatures (52-57°C, Additional file 1: Table S1). Forward primers were labeled at their 5′ end with fluorescent dyes. Differences in amplification efficiency and dye strength of the primers were accommodated by adapting the primer concentrations in these mixes (details given in Additional file 1: Table S1). Each 10 μl multiplex PCR contained 20 – 100 ng DNA, 5 μl of the 2× Qiagen Multiplex PCR Master Mix, 1 μl of one of a primer mix and 3 μl of ddH_2O. Cycling conditions were: 15 min initial denaturation at 95°C, 24–25 cycles (see Additional file 1: Table S1) of 30 s denaturation at 94°C, 90 s annealing at 52-57°C, and 1 min extension at 72°C, followed by 30 min completing final extension at 60°C. 1.5 μl of the PCR product was mixed with formamide containing the GeneScan 500 LIZ Size Standard and heat denatured. Fluorescently labeled PCR products were sized on a 3130 xl Genetic Analyser (Applied Biosystems, Darmstadt, Germany). Subsequently allele lengths were determined using GeneMapper 4.0 software. The most likely set of parents were searched from the pool of available males and females with CERVUS version 3.0.3 (©Field Genetics Ltd). If either the social male or social female was not sampled, the set of alleles from the nestlings and the available parent was used to infer the genetic father/mother. Because males and females were observed provisioning the nestlings, it was possible to recognize the social parents. Nestlings that had more than two mismatches with the social father were assigned to be extra-pair young, none of the nestlings had mismatches with the mother. The number of mismatched loci among extra pair nestlings was 6.4 ± 2.1 (mean ± sd). Only one mismatch loci with the social father (n = 2, after repeated genotyping) was assumed to be due to mutation [79].

Hormone analysis

Testosterone and corticosterone concentrations were determined by radioimmunoassay following the procedures described in [71]. Samples were assayed in duplicate and distributed randomly between two assays. The extraction recovery for testosterone was 88.0% ± 5.5% (mean ± sd). Hormone concentrations were calculated with Immunofit 3.0 (Beckmann Inc., Fullerton, CA, USA). The lower detection limits of the testosterone assays were 0.35 pg/ml and 0.45 pg/ml, respectively, and all samples were above the detection limit. The intra-assay coefficients of variation were 8.7% and 13%, respectively; the intra-extraction coefficients of variation of a chicken plasma pool were 0.02% and 5.6%, respectively. The inter-assay coefficient of variation between the two assays was 14.8% and the inter-extraction coefficient of variation between the two assays was 11.1%. For corticosterone, extraction recovery was 83% ± 6% (mean ± sd). Samples were measured in duplicate in one assay. The lower detection limit of the assay was 4.78 pg/ml and all samples were above the detection limit. The intra-assay coefficient of variation was 4.1%; the intra-extraction coefficient of variation of the chicken plasma pool was 2.5%.

Data analysis

Data were analyzed using the R (2.13.0; R Development Core Team) package “arm” [80]. To determine the relationship between parental care and testosterone, two models were used: one including post-capture testosterone and another one using GnRH-induced testosterone as independent variables. Paternal care, expressed as the proportional feeding rate of the male (male feeding rate/total feeding rate), was the dependent variable. We used the proportion to reduce the influence of other factors (i.e. chick age). In addition, we calculated the same model using the absolute male feeding rate. Independent variables included post-capture testosterone (or log transformed GnRH-induced testosterone concentrations for the second model), breeding stage during sampling (mating or feeding) and its interaction with testosterone, brood sequence (1st or 2nd brood), rain, temperature, cloud cover and song frequency using a generalized linear mixed model (glmer) with a binomial distribution. We did not find any age-related difference throughout the analysis, and thus did not include male age as a factor in these models. Because we measured the feeding rate on 3 consecutive days, bird ID was included as a random effect to account for repeated measures. For the inferences of the model we used the Bayesian approach and obtained 95% credible intervals for the model parameters [81].

As a second step we assessed the relationship of paternal care and paternity loss. For this we used a sub set of individuals for which both paternal care and paternity data were available. Paternal care, expressed as the proportional feeding rate of the male was used as the dependent variable. In addition, we used the absolute feeding rate in a separate model. Paternity loss (yes/no), brood number (1st/2nd), age and number of nestlings served as independent variables. We also included air temperature and song frequency in the model because they were related to paternal care in the previous model (see Results section). A generalized linear mixed model (glmer) with binomial distribution was used. Bird ID was included as a random effect to account for repeated measures. For the inferences of the model we used the Bayesian approach to obtain 95% credible intervals for the model parameters using an uninformed prior distribution, which is the equivalent of null hypothesis testing. Currently, the Bayesian approach is the only method that allows drawing exact inferences and avoids the difficulties of determining the degrees of freedom in mixed model analyses [81].

In addition, we assessed if male and female feeding rates were correlated. Using a linear mixed model, we contrasted these two parameters. Because both parent were always observed on the same days we included the identity of the couple as a random effect to account for repeated measures. We also did a Pearson's correlation using the mean of all feeding observations.

As a third step, to assess which factors may have affected testosterone concentrations, the effect of paternity loss (yes/no), the GnRH treatment (before/after), the breeding stage during which the blood sample was taken (mating/feeding) and the brood sequence (first or second) on testosterone levels was tested using a linear mixed model (lmer). Bird ID was included as a random effect to account for repeated measures (because (a) all males were sampled twice to measure post capture and GnRH-induced testosterone concentrations, (b) 8 individual males were sampled during both breeding stages and (c) we included data from nestlings of first and second broods for 7 males).

Finally, we assessed which factors may have affected corticosterone concentrations. First, we tested whether corticosterone concentrations were related to the feeding proportion of males, the breeding stage (mating/feeding) and the brood sequence (first/second) using a linear mixed model (lmer). Bird ID was included as a random effect to account for repeated measures, because 3 males were sampled twice (during mating and feeding). In addition, separately we assessed if corticosterone was related to testosterone concentrations using a linear mixed model. Finally, in the subset of males for which data regarding paternity loss were available, we tested if corticosterone levels differed depending on paternity loss (yes/no) using a linear mixed model.

Additional file

Additional file 1: Table S1. Characterization of 13 microsatellite loci for *Phoenicurus ochruros*. Primer sequences include information on fluorescence labels used and details of the multiplex PCR conditions per mix (temperature and cycles). C is the primer concentration in multiplex primer mix and NA is the number of alleles.

Abbreviation

GnRH: Gonadotropin releasing hormone.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CPV and WG conceived the study and design the experimental set-up. CPV, WG and BA executed the experiments. CPV analyzed the data and wrote the first draft of the manuscript. CPV and WG wrote the final version of the manuscript. All authors read and approved the final manuscript.

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Chapter 4

Associated and disassociated patterns in hormones, song, behavior and brain receptor expression between life-cycle stages in male black redstarts, *Phoenicurus ochruros*

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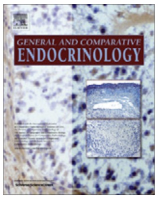

Associated and disassociated patterns in hormones, song, behavior and brain receptor expression between life-cycle stages in male black redstarts, *Phoenicurus ochruros*

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ABSTRACT

Testosterone has been suggested to be involved in the regulation of male territorial behavior. For example, seasonal peaks in testosterone typically coincide with periods of intense competition between males for territories and mating partners. However, some species also express territorial behavior outside a breeding context when testosterone levels are low and, thus, the degree to which testosterone facilitates territorial behavior in these species is not well understood. We studied territorial behavior and its neuroendocrine correlates in male black redstarts. Black redstarts defend territories in spring during the breeding period, but also in the fall outside a reproductive context when testosterone levels are low. In the present study we assessed if song output and structure remain stable across life-cycle stages. Furthermore, we assessed if brain anatomy may give insight into the role of testosterone in the regulation of territorial behavior in black redstarts. We found that males sang spontaneously at a high rate during the nonbreeding period when testosterone levels were low; however the trill-like components of spontaneously produced song contained less repetitive elements during nonbreeding than during breeding. This higher number of repetitive elements in trills did not, however, correlate with a larger song control nucleus HVC during breeding. However, males expressed more aromatase mRNA in the preoptic area – a brain nucleus important for sexual and aggressive behavior – during breeding than during nonbreeding. In combination with our previous studies on black redstarts our results suggest that territorial behavior in this species only partly depends on sex steroids: spontaneous song output, seasonal variation in trills and non-vocal territorial behavior in response to a simulated territorial intruder seem to be independent of sex steroids. However, context-dependent song during breeding may be facilitated by testosterone – potentially by conversion of testosterone to estradiol in the preoptic area.

