University of Groningen

Testosterone a female hormone

de Jong, Berber

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2013

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
de Jong, B. (2013). *Testosterone a female hormone: Testing the function and evolution of testosterone in female birds*. s.n.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 01-02-2024

Testosterone a female hormone

Testing the function and evolution of testosterone in female birds

This PhD project was carried out at:
1) the Behavioural Ecology and Self-Organization group, which is part of the Centre for Ecological and Evolutionary Studies.
2) the Behavioural Biology group, which is part of the Centre for Behaviour and Neurosciences. Both groups are of the University of Groningen, The Netherlands.
3) the Terrestrial Ecology Unit of Ghent University, Belgium.

This PhD project was implemented according to the requirements of the Graduate School of Science (Faculty of Mathematics and Natural Sciences, University of Groningen) and the Doctoral School of the Ghent University.

The research was financially supported by a the promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen) and the Dr. J.L. Dobberke foundation to Berber de Jong.

The printing of this thesis was partly funded by the University of Groningen and the Faculty of Mathematics and Natural Sciences.

Lay-out and figures:	Dick Visser, Berber de Jong
Cover design:	Berber de Jong
Photo's:	Wouter Moerland, Toke Egberts, Berber de Jong
Printed by:	GVO drukkers & vormgevers B.V., Ede

ISBN: 978-90-367-6564-0
ISBN: 978-90-367-6563-3 (electronic version)

RIJKSUNIVERSITEIT GRONINGEN

Testosterone a female hormone

Testing the function and evolution of testosterone in female birds

proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
vrijdag 22 november 2013
om 12:45 uur

door

Berber de Jong

geboren op 10 januari 1982
te Ooststellingwerf

Promotores:	Prof. dr. ir. J. Komdeur
	Prof. dr. A. G. G. Groothuis
	Prof. dr. L. Lens
Beoordelingscommissie:	Prof. dr. E.D. Ketterson
	Prof. dr. J.M. Koolhaas
	Prof. dr. B.I. Tieleman

Contents

CHAPTER 1

General introduction

Berber de Jong

In the majority of animal taxa, males and females differ profoundly in their morphology, behaviour and/or physiology. When Darwin observed the prevalence of sexual dimorphism across the animal kingdom, he concluded that it could not easily be explained by natural selection, as he found that both sexes typically encounter similar environmental and ecological conditions throughout their lives (Darwin, 1859). Therefore, Darwin proposed that sexual selection may be a more likely explanation for the extent of sexual dimorphism among taxa. He considered sexual selection as "the *advantage which certain individuals have over others of the same sex and species solely in respect of reproduction*" (Darwin, 1874). In addition, Darwin made the distinction between intrasexual and intersexual selection; where intrasexual section is the competition for mates within the same sex – for example, male-male competition for the possession of females. Intersexual section is the competition between individuals of the same sex, in order to excite or charm those of the opposite sex (Andersson, 1994). The study of sexual selection is today the study of those traits that yield an advantage in reproductive competition and, therefore, the fitness of their bearers. Darwin realized that competition over mates is typically much stronger in one sex than the other. Consequently, the divergence in selection pressures between the sexes arising from sexual selection provides a much more straightforward explanation for the evolution of sexually dimorphic traits than natural selection, for which selection pressures are generally (but not always, see Hedrick and Temeles, 1989; Slatkin, 1984) similar between the sexes.

Sexual dimorphism and intralocus sexual conflict

In general, males and females show substantial sexual dimorphism in their reproductive strategies (Anderson, 1994). For example, males are generally selected for high mating frequency and females for low mating frequencies (Davies et al., 2012; Trivers, 1972). The intersexual conflict between reproductive strategies can generate different selection pressures on many traits between sexes, which can cause sexually antagonistic selection (Cox and Calsbeek, 2009). Because males and females share most of their genome (intersexual genetic correlation), natural or sexual selection on a trait in one sex can result in a correlated response of that trait in the same direction in the other sex (Lande, 1980). When the optimum value of that trait, which is encoded by the same gene(s), diverges between the sexes either due to natural or sexual selection, then intralocus sexual conflict occurs (Bonduriansky and Chenoweth, 2009; Chapman, 2006; Rice and Chippindale, 2001). The optimal trait value is the value that has the highest fitness for its bearers, due to either natural or sexual selection. If a correlated response of the same trait in males and females is maladaptive, the evolution of that trait would then result in a situation that is suboptimal for both sexes, with one sex benefitting from even higher levels of trait

expression and the other sex from lower levels than currently observed (Arnqvist and Rowe, 2005). For example, one trait which has been demonstrated to be constraint in one sex through intralocus sexual conflict is human height (Stulp et al., 2012): When sibling pairs (sister-sister pairs or brother-brother pairs) were compared, a sister of an on average shorter sibling pair had a higher reproductive success compared to a brother of such a pair. By contrast, when the sibling pairs were of average height, then the brother had a higher reproductive success relative to the sister of that pair. Thus, the optimal height for more reproductive success differs between males and females and the possible intersexual genetic correlation might constrain the sexes to reach their optimum height. In general, intralocus sexual conflict leads to a phenotype that is suboptimal for both sexes and, due to the genetic correlation, will slow down the evolution towards the optimal trait value in each sex.

Neutral hypothesis

The evolution of the same trait in males and females does not always have to be constraint by its intersexual genetic correlation. When the level of the trait in one sex does not affect its fitness then that trait is selectively neutral for that sex. This is because traits that do not have any fitness benefits or costs in one sex cannot be selected for or against and that trait will then be free to evolve in the direction which is optimal for the other sex. For example, if men with blue eyes (the heritability of eye colour in humans is high; Bräuer and Chopra, 1980) have a higher fitness, but women's fitness is un-affect by their eye colour, then selection will favour blue eyes in men. Also, by choosing to mate with blue-eyed men, females can indirectly increase their fitness through their sons since they will have blue eyes too. In the end the high fitness of blue-eyed men, and the related female preference for them, will lead to a population of only blue-eyed men and women.

The resolution of intralocus sexual conflict

In theory, when a trait experiences a constant sexually antagonistic selection pressure, then ultimately selection will favour those mutations that reduce intersexual genetic correlations (Bonduriansky and Chenoweth, 2009; Van Doorn, 2009). When the same trait in both sexes ceases to be genetically constrained, then each sex can independently evolve their own optimum for that trait (Lande, 1980). Future evolution of that trait will then be under direct natural or sexual selection for each sex. An intralocus sexual conflict may have been resolved when, for example, the observed distribution of a sexually dimorphic trait corresponds to the phenotypic optima in each sex (Cox and Calsbeek, 2009), meaning that the behaviour, morphology and

physiology of each sex is adapted to its own ecological and social condition. An example of a highly sexual dimorphic trait where intra-sexual conflict may have been resolved are cuticular hydrocarbons profiles (CHC; which, play an important role in sexual communication and partner choice) in *Drosophila melanogaster* (Bedhomme et al., 2011). Bedhome et al. (2011) used selection lines of *D. melanogaster* in which the transmission of genetic material was restricted to males. In these selection lines, females were limited in the transmission of genomic haplotypes (the X chromosome and all major autosomes) from father to son. Thus, sons received almost no genetic material from their mother, resulting in single, male-limited chromosomes (Rice, 1996). Even after 82 generations CHCs were still not changed. This was unexpected because, if the evolution of CHCs is constraint by sexual antagonistic selection, then male CHC profiles would evolve to their optimal trait value intersexual genetic correlation was now absent. This study provides evidence that intralocus sexual conflict over CHCs is currently absent (Bedhomme et al., 2011).

Underlying mechanisms that regulate sexual dimorphism

The main objective of this thesis is to study mechanisms that may lead to the evolution of sexual dimorphism. Morphological and behavioural differences between the sexes are the outcome of many different genetic, developmental and physiological mechanisms (Williams and Carroll, 2009), such as the presence of sex chromosomes or the expression of sex-specific transcription factors. For instance sex hormones are one mechanism involved in the regulation of sexually dimorphic traits (Williams and Carroll, 2009). Because hormones are organic chemical messengers that are secreted into the bloodstream and can therefore reach the whole organism, many phenotypic traits of an organism are regulated by hormones (Adkins-Regan, 2005). They can synchronize the physiology, morphology and behaviour of an animal to variations in the environment by regulating, integrating, and controlling its bodily functions (Nelson, 2011). In vertebrates, the steroid hormone testosterone (hereafter, "T", see **box 1.1.**) has been shown to be one of the major hormones that mediates phenotypic differences between sexes (Adkins-Regan, 2005). In the next section I will explain how T can play a role in sexual dimorphism, how this can be tested experimentally, and what the limitations are of current research that has investigated the role of T in sexually dimorphic traits and sexual selection.

Testosterone and sexual dimorphism

Many species show sexual dimorphism in T levels, with males generally having higher T levels than females (birds: Ketterson et al., 2005; Møller et al., 2005; mammals:

Adkins-Regan, 2005; Nelson, 2011; fish: Mank, 2007). In seasonally breeding vertebrates T levels vary throughout the year, with a peak during the breeding season (Kempenaers et al., 2008; Wingfield et al., 1990; see figure 1.1). This seasonal profile co-varies between the sexes (Ketterson et al., 2005; Møller et al., 2005).

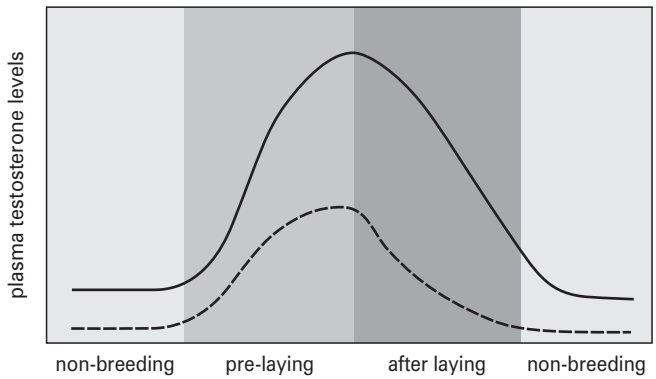


Figure 1.1. General pattern of circulating testosterone levels in seasonal breeding male and female song birds. The straight line represents the males and the dashed line the females.

I will focus mainly on studies of birds. Birds are an excellent class of vertebrates with which to study the evolution of sexual dimorphism because the sexes of many bird species differ profoundly in their morphology, behaviour and/or physiology: For example, the extreme tail lengths observed in Birds-of-Paradise (Paradisaeidae) or the fact that song is so universally performed by males, but absent in most females. Also, most bird species are diurnal and are therefore easier to observe and follow than mammals, especially in free-living situations. In addition, the role of T is well studied in many different species of birds (Adkins-Regan, 2005; Ketterson et al., 2005; Wingfield et al., 1990). Such knowledge is critical to understanding the evolution of a sexual dimorphic trait, because it is essential to know the fitness costs and benefits of the trait in both sexes.

In adult male birds T is an important mediator of reproductive behaviour, reproductive success and survival (Adkins-Regan, 2005; Garamszegi et al., 2005), which is also reflected in its seasonal profile. In most temperate song birds plasma T levels rise at the beginning of the breeding season, when males frequently engage in territorial aggression and mating behaviours. During the breeding season T levels decrease again to their baseline levels and remain low until the next breeding season (Goymann et al., 2007; Wingfield et al., 1990). Previous studies have shown that the height of an individual's natural peak in T levels at the beginning of the breeding season are positively associated with the number of offspring a male has sired with

his own social partner (within pair paternity) and in other nests (extra-pair paternity, (Garamszegi et al., 2005). Experimentally elevated T levels, however, increase extra-pair paternity, but not within pair reproductive success (Raouf et al., 1997). Experimentally elevated T levels also affects mating behaviour in males later in the breeding season. For example, experimentally elevated T later in the breeding season increased male singing behaviour to attract additional mates (De Ridder et al., 2000), increased courtship behaviour (Edler et al., 2011; Hegner and Wingfield, 1987), and attractiveness to females (Enstrom et al., 1997). However, this prolonged sensitivity to T levels can come at a cost, since T can supress parental behaviour, such as incubation (Alonso-Alvarez, 2001a; Oring et al., 1989) and the provisioning of nestlings (Hegner and Wingfield, 1987). In general, sustained elevated levels of T appear to enhance sexual behaviour but reduce parental care in male birds (Adkins-Regan, 2005; Lynn et al., 2002). Despite the fact that T reduces paternal care, T can still increase male fitness, especially through extra-pair paternity (Reed et al., 2006). Yet in some species for whom bi-parental care is essential for the survival of offspring, males may become insensitive to T during the period of increased paternal care (the behavioural insensitive hypothesis; (Lynn et al., 2002 & Lynn, 2008).

Similarly, females also show an increase in T levels at the beginning of the breeding season, although the peak in T levels is generally lower in females than in males (Ketterson et al., 2005). Following the peak, T levels typically decrease after the onset of egg laying and remain at low levels throughout the rest of the year. Thus females also have significant levels of T at the beginning of the breeding season, but less is known about its functional role than in males. There are three potential explanations for the currently observed T levels in females; first, peaks in T levels could be the result of selection on T levels in males, where the presence of an intersexual genetic correlation causes sexually antagonistic selection on female (and male) T levels (Clotfelter et al., 2004; Ketterson et al., 2005). Alternatively, both male and female levels of T might, in fact, be optimal and therefore the result of direct selection on females (Cain and Ketterson, 2012). Lastly, T levels may be selectively neutral in females (Adkins-Regan, 2005). Which, if any, of these mechanisms explains the evolution of female T levels is currently unknown.

In addition to the question regarding the function of the short seasonal peak in T levels in females, the timing of this peak might also be important. In many seasonal reproducing female birds, T levels decline during parental care, just as they do in males (Ketterson et al., 2005; Wingfield et al., 1990). Although T levels decline during the period of parental care in males, in the majority of birds, males remain sensitive to elevated T levels through much of the breeding season (Lynn et al., 2002 and Lynn, 2008). This prolonged sensitivity can be beneficial, for example, for territorial behaviour or male reproductive behaviour (obtaining extra pair fertilizations, see above, (De Ridder et al., 2000; Edler et al., 2011; Hegner and Wingfield, 1987). Several studies have shown that females of several bird species also remain sensitive to prolonged

elevated T levels (Gerlach and Ketterson, 2013; Lopez-Rull and Gil, 2009; O'Neal et al., 2008; Veiga et al., 2004; Veiga and Polo, 2008). Unlike males, however, the potential benefits of elevated T during maternal care are less clear for females, as in most species they typically do not strive for more fertilizations throughout the breeding season. Because it is unknown whether such continued sensitivity is beneficial or costly for female reproductive success, it is also unclear it is adaptive. In this thesis I will address the question of whether currently observed T levels during the female reproductive period are selectively neutral in females, or are the result of sexually antagonistic selection, or direct selection. In addition, I will address the question of whether seasonal T profiles in temperate zone birds are adaptive for females.