1. Introduction

Most animals live in highly seasonal environments that vary, for example, in temperature and precipitation and consequently in the availability of resources such as food. To survive and maximize their reproductive success animals need to cope with these largely predictable changes and time their life-cycle accordingly. Hormones play a major role in the development and transition between life-cycle stages (e.g. Jacobs and Wingfield, 2000; Wingfield et al., 2001) and testosterone has been identified as an important player in regulating the breeding life-cycle stage of male vertebrates: it is required for spermatogenesis, the development of secondary sex characteristics and it facilitates sexual and territorial behaviors (Adkins-Regan, 2005; Nelson, 2005). Accordingly, testosterone levels are often highest at the beginning of the breeding season when interactions among males, song and sexual activities are most intense (Ball and Wingfield, 1987; Dawson, 1983; Morton et al., 1990; Silverin, 1993; Silverin et al., 1986; Van Duyse et al., 2003; Wingfield et al., 1990). However, the correlation between behaviors expressed in a territorial context and testosterone levels is not always that straight-forward: many temperate-zone songbird species, for example, defend territories and sing outside the breeding season when testosterone levels are low (Apfelbeck and Goymann, 2011; Canoine and Gwinner, 2002; Landys et al., 2010; Schwabl, 1992; Wingfield, 1994). Testosterone could still facilitate territoriality in these species: testosterone precursors may be derived from non-gonadal sources and metabolized to testosterone

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directly in the brain (e.g. Soma et al., 2000; Soma and Wingfield, 2001) or the brain may have a higher sensitivity for low levels of the hormone (Canoine et al., 2007). In some species, however, testosterone facilitates territorial behavior during the breeding period, but does not appear to facilitate territorial behavior during the nonbreeding season (Canoine and Gwinner, 2002; Hau and Beebe, 2011; Hau et al., 2000; Landys et al., 2010; Marasco et al., 2011; Schwabl and Kriner, 1991). In rufous-collared sparrows (*Zonotrichia capensis*) territorial behavior seems to be independent of testosterone even during breeding (Moore et al., 2004; Moore et al., 2004). In other species, that defend territories and sing both in- and outside the breeding season, these behaviors seem to differ in these two contexts (Moore, 1988). For example, during breeding song may contain more repetitive elements (DeWolfe et al., 1974; Leitner et al., 2001; Smith et al., 1997; Voigt and Leitner, 2008), longer songs (Riters et al., 2000) or song may be more stereotyped (Smith et al., 1997) than during nonbreeding. In the latter cases testosterone during breeding may activate these changes in territorial behaviors and song. In song sparrows (*Melospiza melodia*), for example, seasonal changes in song have been correlated with the size of the HVC, a brain nucleus of the song control system in songbirds (Nottebohm et al., 1976) that is considered to control motor output during singing (Brenowitz et al., 1997; Yu and Margoliash, 1996). The HVC is sensitive to androgens (Gahr and Metzdorf, 1997) and its size is thought to depend at least partly on circulating testosterone levels (Nottebohm, 1980; Sartor et al., 2005). Furthermore, the sensitivity to testosterone may change within the HVC depending on season (Gahr and Metzdorf, 1997; Soma et al., 1999). It has been argued that a larger HVC during breeding is related to a larger song repertoire, a higher song rate and facilitation of a more complex song in song sparrows (Brenowitz, 1997; Smith et al., 1997, but see (Gahr, 1997). In other species such as canaries (*Serinus canaries*) and black-capped chickadees (*Poecile atricapillus*), however, seasonal changes in song are not related to HVC size (Fusani et al., 2000; Smulders et al., 2006). Furthermore, testosterone may influence the motivation to sing and the song rate in a reproductive context by activating song areas outside the song control system, e.g. by aromatization of testosterone to estrogens in the preoptic area (Foidart et al., 1998; Riters et al., 2000; Soma et al., 2003). The preoptic area has been shown to play an important role in the regulation of estrogen-dependent aggressive behavior (Schlinger and Callard, 1990; Silverin et al., 2004).

Hence, although it is well accepted that testosterone plays a role in the organization and activation of song (Bolhuis and Gahr, 2006) and territorial behavior (Wingfield et al., 2006) in the breeding season, it is still unclear to what extent testosterone facilitates these behaviors in species that sing and defend territories outside the breeding season.

The black redstart (Phoenicurus ochruros) is a temperate-zone song bird species that defends a territory and sings during the breeding season in spring and also during nonbreeding in fall. Black redstarts are socially monogamous and both females and males provide parental care (Draganoiu et al., 2005; Landmann, 1996). Males defend a territory and sing during the breeding season in spring and early summer. During late summer (mid-August–mid-September) they molt and show a decrease in singing activity. Afterwards they express a pronounced period of fall territoriality and song activity until the end of October just before they start migration (Nicolai, 2005; Weggler, 2000).

To investigate whether testosterone may facilitate song and territorial behavior in different life-cycle stages, we compared testosterone (obtained via blood samples), territorial behavior, spontaneous song, and the distribution of hormone receptors in the brain of male black redstarts during breeding and nonbreeding territoriality. We focused on brain areas relevant for singing (forebrain song control nuclei) and aggressive behavior (diencephalon). We describe the expression pattern of androgen receptor-, estrogen receptor- and aromatase mRNA of male black redstarts during breeding and nonbreeding territoriality and determine whether HVC size and aromatase expression in the preoptic area differ between life-cycle stages and correlate with testosterone levels, territorial behavior and spontaneous song. If song output and structure differ with life-cycle stage, we expected males to have a larger HVC during breeding than outside the breeding season (e.g. Smith et al., 1997). As aromatase expression in the preoptic area has been shown to play an important role in the expression of reproductive behaviors (Balthazart et al., 2010), we expected a higher expression of aromatase mRNA in that area during breeding compared to the nonbreeding fall territorial phase.

We compared song output and song structure between breeding and nonbreeding territoriality to test if they differ between life-cycle stages. If testosterone activates song during the breeding season we expected that males produce more spontaneous song during breeding in spring than during nonbreeding in fall. Similar to other species, parts of the song of black redstarts contain repetitive elements and males increase the number of these elements in an agonistic context (Apfelbeck et al., 2012). If testosterone changes the structure of song during breeding, we expected to find significant differences in the structure of spontaneous song between the breeding and the nonbreeding season.

2. Methods

2.1. Study period and study site

Free-living male black redstarts were challenged with simulated territorial intrusions and caught in 2008 (April 1–June 12; September 19–October 6), 2009 (July 3–August 13) and, 2010 (June 28–July 31) in Upper Bavaria, Germany (N 47°, E 11°, 500–600 m above sea level). These males contributed to different experiments (see below); however most of them were bled and contributed to the seasonal testosterone profile presented. Furthermore, some of the hormone data from the early breeding season and the fall nonbreeding period as well as the behavioral responses to simulated territorial intrusions were collected as part of a different study and have been previously published (Apfelbeck and Goymann, 2011). The comparison between the two territorial phases (during breeding in spring and nonbreeding in fall) was conducted in a cross-sectional manner (studying different birds in each phase). Breeding stages for the seasonal hormone profile were determined by behavioral observations of males and females during capture. Before capture, we mapped territories by determining boundaries through frequently used singing posts. After capture, territorial males were followed throughout the breeding season and the presence of focal males in the respective territories was confirmed through observation or the use of playback (i.e. we played back black redstart song until the territory owner responded by approaching the loud speaker or by singing or for at least 10 min). The main focus of this study was a comparison of hormone levels, behavior, song and brain between early breeding season territory establishment and fall nonbreeding territoriality. In addition, hormonal data were obtained in more detail throughout the whole breeding season and during molt. Therefore, for the hormone part of the manuscript, the breeding period was further divided into several substages (incubation, nestlings and fledglings of first brood, nestlings and fledglings of second brood).

2.2. Simulated territorial intrusion experiments

Territorial behavior was assessed by simulated territorial intrusion experiments (STI). For a full description of the territorial intrusion experiments see (Apfelbeck and Goymann, 2011). Briefly, a stuffed decoy (in full adult plumage, three specimens) along with black redstart song was placed into the territory and the behavioral responses of the territory owner were recorded from a distance. We used five different playback strings with species-typical song in random order (wav.files, each repeated at a rate of eight strophes per min) that were played back from a loudspeaker at a sound pressure levels of 65 dB SPL at 1 m (as measured with a CEL 573.B1 Sound Level Analyser) directly below the decoy. We recorded the following behaviors of the territory owner during 10 min of vocal playback: (1) latency to respond to the STI either by singing or approaching the decoy, (2) the first time the male was in a 5 m radius around the decoy, (3) the total time the male spent in this 5 m radius, (4) the total time the territory owner fluffed its feathers, and (5) the number of head nods. Furthermore, we noted whether the male attacked the decoy or sang at any time during the STI.