One method to test the adaptive significance of a phenotypic trait, such as T, is through phenotypic engineering (Ketterson et al., 1996). Phenotypic engineering entails the experimental modification of some aspect(s) of an individual phenotype (in our case the levels of T). Subsequently, the fitness of this altered phenotype can be compared with the fitness of the sham manipulated (control) individual, which has natural levels of the phenotype in question. If reproductive success is similar between the groups, one can conclude that experimentally altered levels of T have little effect, leading to the conclusion that selection is currently absent on the altered phenotypes (i.e. levels of T are selectively neutral in females, see figure 1.2A). On the other hand, if the reproductive success of the manipulated individuals is higher than

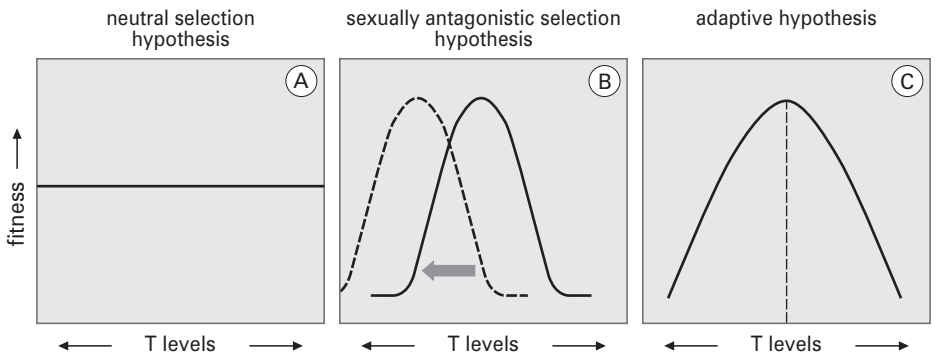


Figure 1.2. Three hypothesis relating testosterone (T) levels and female fitness. In (A) the level of T does not affect female fitness and therefore is selectively neutral. In (B) T levels in females are under sexually antagonistic selection pressures. The straight line represents the fitness levels for the currently observed T levels and the dotted line the optimal fitness curve for females. In this situation females with lower T levels are predicted to have a higher fitness compared to females with intermediate or high T levels. Hence, if sexually antagonistic selection would be resolved, the optimal T values for females would shift to the left. In (C) current T levels in females are expected to be adapted to the ecology of the female and these females have the highest fitness. The dotted line represents the optimal T level for females. In contrast, females with lower or higher levels have lower fitness and will be selected against.

that of the control, then one can assume that observed natural levels of female T do not correspond to the optimal levels of female T (see figure 1.2B; Ketterson et al., 1996). Such a pattern would highlight the existence of evolutionary constraints, such as the presence of an intersexual genetic correlation. For example, assuming that females with lower T levels than currently observed would have a higher fitness, than the manipulated phenotype with lower T levels would do better compared to the controls. Lastly, in case the reproductive success of the manipulated phenotype is lower than that of control, one would expect that T levels are currently near the optimal female levels of T, indicating that sexually antagonistic selection pressures are currently absent and that observed T levels are adjusted to the ecology of the female (see figure 1.2C).

Current state of knowledge on the effects of experimentally elevated T levels in females

Conventional studies on T have mainly focussed on males because T was considered as a typical male hormone, and less relevant for females (Staub and DeBeer, 1997). In the mid-1990s an increasing number of biologists realized that T may not only play a central role in the male, but also be of importance in females (Staub and DeBeer, 1997). For example, in female domestic chickens (*Gallus domesticus*), T levels show a significant peak 6-10 hr prior to ovulation (Johnson, 2000) and ovulation is induced by T (Croze and Etches, 1980). Since the 1990s, several studies have therefore been conducted to investigate the role of T in females.

Recently, T has been shown to affect many aspects of the female phenotype, i.e., morphology, behaviour, and physiology. For instance, experimentally increasing T levels in females, can lead to the development of male-like features, such as male-like plumage in both ruffs (*Philomachus pugnax*; Lank et al., 1999) and superb fairy-wrens (*Malurus cyaneus*, (Peters, 2007). Changes in behaviour after T elevation are also common. Elevated T levels increase female aggression in numerous bird species (Searcy, 1988; Zysling et al., 2006; Sandell, 2007), and induced and male-like courtship in budgerigars (*Melopsittacus undulates*; Lahaye et al., 2012). T is also known to play an important role in reproductive physiology. For example, experimentally elevated T levels reduced clutch size in zebra finches (*Taeniopygia guttata*; Rutkowska et al., 2005); negatively affected maternal care in dark-eyed juncos (O'Neal et al., 2008); delayed breeding in red-winged blackbirds (Searcy, 1988) and dark-eyed juncos (Clotfelter et al., 2004); and decreased hatching success, but not fledging success in the spotless starling (*Sturnus unicolor*; Lopez-Rull and Gil, 2009; Veiga and Polo, 2008).

Although the above studies are informative on the role of T in females, it remains to be seen whether their conclusions are relevant to extant selection pressures in wild

populations. Many of these studies experimentally elevated T for a longer period than would occur naturally, since levels of female T typically decrease after the onset of egg laying, and remain at low levels throughout the rest of the year (Gerlach and Ketterson, 2013; Lopez-Rull and Gil, 2009; Searcy, 1988; Veiga and Polo, 2008), and thus their relevance to wild birds is uncertain. A prolonged elevation of T levels is useful for understanding the adaptive value of the seasonal profile of T levels in females, as prolonged sensitivity to T in females might have negative effects on their reproductive success (Gerlach and Ketterson, 2013; Lopez-Rull and Gil, 2009), but it does not allow us to draw conclusions about the adaptive value of the natural peak in T levels at the beginning of the breeding season in females. Additionally, T implants often result in T levels beyond the typical physiological range found in wild females (Peters, 2007; Rutkowska et al., 2005; Searcy, 1988). Such unnatural levels and profiles of T make it difficult to assess whether measured costs of T have been relevant for selection in extant populations. Finally, no study has simultaneously investigated the effects of both an experimental increase of circulating T concentrations as well as a decrease of exposure to T. The latter is needed for testing the adaptive hypothesis because by only elevating T levels, one cannot distinguish between the effects of the deviations and the costs of an elevation of T per se. If experimentally elevated T levels are costly for females, this does not necessarily mean that females with lower levels than currently observed would have a higher fitness (see also figure 1.2B & C). To assess whether observed levels of female T in natural populations are indeed adaptive, an experiment is needed that will allow us to disentangle the three hypotheses mentioned above. In **chapters 2**, **3** and **4** I undertake such experiments.

General experimental set-up

To circumvent the shortcomings of the experimental design used in previous experiments, I used a different experimental set-up than commonly applied. To investigate the functional role of testosterone on female behaviour in blue tits (*Cyanistes cearuleus*, **Chapters 2**,**3**,**4**) I manipulated T levels within the physiological range of females in this species, and used another group of females in which we blocked testosterone effectiveness with flutamide, and compared these two altered phenotypes with control females (figure 1.3). Flutamide is an androgen receptor blocker, which inhibits nuclear binding of T to the androgen receptor in its target tissue (Adkins-Regan and Garcia, 1986; Hegner and Wingfield, 1987; Scanes, 2000; Sperry et al., 2010). The manipulations took place during the onset of the breeding season, when females elevate their T production and were building nests and defended territories, and ended shortly after the onset of egg laying, when circulating T concentrations normally decrease. However, in one study, using great tits (*Parus major*, **chapter 5**), T was elevated to for a longer period than would occur in nature. This allowed us

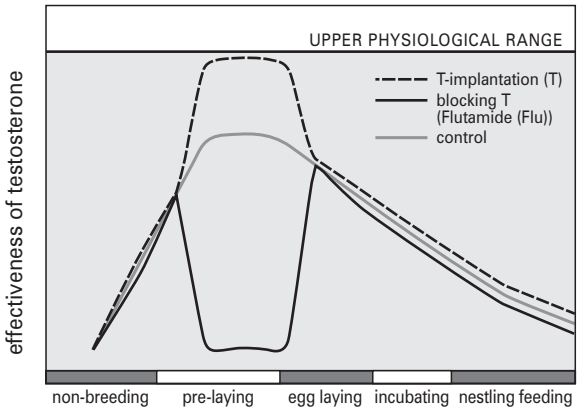


Figure 1.3. Set-up of the experiment that was used in to examine the functional role of testosterone (T) in female blue tits. The y-axis represents the assumed level of effectiveness of T and the x-axis the different stages during the breeding period and outside this period. The high horizontal line displays the upper physiological range of T levels for females. Females were implanted with either T, an androgen receptor blocker (flutamide (Flu)), or with an empty implant (control (C)) during the pre-laying period, when females were building their nest. As soon as the females started egg laying they were recaptured to remove their implants. After implant removal T levels will decrease (in case of the T females) and increase (in case of the Flu females) to normal levels.

to examine if females are still sensitive to elevated T levels during the period when T levels are naturally decreased, and to test whether the current seasonal profile in T levels is adaptive in female great tits.

Aims of the thesis

In this thesis I will focus on observed T profiles, in terms of peak levels and temporal profiles over the season, with the following questions: i) whether observed peak testosterone levels in females are the result of sexually antagonistic selection or direct selection in females, ii) and whether seasonal profiles of T levels in females are adapted to their ecology. During my study I used an experimental approach (see above) to estimate the effects of testosterone manipulation on reproductive behaviour, reproductive success and survival. I studied these questions in two study systems. The first question I studied in a nest box population of free- living female blue tits near the city of Groningen, The Netherlands (see **box 1.2.**), and the second question in a nest box population of free-living female great tits near the city of Mortsel, Antwerp, Belgium (see **box 1.2.**).

Study species: The great and blue tit

The great tit and blue tit are excellent model species to study the functional role of T in females for a number of reasons. First, the functional role of T has been extensively studied in both male great and blue tits. Studies on the functional role of T in males are essential to be able to conclude whether T in females is the result of sexually antagonistic selection. As in most bird species, in male great and blue tits, natural plasma levels of T increase at the beginning of the breeding season and drop to values close to zero during the period of nestling feeding (Van Duyse et al., 2003; Foerster et al., 2002).

In male blue tits experimentally elevated testosterone during the period of nest building and egg laying had no effect on male reproductive success (Foerster and Kempenaers, 2004). Elevated T males were equally likely to become cuckolded and did not gain more extra-pair paternity than control males. However, in another study male blue tits with experimentally elevated T levels lost more paternity than control males (Foerster and Kempenaers, 2004), which contrasts with findings in other bird studies (Raouf et al., 1997; Reed et al., 2006). These males, however, did interact more with potential extra-pair mates than control males (Foerster and Kempenaers, 2005). Elevated levels of T in blue tit males did not affect aggressive behaviour or mate guarding (Foerster and Kempenaers, 2005). Females paired with a male with elevated T levels did not change their behaviour during egg laying and the treatment of T implanted males did not significantly affect male and female feeding behaviour. Nevertheless, nests of T males produced heavier fledglings (Foerster and Kempenaers, 2005), which can increase their survival (Magrath et al., 2009; Schlicht et al., 2012), and subsequently could increase the fitness of males treated with T.

Male great tits implanted with T at the beginning of their breeding season, increased the number of aggressive calls during a simulated territorial intrusion (Van Duyse et al., 2002). However, elevated T levels did not affect aggressive behaviour towards a decoy (e.g. the time spent close to the decoy; Van Duyse et al., 2002). Experimentally elevated T levels in male great tits did not affect parental care during the nestling phase since there was no effect of increased T on nestling feeding rate (Van Duyse et al., 2000 & 2002). When males were implanted with T during the period of nestling feeding they sang more than control males (van Duyse et al., 2000).

In conclusion, it remains unclear if elevating T levels at the beginning of the breeding season increases male fitness in great and blue tits (Foerster and Kempenaers, 2004; Van Duyse, 2004). Nevertheless it is clear that T plays an important role in several different reproductive behaviours in male great and blue tits.

Second, both great tits and blue tits are excellent model species for my study because they show sexually differentiated behaviours. For example, in both species only the females undertake nest building, incubation, and brooding of young (great

tit: Gosler, 1993; blue tit: Cramp and Perrins, 1993). But some behaviours are similar in both sexes. For example, males and females of both species defend a multi-purpose territory, which is important for successful breeding; the feeding of young and care of fledged young is also executed by both parents (Gosler, 1993; Cramp and Perrins, 1993). These similarities and dissimilarities in reproductive behaviour allow me to examine which behaviours would be more likely to be under sexually antagonistic selection, such as aggression and extra-pair copulations, and compare them to behaviours which are expected to be under direct selection in females, such as incubation.

Third, both species are cavity-nesting birds that breed in both natural cavities and nest boxes. Their use of nest boxes makes it possible to gather long term data, as the reproductive success and survival of individuals can be monitored for many generations. This is important when investigating the fitness consequences of elevated T levels. In addition, due to the supply of extra nesting opportunities in the form of nest boxes, the number of breeding pairs can be increased, leading to a larger sample size. Also, birds that roost and breed in nest boxes are easier to monitor, because they are easily captured inside their nest box when roosting or feeding their young.

Fourth, sexual selection pressures occur in both monogamous and polygynous mating systems, with these pressures being stronger in the later because there is more competition among males, as fewer males will the opportunity to mate, and more attractive males may attract more females (Darwin, 1874). Each of my model species represents one of the mating systems, where the great tit has a social monogamous mating system and polygyny rarely occurs. Blue tits also have a social monogamous mating system, but in contrast to great tits, polygyny occurs (Kempenaers, 1994; Vedder, 2011). Pair bonds are rarely broken during the breeding season in either species. Pairs dissolve after the end of the breeding season and prior to the formation of winter flocks. In subsequent seasons, pairs from previous breeding seasons usually stay together, but new pairs may form in the period between the end of the winter flocking period and onset of the breeding season (75% pair-retention rate in blue tits: Winkel and Winkel, 1980 and 77% in great tits: Kluijver, 1951). Although pair bonds are stable throughout the breeding season, extra-pair fertilizations are common in both species (Strohbach et al., 1998; Kempenaers et al., 1992). Extra-pair fertilizations are copulations outside the pair bond, which can lead to extra-pair offspring (EPO). Forty percent of all great tit nests and 45% of all blue tit nests have at least one chick resulting from an EPC (Strohbach et al., 1998; Magrath et al., 2009).

Outline of the thesis

The result section of this thesis is divided into two parts: Part I focuses on the role of T in reproductive behaviour (**Chapter 2**), female promiscuity (**Chapter 3**) and reproductive success and survival (**Chapter 4**). Part II looks more in detail at the

behavioural insensitive hypothesis with a focus on incubation behaviour (**Chapter 5**). The results are subsequently synthesized and discussed in a general discussion (**Chapter 6**).

Part 1 What is the role of current Testosterone levels in female blue tits?
To investigate the role of T in female blue tits, I manipulated circulating T concentrations by either implanting females with a silicone tube filled with crystalline T, or filled with crystalline flutamide. A third group of females was implanted with empty implants (the control group). Subsequently, we measured the effects of our manipulation on reproduction behaviour, reproductive success and survival in comparison to those of control females. In **Chapter 2** I examined the effects of this manipulation on nest building behaviour, aggression, egg laying, nestling characteristics and number of fledged young. In **Chapter 3** I investigated the role of testosterone in female mating behaviour by measuring the number of extra-pair offspring (EPO) per nest of females treated with T or Flu compared to control females. The number of EPO was measured in the short term (during the year of the hormone manipulation), and in the long term (one year after the manipulation). To be able to disentangle whether the decline in the number of EPO by T treatment was caused by a decrease in female attractiveness or caused by differences in mate seeking behaviour, I conducted a mate choice experiment where male blue tits had to choose between Flu and T females. In **Chapter 4** I analyse the short- and long-term effects of our hormone manipulation on reproductive success and survival. In all of these chapters I discuss my results in the context of intrasexual genetic conflicts and adaptive selection.

Part 2 Investigating the seasonal testosterone profile; testing the insensitive hypothesis
To gain more insight into the seasonal profile of T, in **Chapter 5**, I investigated the effects of long-term elevated T levels on incubation behaviour and its subsequent effect on fitness in female great tits in.