2.3. Capture and blood sampling

Males were caught after the STI experiments or while searching for food to obtain blood for hormone analysis (see also Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011). They were caught after STI experiments to obtain brains for mRNA expression analysis. Birds were caught in mealworm-baited ground traps or tree traps (in the case of simulated territorial intrusion experiments, some traps were attached close to the decoy and were opened after ten minutes and the playback continued until capture of the territory holder). Immediately upon capture (178 ± 112 s), a blood sample (~120 μl) was taken from the wing vein and collected into heparinized capillaries. We checked if males were molting body, wing, and/or tail feathers and banded them with a numbered aluminum ring (Vogelwarte Radolfzell) and a combination of two color rings. All experimental procedures were approved by the governmental authorities of Upper Bavaria.

2.4. Recording and analysis of spontaneous song

Adult ($\geqslant$2 years) territorial male black redstarts were recorded with a Sennheiser directional microphone (ME66/K6) connected to a Marantz solid state recorder PMD 660 (sampling frequency: 44.1 kHz; resolution: 16 bit) during breeding territorial establishment in spring 2009 (April 9–27, $n = 20$) and a different set of individuals during the nonbreeding territorial phase in fall 2009 (September 22–October 7, $n = 12$). Songs were analyzed using Avisoft-SASLab Pro software, version 4.51. Recordings were visualized in spectrograms (settings: sample rate 22,050 Hz, FFT = 256 points, Hamming-Window, Overlap: 50%). We determined the number of songs by visual inspection and selected songs of sufficient quality (low background noise) for further sound analysis. Each song of black redstarts can be divided into three distinct parts (part A, B, and C, e.g. (Cucco and Malacarne, 1999) with a pause of varying length between part A and B. We measured the duration of part A, B, C, the total song and the duration of pauses between A and B. We counted the number of elements of part A and C (mean of max. 20 songs). We also determined the frequency bandwidth and the maximum frequency of part A, B, and C using the automatic parameter measurement function (threshold −20 dB) in Avisoft (mean of max. 10 renditions of high-quality songs).

2.5. Plasma separation and hormone analysis

Plasma was immediately separated by centrifugation with a Compur Minicentrifuge (Bayer Diagnostics). The amount of plasma was measured with a Hamilton syringe and stored in 500 μl ethanol (Goymann et al., 2007). After returning from the field samples were stored at −80 °C. Testosterone concentration was determined by direct radioimmunoassay (RIA, following Apfelbeck and Goymann, 2011; Goymann et al., 2006). Samples were measured in duplicates in four assays. Mean ± SD efficiency of the extraction with dichloromethane was 92.5 ± 0.1%. The lower limits of detection of the assays were determined as the first values outside the 95% confidence intervals for the zero standard ($B_{\max}$) and were 6.7, 6.6, 6.0, and 8.0 pg/tube respectively. The intra-assay coefficients of variation were 4.7%, 4.2%, 3.1%, and 7.9%, respectively. The inter-assay variation was 6.4 ± 1.8%. As the testosterone antibody shows significant cross-reactions with 5α-dihydrotestosterone (44%) our measurements may include a fraction of this potent androgen.

2.6. Tissue collection

Brains and testes were collected between April 14–28 in 2008 ($n = 8$) and between September 21–24 in 2009 ($n = 8$). Upon capture, birds were immediately killed by decapitation and brains removed from the skull, frozen on dry ice and stored at -80 °C until further analysis. We also recorded the length and width of the left and right testis using calipers and calculated testis volume as the volume of an oval body ($4/3*\pi*(\text{width}/2)^2*(\text{length}/2)$). Frozen brains were cut into 20 μm sagittal sections on a cryostat microtome (Leica Microsystems GmbH, Wetzlar, Germany) and collected on Superfrost object slides (Menzel GmbH, Braunschweig, Germany) in five parallel series. One series of brain sections was selected for Nissl staining and used to provide anatomical landmarks for later interpretation of in situ hybridization results. The remaining series were used for in situ hybridization of adjacent sections for androgen receptor, estrogen receptor α and aromatase.

2.7. In-situ hybridization

Riboprobes were synthesized from cDNA previously cloned from zebra finch androgen and estrogen receptor α mRNA (Gahr and Metzdorf, 1997) and canary aromatase mRNA (Metzdorf et al., 1999). Antisense and sense ^{35}S-CTP-labeled probes were transcribed from the T7 and SP6 promoter region of a pGEM7Zf + vector using the Riboprobe System (Promega, Madison, WI). Brain sections were fixed in a 4% formaldehyde solution in phosphate-buffered saline (PBS; 0.01 M; pH 7.4) for 5 min, washed in DEPC-treated PBS, and incubated in 0.25% acetic anhydride in ethanolamine (TEA; 0.1 M; pH 8.0) for 10 min to reduce nonspecific binding. After a washing step in 2× standard saline citrate (SSC), sections were dehydrated in serially increasing percentages of ethanol, and left to dry at room temperature. Sections were hybridized under a cover slide with ^{35}S-CTP-labeled *sense or* antisense riboprobes (0.4×10^6 cpm/slide) in hybridization buffer with 50% formamide and 10% dextran sulfate overnight at 55 °C. After hybridization, slides were immersed in 2× SSC at room temperature to remove the cover slides and incubated in RNase A (20 ug/ml) for 30 min at room temperature. Sections were then consecutively washed for 30 min in 2× SSC at 50 °C, 0.2× SSC at 55 °C, and 0.2× SSC at 60 °C, dehydrated in ethanol containing 0.3 M ammonium acetate, and dried for 1 h at room temperature. Finally, slides were exposed to Kodak BioMax MR film (Sigma–Aldrich Co., St. Louis, MO) in lightproof boxes for 3 weeks at room temperature, developed in Kodak D-19 developer, washed in tap water, and fixed with Kodak fixer.

2.8. Brain data analysis

Brightfield photomicrographs of the Nissl stained brain sections were made with a Leica DM6000B digital microscope (Leica Microsystems), equipped with a Leica DFC420 5 megapixel CCD digital

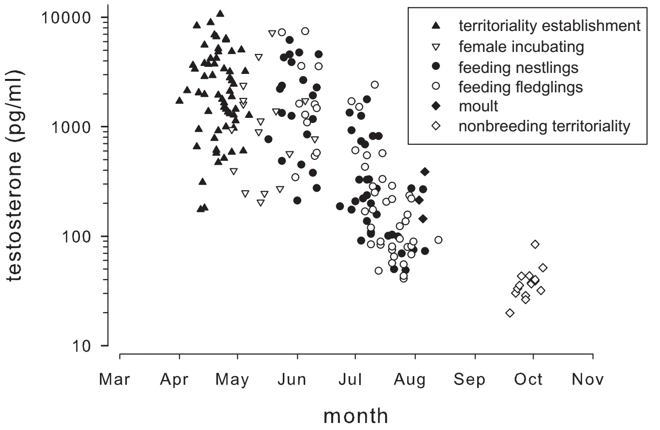

Fig. 1. Post-capture testosterone levels (ng/ml) of male black redstarts caught during different life-cycle stages. Testosterone levels are presented on a log-scale. Territory establishment: $n = 60$, male testosterone levels during female incubation: $n = 17$, nestlings first brood: $n = 21$, fledglings first brood: $n = 12$, from July on: nestlings second brood: $n = 33$, fledglings second brood: $n = 38$, molt: $n = 3$, nonbreeding territoriality: $n = 15$. Most birds caught after the 21 July had already initiated molt of the wing feathers even if they were still feeding young. Each individual is represented only once.

color camera. The Nissl sections were manually co-registered with the in situ hybridization autoradiograms of parallel brain sections to reliably determine the location of brain areas, such as the robust nucleus of arcopallium (RA) and the preoptic area (POA), which were difficult to distinguish from the autoradiograms directly. The data analysis of mRNA expression patterns was carried out similarly as described in Voigt et al. (2009). Autoradiograms were scanned with an Epson scanner using SilverFast Ai software as 16 bit grey values and with a resolution of 2400 dpi for later analysis in ImageJ. The system was calibrated by scanning a calibrated optical density step tablet (part #T2115, Stouffer Graphic Arts Equipment Co., Mishawaka, USA) and a calibration curve was calculated based on the Rodbard function in ImageJ. All autoradiogram images were saved in ImageJ with this calibration. HVC volumes were estimated based on androgen receptor expression (see also Fusani et al., 2000; Gahr and Metzdorf, 1997). For each androgen receptor-labeled brain section we delineated HVC, summed the area measurements and multiplied them with 100 μm (interval between sections). Optical densities of androgen receptor, estrogen receptor α and aromatase mRNA expression levels in the preoptic area were measured in two different ways using an ellipsoid with fixed dimensions for all sections and individuals, and again using an ellipsoid covering most of the stained area (therefore with changing size between individuals). To control for background staining the optical density in a control area just adjacent to the preoptic area was subtracted from the value for receptor expression. Optical density measures were averaged across all sections that were labeled for the respective mRNA. All measurements were carried out blind to the seasonal group of the bird.