In **Chapter 6**, I provide a synthesis in which the most important results of these studies are summarized and placed in a broader context of other research findings. In addition, I discuss possible mechanisms that could have resolved the intersexual genetic constraint caused by sexually antagonistic selection pressures. I end this chapter with a short overview of the limitations of current studies on the adaptive role of T in males and females and how future studies can overcome these.

Box 1.1. Testosterone

Testosterone is a steroid hormone that is synthesized from cholesterol. In vertebrates testosterone is mainly produced by testes and ovaries. But recent research provides strong evidence that cells located outside the gonads can produce testosterone, for example, the adrenals and the brain (Adkins-Regan, 2005). T production is regulated by the so called hypothalamic-pituitary-gonadal (HPG) - axis that plays a role in the production of androgen hormones. The hypothalamus releases a gonadotropin releasing hormone (GnRH) that stimulates the anterior pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). When LH and FSH reach the gonads they stimulate the production of sex steroid hormones (Adkins-Regan, 2008). After T is produced it is secreted into the circulation where it can bind to a transport globulin (in birds: corticosterone-biding-globulin (CBG); in mammals: sex hormone binding globulin (SHBG). In target tissues, T is released from the binding globulin and crosses the cell membranes and bind to receptors within the cytoplasm, where they have access to the nucleus and gene expression. Additionally, T can be converted in to oestradiol by the enzyme called aromatase, as well as to another androgen, dihydrotestosterone, that binds to the same androgen receptor as T but with higher affinity.

During early development steroid hormones play an important role in sex differentiation of the embryo. In mammals females are the default sex and males masculinize under the influence of T. In birds, males are the default sex and females feminize under the influence of oestrogen. This organizational effect of T and oestrogen during early development causes permanently differentiation in morphological, physiological and behavioural traits between the sexes (Nelson, 2011). Although, recently it has been shown that sexual dimorphism of specific cells or tissue is also influenced by the sex chromosomes of the individual, and even come about in the absence of regulation from gonadal hormones during the development (Arnold, 2012).

In addition to organizational effect of steroid hormones, they also have an activational role. Later in live the nervous system stays sensitive for T and oestrogen, to enable the expression of steroid-linked behaviour. For example, an increase in T in adult male birds induces mating behaviour, whereas mating behaviour in adult females in stimulated by oestrogen (Adkins-Regan, 2005).

Box 1.2. Study areas

Vosbergen

"De Vosbergen" is an estate in the North of the Netherlands near the city of Groningen (53°08'N, 06°35'E). The estate was founded in 1890 by the Kraus-Groeneveld couple. They changed the area, which consisted mainly out of heath, sand, and peat, into a park in the English landscape style. The area covers ca 50 ha, consisting out of forest plots which are separated by avenues of tall trees, and open grass land (see figure 1.2.1). The forest is a mixture of coniferous and deciduous trees, mainly oak and beech. In 2001 a blue tit nest box population was established (Korsten, 2006). In total 188 nest boxed designed for blue tits (with an entrance hole of 26 mm thereby excluding great tits (*Parus major*) and Eurasian Nuthatch (*Sitta europaea*) from nesting in these nest boxes) were put

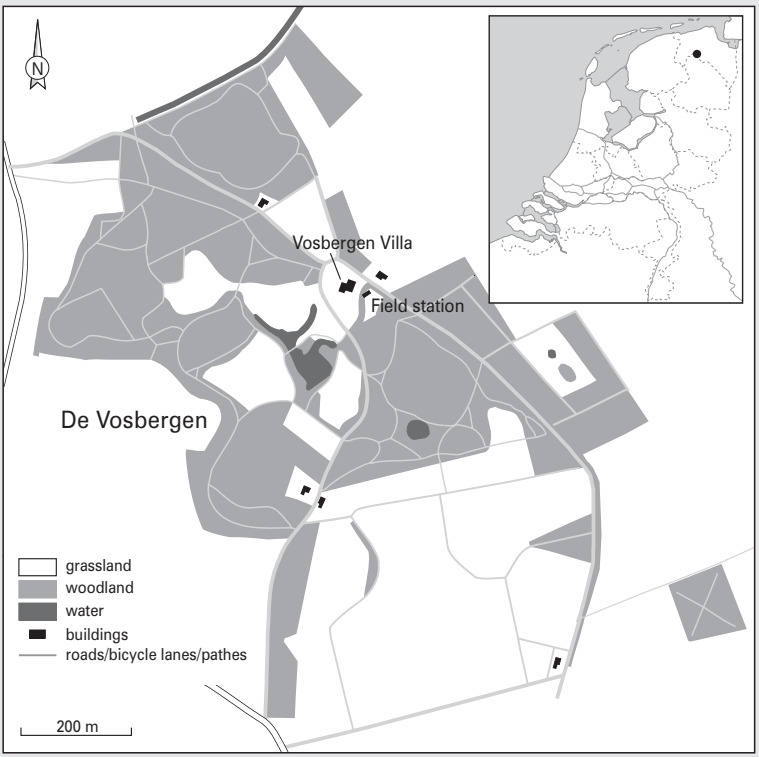


Figure 1.2.1. Study area "De Vosbergen".

up across the whole area. On average between 50-65% of the nest boxes are occupied, resulting in more than a hundred breeding pairs each year. Since the start of this population the blue tits have been monitored every year. All the adult birds that were breeding in the population and their nestlings have been ringed with a metal ring with a unique number. In addition, DNA was collected from adults breeding in the population and their nestlings for parentages analysis since 2006 (Vedder et al., 2010a). The high nest box occupation, together with the long term data set, makes "De Vosbergen" an ideal study population for ecological and behavioural research questions.

Fort 4 - Mortsel

"Fort 4" is an old fortress in the North West of Belgium near the city of Antwerp (51°10'N, 04°27'E). The fort is part of the fortifications around the city of Antwerp and was built between 1860 and 1865. Until 2002 the fort was part of the army and not accessible for the general public. Since 2004 the area has been used as a study area for great tit research. The area has a park landscape, consisting out of forest areas with a grass land in the middle and a fosse surrounding the fort, and covers ca 25 ha (see figure 1.2.2). The trees in the area

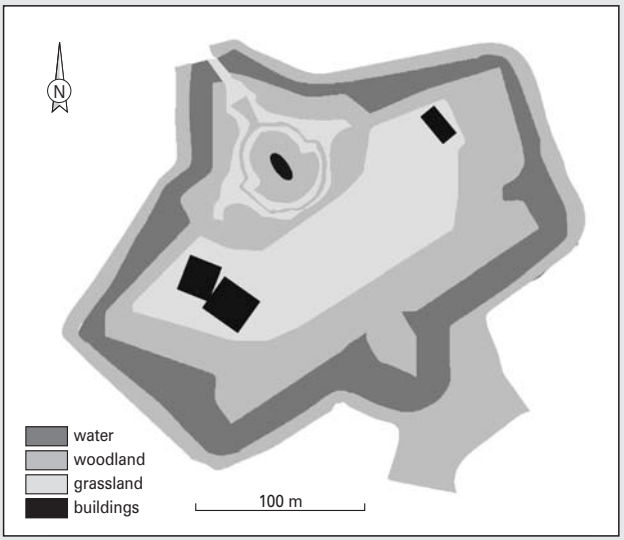
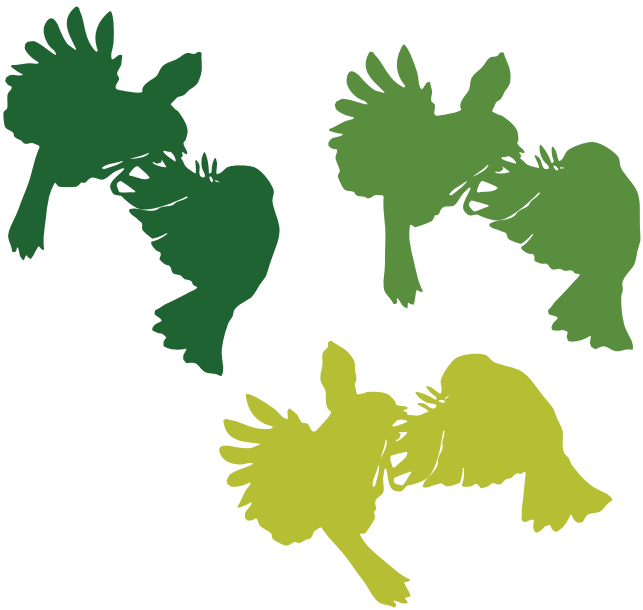


Figure 1.2.2. Study area, "Fort 4".

are a mixture of deciduous trees, such as oaks, beech and maple. The fort is surrounded by a suburban area. In 2004 36 nest boxes were hung at the inside area of the fort. In January 2008 extra nest boxes (N = 23) were put up on the outside border of the fortress, around the fosse, thus in total there were 59 nest boxes in the study area. The boxes were used by great tits and blue tits. In 2009 36% of the nest boxes were occupied by great tits and 34% by blue tits. Since the start of the study population the great tits have been monitored every year. All the adult birds that were breeding in the population and their nestlings have been ringed with a metal ring with a unique number.