2.9. Statistical analysis

Data were analyzed in *R* Development Core Team (2009). Hormonal data were analyzed using linear models. In a first step we used a priori set contrasts to compare the breeding substages (incubation, nestlings, and fledglings) and the nonbreeding period with the breeding substage territory establishment. In a second step we only compared testosterone levels of males caught in different phases within the breeding season (first and second brood vs. territory establishment). Spontaneous song and brain data were only collected during breeding territory establishment and nonbreeding territoriality. Spontaneous song data were tested for seasonal differences using *t*-tests and were transformed, if necessary, to meet assumptions of equality and normality of variances. Brain data did not meet these assumptions and were, therefore, tested for seasonal differences using non-parametric Mann–Whitney-*U*-tests.

3. Results

3.1. Seasonal testosterone profile

Testosterone levels of males caught at various times of the year (see next sentence) differed significantly ($F_{5,193} = 34$, $p < 0.0001$, Fig. 1). A priori set contrasts revealed that testosterone levels of males during all other phases of the life-cycle were significantly lower than during territory establishment (incubation: $t = -2.3$, $p = 0.02$, nestlings (first and second brood combined): $t = -6.4$, $p < 0.0001$; fledglings (first and second brood combined): $t = -8.5$, $p < 0.0001$; molt: $t = -3.1$, $p = 0.002$; nonbreeding territoriality: $t = -11.7$, $p < 0.0001$). Within the breeding life-cycle stage, testosterone levels also differed significantly ($F_{2,178} = 99$, $p < 0.0001$). Testosterone levels of males caught during their first brood did not significantly differ from those of males caught during territory establishment ($t = -1.8$, $p = 0.08$), but males caught during the second brood had significantly lower testosterone levels than males caught during territory establishment ($t = -13.3$, $p < 0.0001$). During early breeding in April testes were fully developed (volume of the left testis (mean ± 95% CI): $76.1 \pm 14.0\ \text{mm}^3$, $n = 8$) and significantly larger than during nonbreeding territoriality in September, when testes were completely regressed ($0.5 \pm 0.5\ \text{mm}^3$, $n = 8$, $U = 0$, $p = 0.0002$).

3.2. Behavioral response to simulated territorial intrusions

Territorial male black redstarts did not significantly differ between life-cycle stages in the time they spent within 5 m of a simulated territorial intruder, the time they spent with feathers fluffed in response to a simulated territorial intruder or the percentage of individuals attacking a simulated territorial intruder (Fig. 2, Apfelbeck and Goymann, 2011). However, a significantly higher percentage of individuals responded to a territorial intruder with song during the breeding life-cycle stages than during nonbreeding territoriality (Fig. 2, Apfelbeck and Goymann, 2011).

3.3. Spontaneous song: differences between life-cycle stages

Territorial male black redstarts sang significantly more songs with shorter pauses between songs during nonbreeding than during breeding (Table 1). Songs during breeding had more elements in part A and C than those sang during nonbreeding (Table 1). All other structural song parameters did not differ significantly between life-cycle stages (Table 1).

3.4. Expression patterns of androgen receptor (AR), estrogen receptor (ERα) and aromatase

3.4.1. Telencephalon

Dense androgen receptor mRNA staining was found in the song control nuclei HVC and in the lateral and medial nucleus magnocellularis (lMAN and mMAN, Fig. 3, Table 2) independent of life-cycle stage. However, in contrast to previous studies in other songbirds, androgen receptor expression in the robust nucleus of arcopallium (RA) was weak (Fig. 4). Instead, RA was surrounded by a band of androgen receptors, as demonstrated by relatively dense androgen receptor expression that was contained to the caudal arcopallium and did not extend rostrally (Fig. 4). Weak estro-

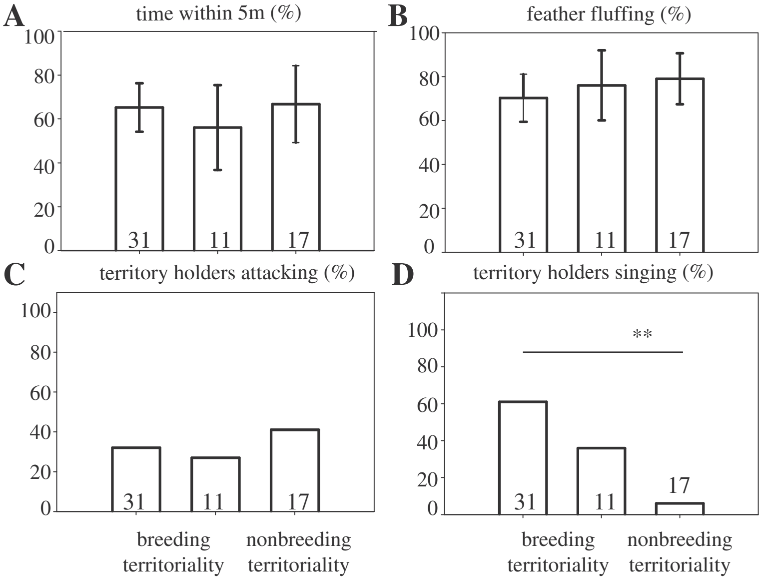

Fig. 2. Time spent within 5 m (as % of time seen, A), time spent feather fluffing (as % of time seen, B), number of individuals attacking (%, C) and number of individuals singing (%, D) in response to a simulated territorial intruder. Horizontal axes depict percent. Breeding territoriality is split into territory establishment (bars to the left) and feeding of nestlings or fledglings of the first brood (bars in the middle). Bars represent means ± 95% CI, numbers within bars represent sample sizes. Asterisks indicate significant differences between breeding stages ($^{**}\underline{p} < 0.01$). For statistics and a description of the territorial intrusion experiments see (Apfelbeck and Goymann, 2011).

gen receptor α mRNA expression was only found in the so called paraHVC (Table 2, see Gahr and Metzdorf, 1997 for a description of this region). Aromatase expression was not found in any of the song control nuclei (Table 2). However, similar to other songbirds, dense staining for aromatase mRNA occurred in the caudo-medial nidopallium (NCM, Table 2, Shen et al., 1995). Similar to canaries, aromatase and estrogen receptor α expression in NCM did not overlap (Metzdorf et al., 1999), but aromatase and androgen receptor expression did. Estrogen receptor α in NCM was specifically expressed around the lateral ventricle.

3.4.2. Diencephalon: preoptic and hypothalamic areas

During both breeding and nonbreeding territoriality co-expression of androgen receptor, estrogen receptor α and aromatase was found in the preoptic area (Fig. 5, Table 2). Androgen receptor, estrogen receptor α and aromatase were also co-expressed in the nucleus lateralis hypothalami posterioris (PLH, Fig. 5, Table 2), but similar to other species no estrogen receptor α expression was found in the nucleus medialis hypothalami posterioris (PMH, Fig. 5, Table 2, (Fusani et al., 2000; Metzdorf et al., 1999). The tuberal region was densely stained for estrogen receptor α and androgen receptor mRNA and weakly stained for aromatase mRNA (Fig. 5, Table 2). Further staining for androgen receptor, estrogen receptor α and aromatase mRNA was found in the bed nucleus of the stria terminalis (BNST, Table 2).

3.5. HVC volume

The HVC volume determined by androgen receptor expression did not significantly differ between breeding and nonbreeding territoriality (mean ± 95% CI: breeding: 0.6 ± 0.1 mm^3, $n = 8$, nonbreeding: 0.8 ± 0.08 mm^3, $n = 8$; Mann–Whitney U test: $U = 50$, $df = 12$, $p = 0.06$).