CHAPTER 2

No evidence for sexually antagonistic selection on testosterone levels: an experiment in free-living female blue tits (*Cyanistes caeruleus*)

Berber de Jong, Jan Komdeur, Oscar Vedder, Luc Lens, & Ton Groothuis

Abstract

The role of testosterone (T) in females is poorly understood. In particular, the question whether circulating T levels in females are (1) the result of sexual conflict, (2) adaptive for females, or (3) selectively neutral, remains unanswered. Studies that experimentally elevated female T levels mainly found adverse effects on female reproductive behaviour and success, inspiring the interpretation of female T as being the result of sexually antagonistic selection. These studies, however, mostly elevated T outside the physiological range for females and/or for a longer period than naturally would occur in the field. In this study, we experimentally elevated T within the physiological range of free living female blue tits (*Cyanistes caeruleus*) during the period in spring when it is naturally increased. In two other groups of females we lowered T effectiveness by blocking androgen receptors with flutamide, and sham treated females as a control group. We found that T females accelerated nest-building and increased aggressive behaviour compared to control females, suggesting a functional effect of T on female reproduction, whereas flutamide females behaved similarly as control females. We did not find any differences in reproductive success among the three treatments. We conclude that the natural elevation of T in female blue tits does not yield clear costs in the short-term for the female, undermining the sexual antagonistic selection hypotheses for T levels.

Introduction

In many animal species the sexes differ in physiology, morphology, and behaviour. Testosterone (T) can act as an important mediator of phenotypic differences between the sexes, as males generally have higher plasma T levels than females (Bubenik et al., 1997; Ketterson et al., 2005; Mank, 2007; Moller et al., 2005; Nelson, 2011; Taylor et al., 2004). Therefore, and because males are often more aggressive and sexually active than females, most studies have focused on the effects of T in males. Experimentally elevated T has been shown to have both potential fitness benefits in females (increasing courtship, aggression and attractiveness to females) as well as detrimental effects (suppression of immune function and paternal care, and in the long term lower survival; for review see Adkins-Regan, 2005; Hau, 2007; Nelson, 2011). This has led to the hypothesis that male's trade off the costs and benefits of T leading to a context-dependent optimum of T production, as also reflected in the seasonal variation of the hormone. In most seasonally breeding species, male plasma T levels are only elevated at the beginning of the reproductive season, the period in which the aforementioned benefits of T are likely to outweigh the costs, since males then have to compete for territories and females (Foerster et al., 2002; Wingfield et al., 1990). Although, T levels in females are in general much lower than in males (Ketterson et al., 2005), these levels show the same seasonal fluctuations as in males (Ketterson et al., 2005; Mank, 2007; Moller et al., 2005; Rubenstein and Wikelski, 2005) and may have functional effects. But, in contrast to males, it remains unclear if the peak of female testosterone levels at the beginning of the reproductive season has any adaptive value.

In a recent and influential review study that inspired the current study, Ketterson et al. (2005) proposed three potential explanations for the occurrence of elevated T levels in females. One explanation is that the seasonal peak of T levels in females is the result of sexually antagonistic selection. Since the genomes of both sexes show a great deal of overlap, natural or sexual selection on a trait in one sex can result in a correlated response of that trait in the same direction in the opposite sex (Lande, 1980). If such a correlated response is maladaptive, the evolution of the trait would then result in a situation that is sub-optimal for both sexes (Arnqvist and Rowe, 2005), with one sex benefitting from even higher levels of trait expression and the other sex from lower levels than currently observed. This may well be the case for testosterone levels, for which in males higher levels have been found to increase reproductive success (Raouf et al., 1997; Reed et al., 2006), but for which the effects in females are not as well known (but see (Clotfelter et al., 2004; Ketterson et al., 2005). A simpler alternative explanation is that current levels of T in females are adaptive, with higher and lower levels being suboptimal (Cain and Ketterson, 2012). The third hypothesis is that female T levels are selectively neutral and higher or lower levels do not influence fitness.

To distinguish between these predictions, one needs to both elevate and suppress female T levels and assess its consequences for estimates of female fitness. Until now, studies in birds have shown that experimentally elevated T levels in female birds not only resulted in enhanced female aggression (Sandell, 2007), but also in delayed breeding (Clotfelter et al., 2004; De Ridder et al., 2002; Searcy, 1988), reduced clutch size (Rutkowska et al., 2005), decreased time spent brooding (O'Neal et al., 2008), lower attractiveness for the other sex (Ketterson et al., 2005), and reduced reproductive success (Gerlach and Ketterson, 2013; Lopez-Rull and Gil, 2009; Veiga and Polo, 2008). In general, these studies indicate that elevated T levels may decrease female reproductive success, consistent with the hypothesis that current T levels in females are the result of sexually antagonistic selection.

Although these previous studies are informative, they are not conclusive. First, many of these studies elevated T for a longer period than would occur in natural situations (Clotfelter et al., 2004; De Ridder et al., 2002; Gerlach and Ketterson, 2013; Lopez-Rull and Gil, 2009; O'Neal et al., 2008; Searcy, 1988; Veiga and Polo, 2008). Second, T manipulations often resulted in T levels outside the physiological range for females of that species (De Ridder et al., 2002; Rutkowska et al., 2005b; Searcy, 1988). Therefore, the costs of elevated T demonstrated in these studies may be difficult to interpret in an evolutionary context. Third, none of these studies have simultaneously investigated both an experimental increase as well as a decrease of T levels, and thus cannot distinguish between effects of deviations from normal (needed for testing the adaptive hypothesis) and of an increase in T levels *per se*. Finally, some of these studies (DeRidder et al., 2002; Rutkowska et al., 2005) were performed in captivity, an environment unlikely to reflect the natural context.

In this study we mimicked a more realistic situation by experimentally elevating T only within the physiological range of females in a wild species. Moreover the manipulation was only during a short period, from the start of nest-building until the onset of egg-laying, when it is naturally increased. In another group of females, we lowered T effectiveness by blocking androgen receptors with flutamide (Flu). Flu does not affect circulating plasma T levels *per se* but it does inhibit androgen uptake by binding to testosterone and dihydrotestosterone intracellular receptors, thereby blocking the effects these androgens have on behaviour and physiology (Adkins-Regan and Garcia, 1986; Hegner and Wingfield, 1987; Nelson, 2011; Neri and Peets, 1975; Peets et al., 1974; Sperry et al., 2010). We performed our study in free-living blue tits (*Cyanistes caeruleus*), a socially monogamous passerine bird that reproduces once per year in spring. Males and females both defend their territory and feed their offspring in the nestling phase, but only the female builds the nest and incubates the eggs (Cramp and Perrins, 1993a).

We predict that if current T levels are the result of sexually antagonistic selection, females treated with Flu will perform better than control and T females. In contrast, if current T levels in females are adaptive, we expect that control females will

perform better than T and Flu females. If T levels are selectively neutral, we would expect no effects of our manipulation on female performance. To test these predictions we investigated the effect of our manipulation on different breeding parameters, including nest-building, egg-laying, territorial behaviour and reproductive success.

Materials and methods

Study population

The study was performed during the breeding season (March-June) of 2010 and 2011. In 2010 we measured reproductive behaviour and reproductive success. In 2011 we tested the effect of the hormone implants on circulating hormones levels. The study area was "de Vosbergen" estate in north-eastern Netherlands (53° 08' N, 06° 35' E). The estate of 54 ha consists of mixed deciduous and coniferous forest interspersed with areas of open grassland and contains 188 nest boxes designed for blue tits (Korsten, 2006). At capture all blue tits received a metal ring with a unique combination of numbers and three colour rings for individual identification. In addition, age (first year or older) of the birds was determined based on the colour of the wing feathers (see Svensson, 1992). All procedures used in this study were approved by the animal welfare committee of the University of Groningen.

Implantation procedure

At the beginning, of the breeding season 59 territorial females were caught (between 25 March and 13 April 2010) for implantation. All nest-boxes were checked daily during the nest-building phase starting at the end of March. Once nest material, was present females were caught and randomly assigned either to the testosterone (n = 21), flutamide (n = 20) or control (C, n = 18) treatment group. Females were either caught during the day by placing a trap inside their nest-box (n = 45; C = 13, Flu = 15, T = 17) or at night when sleeping in their nest-box (n = 14; C = 5, Flu = 5, T = 4). Before implantation, females were weighed with a spring balance to the nearest 0.1g, and tarsus length was measured to the nearest 0.1 mm, and the length of the third primary feather (P3) was measured to the nearest 0.5 mm with a stop-ruler. There was no difference in age, body mass, wing- and tarsus length among the females of the three treatment groups (all $p > 0.49$). After the body measurements, each bird received an implant, which was inserted subcutaneously along the left flank under local anesthesia (Xylocaine 10% spray). The small wound was sealed with tissue glue (1 × 0.5 ml Histoacryl, Braun, Germany). The implant consisted of a silicone tube that was sealed at both ends with 1mm of silicon glue. The T- and control-implants were 4.4 mm long (id 0.5; od. 1.0) and filled with crystalline T (Sigma, Germany) or left empty, respectively. The flutamide implants were 7.2 mm long (id 1.47; od 1.96) and filled with crystalized flutamide (Sigma©). Flutamide is

an androgen receptor antagonist that inhibits the binding of endogenous T to the androgen receptor (AR). Time of day and date of implantation were recorded.

After capture, nest material was removed from the nest boxes to stimulate renesting and allow us to measure effects of our treatment on nest-building behaviour. To minimize the departure of females from their territory, we provided all the females with an extra nest box within 1 meter from the nest box in which they captured. After implantation, 31 females moved to another nest box and six females were not resighted. There was no difference in the rate of moving to another nest box among treatments ($\chi^2 = 1.48$, df = 2, $P = 0.92$). When it was clear that females did not use the extra nest box in their territory, the nest box was removed. When females had moved to the extra nest box, the entrance of the original nest boxes was covered to keep the number of available nest boxes for each territory the same.

When females started egg-laying, they were recaptured, weighed and implants were removed. The females were caught with a mist net close to the nest box ($n = 30$) or with a hand net (De Heij et al., 2008) when leaving the nest box in the morning ($n = 15$). The implant was removed by creating a small incision next to the implant under local anesthesia (Xylocaine 10% spray). After removal of the implant, the small wound was closed with tissue glue (1 × 0.5 ml Histoacryl, Braun, Germany). All females were returned to their nest box (when captured at night) or territory within 30 minutes after capture.

Measurements

All nest boxes were checked daily to determine the restart of nest-building (C = 16, Flu = 19 and T = 19), the number of days needed for nest completion (C = 16, Flu = 18 and T = 18), and the onset of egg-laying. A nest was defined to be complete when there was a nest cup lined with feathers and/or hairs, which is the last stage of nest completion. The onset of egg-laying was scored in March days (the 1st of March is 1). On the day of onset of egg-laying, the mass of the complete nest was measured with a spring balance to the nearest 0.1 g. After the second egg was laid, nest checks were temporarily halted to minimize disturbance of the laying female. A week later nest, checks were recommenced to determine clutch size and the start of incubation. The start of diurnal incubation was defined as the first date the female was found sitting on her eggs or when the eggs were found warm. Ten days after the start of diurnal incubation, daily nest checking was resumed to determine hatching date.

The implant could not be removed from one flutamide, six testosterone and two control females because either implants were entwined with the brood patch, or the females could not be recaptured (C = 2, Flu = 1, T = 4). Hence, all analyses on clutch size and hatching success were performed on 12 control, 16 flutamide and 13 testosterone females.

During the nestling phase, all nestlings were ringed on day 8 (day of first hatching is day 0). On day 15 all, nestlings were weighed, tarsus (TL15) length and third

primary length (P3) were measured as described above for the females. From 20 days after hatching onwards, nest boxes were checked daily for remaining nestlings until all had fledged or were found dead. For the analyses of the number of fledglings and fledging success, one flutamide nest was excluded because two nestlings of this nest died of an accident. Hence, the analyses on fledgling number and success were performed on the offspring of 12 control, 15 flutamide and 13 testosterone females.

Aggression test

To test the effect of our treatment on territorial aggression, an intruder was simulated by presentation of a taxidermic mount of a female blue tit. The taxidermic mount was placed in a small cage (11.5×12×16.5 cm), to prevent it from damage, and placed onto a wooden platform of 1 m height. At the start of the test, the small cage was covered with a plastic bag. The set-up was placed approximately 1 m from the tree at which the nest box was attached, together with a speaker. The speaker played a mix of blue tit vocalizations recorded from birds that are no longer present in the population. The observer was about 10-15m away from the set-up with a video camera and binoculars. After the camera and the speakers were turned on, the decoy was uncovered. The reaction time of the bird to the decoy was defined as the time between uncovering the decoy and the first arrival of the female. For the following 5 min, we recorded (1) the number of pecks at the decoy, (2) the number of parachute flights and (3) the number of times a bird showed wing flagging. From these agonistic behaviours, we calculated z-scores for each and added them all up to derive a single aggression score for each individual. Females were tested before implantation (trial 1, n: C = 16, Flu = 16 and T = 14) as well as after implantation (trial 2, n: C = 12, Flu = 15 and T = 15). There was no difference in the number of days between test date and implantation date between treatments (before implantation: $F_{2,43} = 1.09$, $P = 0.85$; after implantation: $F_{2,39} = 0.88$, $P = 0.42$). On average, tests were performed 3.35 ± 0.38 days before and 6.17 ± 0.10 days after implantation. There were no differences in trial date (trial date (1): $F_{2,43} = 0.002$, $P = 1.00$; trial date (2): $F_{2,39} = 1.01$, $P = 0.95$) between the treatment groups. Although the trials were conducted randomly throughout the day, there was a difference in time during the first trial ($F_{2,43} = 3.18$, $P = 0.05$) but not during the second trial ($F_{2,39} = 0,24$, $P = 0.79$). There was a significant difference between the trial time of the C females and Flu females (Tukey test; $P = 0.016$). The time of C females did not differ from T females (Tukey test; $P = 0.14$) nor did T females differ from Flu females (Tukey test; $P = 0.36$). Observations were conducted between 07.30 and 17.30h in dry weather.