3.6. Optical density in the preoptic area

As both methods to measure optical density produced similar results, we only present results for one of the methods (i.e. ellipsoid fitted to area with staining). Aromatase mRNA expression in

Table 1
Comparison of song parameters during bouts of spontaneous song between breeding and nonbreeding territoriality. Significant results are highlighted in bold.

Song parameter	t – Statistic ($df = 30$)	p – Value	Mean ± 95% CI	
			Breeding ($n = 20$)	Non-breeding ($n = 12$)
Song rate (songs/min)	**3.9**	**0.0005**	**3.1 ± 1.1**	**6.1 ± 1.5**
Pauses betw. songs (s)	−**3.2**	**0.004**	**11.3 ± 5.1**	**6.0 ± 1.8**
Duration A (s)	0.7	0.5	0.9 ± 0.1	0.9 ± 0.1
Duration B (s)	0.6	0.6	0.9 ± 0.2	0.9 ± 0.2
Duration C (s)	−1.8	0.09	0.8 ± 0.1	0.7 ± 0.1
Duration pause A–B (s)	−0.4	0.7	1.3 ± 0.4	1.2 ± 0.5
No. of elements in A	**2.0**	**0.05**	**8.4 ± 1.5**	**6.9 ± 1.1**
No. of elements in C	**2.8**	**0.009**	**6.8 ± 1.1**	**5.2 ± 1.0**
Max frequency A (kHz)	−0.6	0.6	6.2 ± 0.2	6.1 ± 0.2
Freq bandwidth A (kHz)	0.5	0.6	3.2 ± 0.3	3.3 ± 3.9
Max frequency B (kHz)	0.1	0.9	8.2 ± 3.7	8.2 ± 2.6
Freq bandwidth B (kHz)	0.4	0.7	6.0 ± 3.9	6.1 ± 3.3
Max frequency C (kHz)	1.7	0.09	5.9 ± 2.7	6.2 ± 2.2
Freq bandwidth C (kHz)	1.9	0.07	3.2 ± 3.5	3.5 ± 3.5

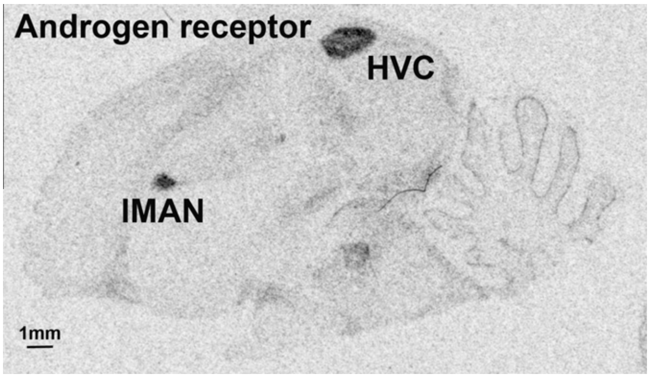

Fig. 3. Example of androgen receptor mRNA expression in two song control nuclei during the early breeding season. HVC: proper name, lMAN: lateral nucleus magnocellularis.

Table 2

Brain areas in the telencephalon and diencephalon expressing androgen receptor (AR), estrogen receptor (ERα) or aromatase (ARO) depending on life-cycle stage. lMAN, lateral nucleus magnocellularis; mMAN, medial nucleus magnocellularis; NCM, caudo-medial nidopallium; POA, pre-optic area; PMH, nucleus posterioris hypothalami medialis; PLH, nucleus posterioris hypothalami lateralis; BNST, bed nucleus of the stria terminalis; RA, robust nucleus of arcopallium. We visually graded the expression in the different areas into ++ strong expression, + weak expression or – no expression. Only in the preoptic area aromatase expression seemed to differ with life-cycle stage (bold areas).

Brain area	AR breeding	AR non-breeding	ARO breeding	ARO non-breeding	ERα breeding	ERα non-breeding
Telencephalon						
HVC	++	++	–	–	++	++
lMAN	++	++	–	–	–	–
mMAN	++	++	–	–	–	–
RA	+	+	–	–	–	–
Arcopallium	++[*]	++[*]	–	–	–	–
NCM	++	++	++	++	++	++
Diencephalon						
POA	++	++	**++**	**+**	++	++
PMH	++	++	++	++	–	–
PLH	++	++	++	++	++	++
Tuberal region	++	++	+	+	++	++
BNST	++	++	++	++	++	++

[*] Expression was restricted to the caudal arcopallium.

the preoptic area was significantly higher during breeding territoriality than during the nonbreeding territoriality ($U = 45$; $p = 0.007$, $n = 8$ per season). Expression of estrogen receptor mRNA ($U = 12$, $p = 0.1$) and androgen receptor mRNA ($U = 26.5$, $p = 0.9$) did not differ significantly between life-cycle stages.

4. Discussion

4.1. Non-vocal territorial behavior and testosterone

In black redstarts, the expression of non-vocal territorial behaviors did not correlate with differences in testosterone levels and aromatase expression in the preoptic area. Because males vigorously defended territories both during breeding and during fall nonbreeding while testosterone levels and aromatase expression in the preoptic area were higher during breeding than during fall nonbreeding territoriality. Thus, territorial behavior does not seem to be maintained by an increased sensitivity for sex steroids during nonbreeding; i.e. upregulation of aromatase (higher levels of aromatase during nonbreeding compared to breeding) metabolizing low levels of testosterone (Canoine et al., 2007). Instead the increased expression of aromatase during breeding in the preoptic area is similar to other temperate-zone bird species (Foidart et al., 1998; Silverin et al., 2000; Soma et al., 2003; Wacker et al., 2010) and suggests that also in black redstarts the preoptic area plays a role in estrogen-dependent regulation of male reproductive behavior (Ball and Balthazart, 2004; Balthazart et al., 2010).

Similar to black redstarts, song sparrows aggressively defend territories in a nonbreeding context and have higher testosterone levels and higher aromatase expression and activity in the preoptic area during breeding than during nonbreeding territoriality (Soma et al., 2003; Wacker et al., 2010). Although song sparrows respond to a simulated territorial intruder during all life-cycle stages, the response is much reduced during molt (Wingfield and Hahn, 1994). Wacker et al. Wacker et al. (2010) propose that in song sparrows aggressive behavior is regulated by aromatase in the ventromedial hypothalamus because a weak response to a territorial intruder during molt correlates with low expression of aromatase in that area. In addition, aromatase inhibitors reduced aggression during nonbreeding in song sparrows and the aggressive response was rescued by estrogen treatment (Soma et al., 2000; Soma et al., 2000). However, although we have not assessed aggressive behavior and receptor expression during molt in male black redstarts we suggest that in redstarts non-vocal territorial behaviors are independent of sex steroidal control as treatment with an androgen receptor blocker and an aromatase inhibitor during breeding did not reduce non-vocal territorial behaviors (Apfelbeck et al., submitted).

4.2. Vocal behavior, brain and testosterone

Similar to black redstarts male canaries spontaneously sing more syllable types with trills (Leitner et al., 2001; Voigt and Leitner, 2008) and male song sparrows sing trills with more elements (Smith et al., 1997) during breeding than during nonbreeding. These changes have been suggested to be mediated by testosterone as they follow seasonal changes in plasma testosterone levels (Leitner et al., 2001; Smith et al., 1997; Voigt and Leitner, 2008). More specifically, these seasonal changes in song structure seem to be regulated by estrogen-dependent mechanisms in HVC in canaries (Fusani and Gahr, 2006; Fusani et al., 2003; Gahr and Metzdorf, 1997; Rybak and Gahr, 2004) and by testosterone-dependent seasonal changes in HVC size in song sparrows and Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*, (Fraley et al., 2010; Smith et al., 1997; Smith et al., 1995; Soma et al., 1999). The expression pattern of androgen receptor, estrogen receptor and aromatase mRNA of male black redstarts is similar to the expression pattern found in canaries and other songbirds (but see expression in the caudal arcopallium, Fig. 4, (Brenowitz, 2008; Fraley et al., 2010; Gahr and Metzdorf, 1997). Thus, similar mechanisms may regulate seasonal changes in song. However, in male black redstarts HVC size and sensitivity for androgens or estrogens do not seem to differ between breeding and nonbreeding territoriality. Furthermore, in another experiment treatment of males with an antiandrogen and an aromatase inhibitor during breeding and fall nonbreeding did not reduce the number of repetitive elements produced (Apfelbeck and Kiefer, 2012). This suggests that the seasonal changes in the production of repetitive elements in the song are not directly induced by sex steroid-dependent changes in nucleus HVC in black redstarts. This demonstrates that although the song system seems to be fairly conserved across song birds, the mechanistic control of song production may still differ between species and more studies especially on wild birds under natural conditions are necessary to understand this diversity of mechanisms in the production of song.

In contrast to the seasonal decrease in repetitive elements in spontaneous song, spontaneous song output was even higher during nonbreeding than during breeding territoriality. During breeding males were sampled during a longer period of time than during nonbreeding and some females had already started to incubate. Males of these females probably decreased their song rate (Landmann, 1996). A high song rate during the fall nonbreeding territo-

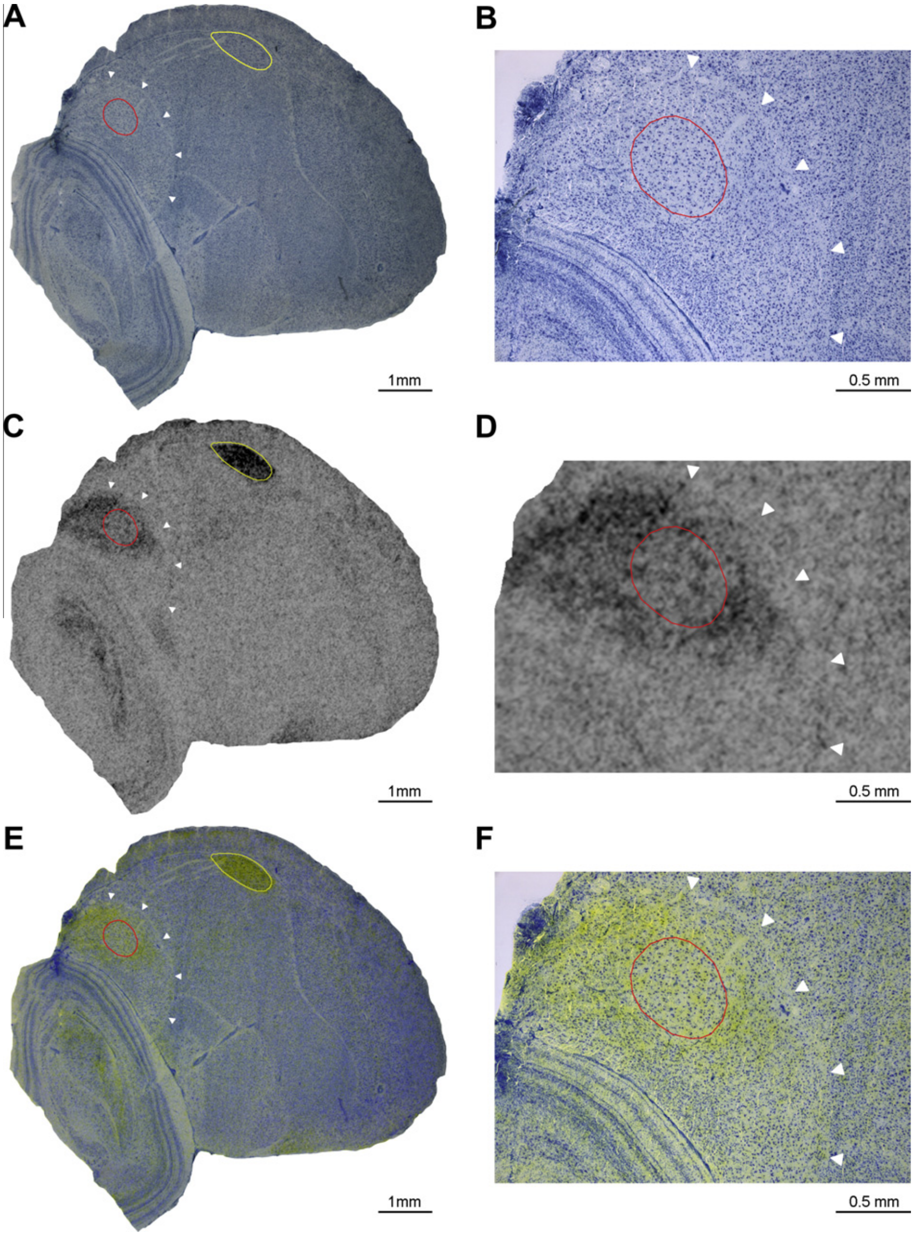

Fig. 4. Androgen receptor expression in the arcopallium and in HVC. (A, B): Nissl stained sagittal brain sections. C, D: In-situ hybridization autoradiogram for androgen receptor expression in brain sections adjacent to sections shown in (A, B). (E, F): Overlay of the Nissl stained sections (A, B) with sections stained for androgen receptor (C, D). Yellow circles indicate the border of HVC and red circles delineate RA as determined in the Nissl stained sections. (B, D, F) show magnifications of the arcopallium of the sections presented in (A, C, E), and closed arrowheads indicate the dorsal border of the arcopallium.

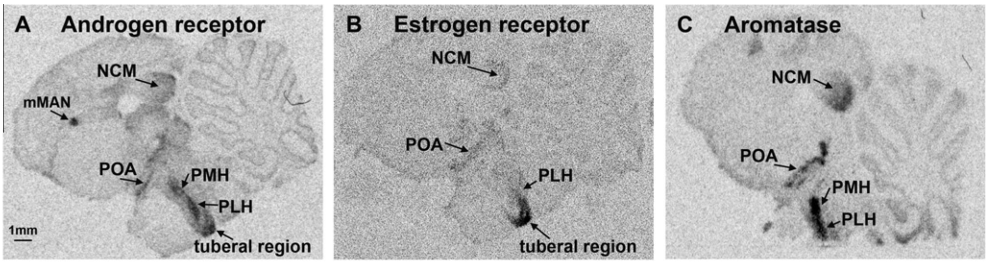

Fig. 5. Example of androgen receptor (A), estrogen receptor (B) and aromatase (C) mRNA expression during breeding territoriality. mMAN: medial nucleus magnocellularis, NCM: caudo-medial nidopallium, POA: preoptic area, PMH: nucleus posterioris hypothalami medialis, PLH: nucleus posterioris hypothalami lateralis.

rial phase also suggests that spontaneous song rate in male black redstarts is independent of testosterone. This is in contrast to most other temperate-zone song birds in which song rate decreases steeply after breeding and in concert with plasma testosterone levels (reviewed in Ball et al. (2002)). However, in contrast to spontaneously produced song, the likelihood of song produced in

Table 3
Summary of physiological, morphological and behavioral results comparing breeding and nonbreeding territoriality of male black redstarts.

	Breeding	Nonbreeding
Endocrine parameters		
Testosterone	High	Low
Testes	Large	Regressed
Response to STI		
Non-vocal	Strong	Strong
Probability of song	High	Low
Spontaneous song		
Song rate	Medium	High
Song structure	More elements in A and C	Fewer elements in A and C
Brain anatomy		
HVC	Large	Large
Aromatase pre-optic area	Strong	Weak

response to a territorial intruder was significantly reduced during nonbreeding territoriality (Fig. 2). This suggests that in male black redstarts seasonal changes in testosterone do not regulate song output in general, but in a context-dependent way. Similarly, European starlings (*Sturnus vulgaris*) sing at a high rate also during nonbreeding (Riters et al., 2000), but respond to females with an increase in song rate only during breeding (Riters et al., 2000). This sexually-motivated change in seasonal song production has been suggested to be facilitated by testosterone through indirect effects outside the song control system: it is correlated with high aromatase activity (Pintér et al., 2011; Riters et al., 2000) and immediate early gene expression in the preoptic area during breeding (Heimovics and Riters, 2005). Thus, the preoptic area supposedly does not only play a major role in the regulation of sexual behavior per se, but also the expression of other sexually motivated behaviors (Riters, 2012).

4.3. Testosterone and the regulation of non-vocal and vocal territorial behaviors

Combined evidence from this and previous studies suggests that testosterone and its metabolites regulate territorial behavior in black redstarts only partly and in a complex context-dependent manner. The non-vocal territorial response and spontaneously produced song seem to be independent of the control by sex steroids (Apfelbeck and Goymann, 2011), Fig. 2, Table 3). However, song produced in an agonistic context is probably facilitated by sex steroids (Apfelbeck and Goymann, 2011, Fig. 2, Table 3). These findings are similar to results obtained from two tropical songbird species. In staged male-male encounters during nonbreeding, captive spotted antbirds (*Hylophylax n. naevioides*) only responded with song when they were implanted with testosterone and during breeding, males implanted with blockers reduced the number of songs during such encounters (Hau et al., 2000). Costa Rican rufous collared sparrows (*Zonotrichia capensis costaricensis*) reacted – similar to male black redstarts – with equal intensity to a territorial intruder across seasons, however, they responded with significantly fewer songs during nonbreeding (Addis et al., 2010). In another rufous-collared sparrow population, *Zonotrichia capensis*, territorial behavior is completely independent of testosterone (Moore et al., 2004). Furthermore, these data corroborate similar findings in other bird species that defend territories outside the breeding season: males can respond to intruders and defend a territory even with low circulating levels of testosterone (Burger and Millar, 1980; Canoine and Gwinner, 2002; Hau and Beebe, 2011; Landys et al., 2010; Logan and Wingfield, 1990; Schwabl and Kriner, 1991; Soma et al., 1999; Wingfield, 1994) In addition to its signal function to other males, song may provide relevant cues for females during breeding. In most songbirds, song is not only important for territory defense, but also to attract and stimulate mates. In many species, including black redstarts (Landmann, 1996), during the breeding season song rate is highest when females are fertile (Gil and Gahr, 2002; Mace, 1987). Furthermore, it has been shown that females pay attention to the vocal output in singing interactions of males during agonistic encounters (Ballentine et al., 2004; Otter et al., 1999). Thus, seasonal changes in social context-dependent song output may be sexually motivated and selected and may be facilitated by the conversion of testosterone to estradiol in the preoptic area.

5. Conclusions

Our studies show that the relationship between testosterone and territorial behavior in male black redstarts is complex: testosterone does not seem to modulate non-vocal territorial behaviors, but the hormone may be involved in the regulation of context-dependent song through aromatization in the preoptic area. Our studies on black redstarts also suggest that there is no one unique mechanism by which sex steroids regulate territorial and song behavior in songbirds. Rather there seems to be a variety of solutions to the control of year round territoriality that we are just beginning to understand.

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General Discussion

The relationship between hormones and behavior has two aspects: hormone secretion modulates behavior and behavior modulates hormone secretion. In my thesis I investigated both aspects. Behavioral modulation of hormone secretion was investigated assessing the effects of social instability and male-male challenges on testosterone concentrations of males black redstarts (chapter 1 & 2). By correlating testosterone with parental care, extra-pair paternity (chapter 3), territoriality and song (chapter 4) I inferred the relation of hormone secretion and behavior. My results showed that social instability and male-male challenges did not modulate testosterone levels in migratory and sedentary populations of male black redstarts. In addition, they suggested that testosterone is not related to territorial and parental care behaviors but to mating behavior (extra pair-paternity) and song.

Hormone secretion can be modulated by the environment in different ways: by seasonal or predictable changes in the environment, by unpredictable changes in the environment and by social interactions. Hormone levels can be socially modulated in response to mates, songs or social instability (Dufty and Wingfield, 1986; Goymann, 2009; Hinde and Steel, 1976; Moore, 1982; Wingfield, 1985). The challenge hypothesis predicts that males will increase their testosterone levels in periods of social instability or when challenged by other males (Wingfield et al., 1990; referred to as $R_{\text{male-male}}$ in Goymann et al., 2007). Opposite to the predictions, previous experiments in black redstarts have shown that males from two migratory populations did not increase plasma testosterone when challenge by single or multiple simulated intruders or by real intruders (Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011). However, it could have been possible that these studies may have missed the right time period (20 minutes STI, see Wikelski et al., 1999 for a species in which it took more than 1 hour to increase testosterone) or the context (dummy with playback; migratory populations) during which males increase testosterone. Here we showed that a longer-term (1-3 days) experimentally-induced natural social instability did not increase testosterone levels in males, even though they were physiologically capable of doing so (after GnRH injection; Chapter 1, Fig. 1). In contrast to song sparrows (Wingfield, 1985) our experiment suggests that longer-term social instability does not alter testosterone levels in black redstarts. In addition, we tested social androgen modulation during early breeding in a sedentary (Spain) and a migratory (Germany) population. Similar to stonechats (Marasco et al., 2011) we found that sedentary males behave less aggressive towards the STI. In contrast to song sparrows and white-crowned sparrows (Wingfield and Hahn, 1994) males did not have higher testosterone levels after an STI compared to control males, regardless of population and even though they were physiologically capable of increasing testosterone (Chapter 2, Fig. 1). Further, testosterone levels did not differ between populations. My results confirm previous work showing that black redstarts do not modulate testosterone during social instability or male-male aggression.

Research on modulation of hormones due to social instability has been extended after the proposal of the challenge hypothesis among vertebrates and recently even applied to invertebrates (reviewed in (Goymann, 2009; Tibbetts and Crocker, 2014). The challenge hypothesis provided a useful background to study the relationship between behavior and hormones. However, it seems that it is not as general as it has been thought before (see discussion chapter 2; Goymann, 2009). For example, until recently dark-eyed juncos were thought to increases testosterone after male-male challenges (McGlothlin and Ketterson, 2008). However, recent experiments revealed that this increase occur only if it goes together with simulated predation of their chicks (Rosvall et al., 2014). The latter study suggests that males that lost their nest increase testosterone to re-activate their reproductive system and start nesting again, independent of social modulation. Accumulative evidence shows no increase of testosterone in response to social instability in many bird species (see discussion of chapter 2), suggesting that social modulation of testosterone ($R_{\text{male-male}}$ Goymann et al., 2007) is the exception rather than the rule in birds. Therefore, in black redstarts, the huge variability that we found in plasma levels of testosterone (Chapter 4, Fig 1) cannot be explained by social modulation due to male-male challenges. However, it is possible that other types of modulation account for the variability observed, for example, female solicitation or other environmental influences such as climate conditions, predation risk or the availability of appropriate places to nest (Goymann et al., 2007).

In black redstarts, testosterone is seasonally modulated, being high at the beginning of the breeding season and declining after the first brood (Chapter 4, Fig. 1). Seasonal modulation of testosterone has been reported for many temperate and even tropical bird species (Goymann and Landys, 2011). This suggests that males black redstarts have a stable testosterone profile in response to predictable environmental cues. The main environmental cue proposed to modulate hormone dynamics is the photoperiod, because it is entirely predictable (Dawson, 2008). In contrast to bird species that live in unpredictable environments and time reproduction in response to unpredictable variables (e.g., rain; Hau et al., 2004), photoperiod is used to time the physiological preparations for breeding, molt and migration in most temperate birds (Dawson, 2008). However, the way that animals integrate environmental cues could change between species, or even between populations. We compared migratory and sedentary populations and found differences in the timing in which migratory and sedentary black redstart males express the highest levels of testosterone (Chapter 2). Testosterone levels of males during migration were lower than those of breeding sedentary males, even though they were measured at the same time of the year and at a similar latitude (March; Chapter 2, Fig. 3). This suggests that similar environmental cues (e.g. photoperiod) affect sedentary and migratory populations in a differential manner. However, how environmental cues can differentially affect the organization of life-history stages between migratory and sedentary birds has been scarcely studied (Cornelius et al., 2013; Ramenofsky, 2011).

In general, high levels of testosterone at the beginning of the breeding season have been correlated with social instability, when males are establishing new territories, actively singing and looking for mates (R_{season}, Goymann et al., 2007). However, in some species territoriality, song and social instability can also be expressed outside the breeding season. In black redstarts territorial behavior is stable for most of the year even when testosterone levels are low. Furthermore, they actively sing not only during breeding but also outside the breeding season (e.g. autumn; Chapter 4, Apfelbeck and Goymann, 2011). This suggests that high plasma levels of testosterone are not necessary to regulate aggressive or singing behavior. One possibility to explain the lack of relation between aggressive behavior and testosterone is that conformational levels of testosterone influence behavior, but variation in hormone levels (high levels of testosterone) does not influence the expression of behavior. Alternatively, the androgen requirement to modulate aggressive behavior could be less than expected, as is has been proposed for sexual behaviors in rats (Damassa et al., 1977). If this is the case, low levels of testosterone during autumn would be enough to regulate aggressive behaviors. However, this seems unlikely because a combination of blocking androgen receptors and the conversion to estrogens did not decrease aggressive behavior in male black redstarts (Apfelbeck et al., 2013). Nevertheless, we found that song production in response to STI was higher during breeding compared to non-breeding territoriality (Chapter 4). This suggests that the probability to respond to song during a male-male challenge is higher during the breeding season and may be facilitated by plasma levels of testosterone. In addition, song characteristics change between spring and autumn, suggesting that testosterone is involved in qualitative song parameters. In general, our results suggest that testosterone can be decoupled from territorial behavior in species that are territorial year-round. If a behavior is stable between stages, there may be no need to modulate it through a hormone – dependent mechanism (Adkins-Regan 2005). However, testosterone may be involved in the regulation of context-dependent song (see also Apfelbeck et al., 2013).

Although it has been suggested that brain production of testosterone would account for non-breeding territoriality (e.g. in song sparrows, Pradhan et al., 2010; Wingfield and Soma, 2002) the mechanism underlying non-breeding territoriality remains unclear (e.g. in black redstarts; Apfelbeck et al., 2013). In an attempt to investigate the mechanisms of breeding and non-breeding territoriality, we assessed the expression of hormone receptors in brain areas that are related to social and sexual behaviors (preoptic and hypothalamic areas) and to song (arcopallium and HVC). We also measured aromatase expression in the preoptic area (POA), because the conversion of testosterone to estrogen by the enzyme aromatase may have important effects on how steroids affect aggressive behavior (Trainor et al., 2006). Previous studies in the spotted antbird showed high expression of estrogen and androgen receptors during the non-breeding season in nuclei that are related to reproductive and aggressive behaviors (e.g. POA and *nucleus taeniae*; Canoine et al., 2007). The later study suggests that a higher sensitivity for testosterone would regulate aggressive behavior during non-breeding. We found that black redstarts show a seasonal change in the expression of aromatase (Chapter 4) and androgen receptors (Villavicencio, unpublished data) in the POA, but contrary to the spotted antbird the expression was highest during breeding compared to non-breeding. Therefore our results suggest that high expression of aromatase (and androgen receptors) in the POA may be mainly related to sexual behavior and not to aggressive behavior.

High levels of testosterone do not only correlate with territorial and aggressive behaviors in many wild animals. In socially monogamous birds with biparental care testosterone decreases following the mating phase, leading to the suggestion that high levels of testosterone would interfere with parental care

(Wingfield et al., 1990). In black redstarts, this observation does not hold. In males of this species testosterone concentrations start to decline only after the first brood has fledged (Chapter 4, Apfelbeck & Goymann, 2011) and we found no differences in paternal care between the first and the second brood (Chapter 3, Fig. 2). Therefore, our results indicate that high levels of testosterone during the first brood did not reduce the degree of paternal care. Furthermore, circulating levels of testosterone (including GnRH induced testosterone) and baseline corticosterone sampled during mating and while feeding their chicks did not correlate with paternal care (Chapter 3, Fig. 1 & 4). Together, these results suggest that circulating levels of testosterone are not related with the degree of paternal care in male black redstarts. Nevertheless, we found that males that had higher levels of testosterone at the end of the breeding season (while feeding the second brood) had fewer extra-pair chicks in their nests (Chapter 3). Circulating testosterone sampled during mating was not related to paternity loss. This implies that the testosterone profile may be related to effectiveness in mate guarding, influencing the likelihood of losing paternity. Possibly, males that did not lose paternity invest more in mating behavior maintaining high levels of testosterone throughout the breeding season and regresses their reproductive system slower preventing their female to engage in extra-pair fertilizations. Taken together our results stress the importance to investigate the hormone-behavior relationship in different stages or contexts (e.g. during mating and while feeding their chicks). Consequently, to study the seasonal hormone dynamics of individuals in correspondence to behavior is important in order to understand the relationship of hormones and behavior.

To conclude, in my thesis I found no direct relationship between testosterone and territoriality in male black redstarts. My results indicate that high plasma levels of testosterone do not influence aggressive behavior and that aggressive behaviors do not modulate testosterone secretion. Therefore, we propose that the control of territorial and parental care behaviors in species that are territorial throughout their annual life cycle can be decoupled from testosterone. However, we found that song output in response to STI changes from breeding to non-breeding suggesting that is probably facilitated by sex steroids. My thesis adds to growing evidence that the hormonal basis of behavior is not so simple. Hence, the relationship of testosterone and territorial behavior needs to be revised and probably reconsidered in a way that integrates its complexity. Yet, my results suggest that testosterone may be mainly related to sexual behavior. Testosterone levels correlate with mating; testosterone levels stay high after the first brood (June), allowing males to mate for a second time (Chapter 4, Fig. 1,). In addition, aromatase expression in the POA was higher during breeding compared to non-breeding. Aromatase expression has been widely related to sexual behavior in different bird species (Ball and Balthazart, 2004; Ubuka and Tsutsui, 2014). Therefore, aromatase expression correlates with the reproductive behavior in black redstarts. Moreover, we found that males that maintain higher levels of testosterone towards the end of the breeding season are less prone to have extra-pair chicks. Thus, the relationship between hormones and behavior in free living birds may differ among bird species with different life styles; in multi-brooded species the relationship between testosterone and sexual behaviors may be more easily separated from territory establishment than in single-brooded species, and in species with year-round territoriality testosterone may be decoupled from aggressive behavior.

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I would like to dedicate my work to my son; your smile illuminates my world.

Curriculum Vitae

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BIRTH DATE:	May 13 of 1979
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ACADEMIC DEGREES

Primary and secondary Studies:

1984-1997:	Saint George' College, Santiago, Chile

Superior Studies:

1999-2002:	Science degree in Biology, Science Faculty, Universidad de Chile, Chile.
2002-2006:	Master in Ecology and Evolution Biology, Science Faculty, Universidad de Chile, Chile
2009 -2014:	PhD in Biology, Ludwig - Maximilian Universität München, and Max Planck Institute for Ornithology, Seewiesen, Germany

TEACHING ACTIVITIES PERFORMED

2002: Teaching assistant of Cell Biology, Prof. Dr. Julio Alcayaga, Faculty of Science, Universidad de Chile

2002: Teaching assistant of Zoology of Invertebrates, Prof. Dr. Michel Sallaberry, Faculty of Science, Universidad de Chile

2002: Teaching assistant of Zoology of Vertebrates curse, Prof. Dr. Fernando Novoa, Faculty of Science, Universidad de Chile

2003: Teaching assistant of System Physiology, Prof. Dr. Julio Alcayaga, Faculty of Science, Universidad de Chile

2003: Teaching assistant of Ecology, Prof. Dr. Rodrigo Vásquez, Faculty of Science, Universidad de Chile

2007-2009: Teacher of Cell Biology, in Pedagogy in Biology and Science career, at Science of Education Faculty, Universidad Central

2008-2009: Teacher of Ecology, in Pedagogy in Biology and Science career, at Science of Education Faculty, Universidad Central

RESEARCH ACTIVITY PERFORMED

2002: Technician, of the project FONDECYT 1020550. Titled: The ecologic-evolutive context of learning: foraging and information use. Responsible researcher: Dr Rodrigo A. Vásquez.

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PUBLICATIONS

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TALKS

Camila P. Villavicencio, Beate Apfelbeck, Bart Kempenaers, Wolfgang Goymann. Relating testosterone, parental care and paternity loss in a monogamous song bird. *33th International Ethological Conference*, August 4-8, 2014 Newcastle, England

Camila P. Villavicencio. Social and environmental modulation of hormones and behavior in the black redstart. *EES Conference* 2.10.2014, Biocenter, LMU, Munich, Germany

Camila P. Villavicencio & Wolfgang Goymann. Mating, but not aggressive behavior or parental care correlates with testosterone in a socially monogamous territorial songbird. *10th topical meeting of the Ethological Society* February 11-14, 2015 Hamburg, Germany

POSTERS

Camila Villavicencio, Beate Apfelbeck, Wolfgang Goymann. Social instability and testosterone in black redstarts. *8th International Conference on Behaviour, Physiology and Genetics of Wildlife*, September 14th – 19th 2011, Berlin, Germany

Camila P. Villavicencio, Beate Apfelbeck, Wolfgang Goymann. Testosterone and its correlation with behavior during the breeding season in black redstarts. *10th International Symposium on Avian Endocrinology*, June 5th–9th 2012*,* Gifu, Japan.

Camila P. Villavicencio, Julio Blas, Wolfgang Goymann. Testosterone differences in migratory and sedentary populations and during migration in males black redstart (*Phoenicurus ochruros*). *International Congress of Comparative Endocrinology* July 15th-19th, 2013, Barcelona, Spain.

Camila P. Villavicencio, René Quispe, Harriet Windley, Manfred Gahr, Wolfgang Goymann. Testosterone & Territorial Behavior when Territoriality is a Default State. *Society for General and Comparative Biology Annual Meeting 2014* January 3rd-7th, 2014 Austin, TX, US.

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