Effectiveness of the hormone implants

To test the effect of our hormone implants on circulating hormone concentrations, we took blood samples, but due to a freezer defect our samples were lost. Therefore, we repeated our implantation procedure in 2011 to test our implants using the same

protocol and with the following sample sizes: T = 20, C = 17, Flu = 21. The implantation procedure was identical to that of 2010 (see above). Before implantation, a blood sample was taken to determine basal testosterone levels (sample 1). A second sample was taken when the birds were recaptured at egg-laying (as in 2010) to remove the implants. To collect blood, a wing vein was punctured with a sterile needle (Terumo, 27g × ¾; 0.4 × 20mm) and approximately 100–120 μl of blood was collected with heparinized microhematocrit capillaries and transferred into an Eppendorf tube. Within 2 hours of sampling, the blood was centrifuged for 10 min at 6000 revolutions per minute. Plasma was removed and stored in a –20°C freezer until analysed.

Hormone analysis

Plasma samples were thawed and hormone concentrations were measured using radioimmunoassays (RIAs). After measuring plasma volume of all samples, 50 μl radio-actively labelled testosterone (Perkin Elmer Life and Analytical Science BV) was added to all samples to measure the accuracy of the extraction process (recovery). After an incubation time of 1 hour, 2.5 ml diethyl ether/petroleum benzine (70:30) was added and samples were vortexed and centrifuged. Samples were snap frozen by a mixture of ethanol and dry ice and decanted. The supernatant was dried under streaming nitrogen, the remaining pellet was again dissolved in 1.5 ml 70% methanol and samples were stored over night at –20ºC. In the morning, samples were centrifuged, the methanol phase was decanted and the samples dried again under streaming nitrogen. The pellet was re-suspended in 200 μl PBS buffer. 30 μl of this mixture was used for measuring recoveries average recovery rate for testosterone (82%).

Plasma samples were analysed in one assay a using commercial kit (Orion Diagnostica, Spectria Testosterone RIA kit, Espoo, Finland) with a sensitivity of 0.04 ng/ml testosterone and cross-reactivities of 4.5% with 5α Dihydrotestosterone (DHT) and 0.01% with Androstenedione (A4). The dilution curve ran parallel to the standard curve. In total 56 (T = 20, C = 15 and Flu = 21) baseline plasma T samples could be analysed and 37 samples (T = 14, C = 8 and Flu = 15) which were collected during recapture.

Statistical analysis

All data were checked for normality. Data that were not normally distributed (i.e., plasma T levels, hatching and fledging success and the number of hatchlings) were either transformed (see below) or a non-parametric test was performed. Before analysing plasma T- concentrations, the data were transformed with a $\log_{10}$ transformation. To check, if natural T levels were elevated early in the breeding season, and decreased towards egg-laying, we compared the T levels of all females just before implantation with those of the C females after implantation by using these T values

as dependent variable and the number of days between taking the first and second blood sample in a regression analysis.. A mixed model (Mixed procedure) including female as a random factor (Littell et al., 1998) was used to test if our implants had an effect on plasma T concentrations, and on female body mass after implantation. For the model on plasma T-concentrations, treatment, sample (first or second sample) and the interaction between these factors was included.

A General Linear Model (GLM) was used to analyse the differences among treatments in the number of days until the restart of nest-building, the number of days until nest completion, the number of days between nest completion and the onset of egg-laying, nest weight, onset of egg-laying, and clutch size. For testing the number of days until nest completion, the date of restarting nest-building was included because females that started later in the breeding season had a shorter nest-building time, regardless of treatment ($r = -0.62$, $r^2 = 0.39$, $P < 0.001$). For testing the number of days between when a female had finished her nest and the onset of egg-laying, we included the date when the nest was completed. Females that completed their nest later in the season had fewer days between nest completion and the onset of egg-laying, irrespective of treatment ($r = -0.67$. $r^2 = 0.45$, $P < 0.001$).

For the aggression test data, we used a mixed model (MIXED procedure, see above) to test the effect of the treatments on the change in aggression over time (before vs after implantation). Furthermore, trial, treatment and trial × treatment interaction were included in the model as fixed effects and time as a covariate. As a post-hoc tests, differences between treatments were tested with the same mixed model, but now only including two of the three treatments per analysis.

Hatching and fledging success were analysed using a logistic regression, including nest as a random factor to account for non-independence of nestlings within nests. The total number of hatchlings was analysed with a Kruskal-Wallis test and the number of fledglings with a one-way ANOVA.

To test whether hormone treatment of the mother affected nestling characteristics (body mass, tarsus length and wing length on day 15), a linear mixed model (LMM) was conducted, including nest as a random effect to account for non-independence of nestlings within nests. To correct for time of day, seasonal effects and clutch size, we included date and time of measurement and number of nestlings per nest as covariates in all the analyses on nestling characteristics.

We analysed nest-building behaviour (restart nest-building, days until nest completion, nest mass), and egg-laying behaviour (onset of egg-laying, clutch size, time between nest finished and 1st egg), and number of hatchlings and fledglings using STATISTICA 7.0 (StatSoft, Inc.). The analyses of plasma T concentrations, female body mass, the aggression test, nestling characteristics and hatching and fledging success were performed in SAS (SAS® 9.2). Means are presented ± SEM or median ± range (minimum and maximum value, or quartiles). Tests were two-tailed and differences were considered to be significant with a P-value < 0.05.

Results

Hormone implants

As expected, natural circulating T concentrations were higher at the beginning of the season compared to when the females were recaptured later in the season, ($r = –0.42$, $r^2 = 0.18$, $P = 0.002$, see figure 2.1). The median baseline T-level before implantation was 0.49 ng/ml. Although plasma T levels were higher early in the breeding season, they were still much lower than in males in this species; the plasma T levels of male blue tits during the mating period (nest building and egg laying period combined) is 1.61 ± 0.71 with a range of 0.07–4.82 ng/ml (Foerster and Kempenaers, 2005). The implants significantly increased plasma T concentrations in T females but did not affect T concentrations of Flu and C females (treatment x sample interaction: $F_{2,32} = 13.53$, $p < 0.0001$). Before implantation, there was no significant difference in plasma T levels among the treatments ($F_{2,53} = 0.39$, $P = 0.68$, see figure 2.1). After implantation, T females (3.03 ± 0.39 ng/ml) had significant higher plasma T concentrations than C (0.59 ± 0.10 ng/ml) and Flu (0.53 ± 0.06 ng/ml) females (T vs C: Tukey test; $P < 0.001$; T vs Flu: Tukey test; $P < 0.001$). The plasma T concentrations of Flu and C females did not significantly differ after implantation (Tukey test; $P = 0.997$). All values after T implantation where below the maximum T level before implantation.

Individual characteristics

None of the treatments had a significant effect on female body mass (table 2.1). Body mass before implantation, but not hormone treatment, was significantly associated with body mass at the time of recapture (table 2.1).

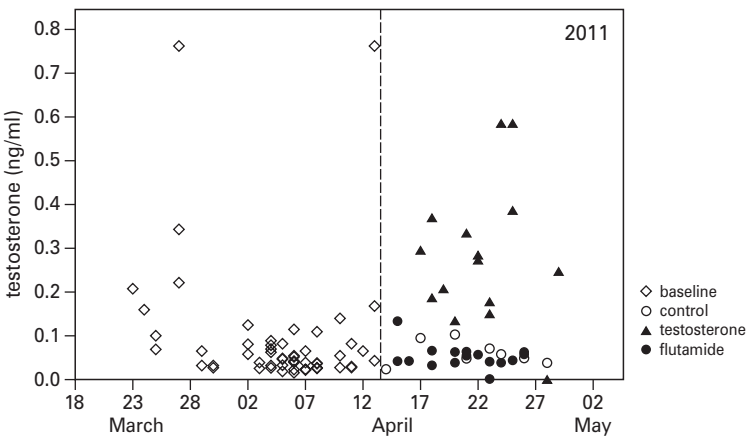


Figure 2.1. Levels of plasma T concentrations (ng/ml) before (baseline, plotted left of the dashed line) and after implantation (right from the dashed line).

Table 2.1. Summary of the overall treatment effects on female blue tit characteristics, different breeding parameters and fledgling characteristics.

Reproductive parameters	T nest	C nests	Flu nests	Statistics
Female body mass after implantation	13.21 – 0.19 (14)	13.10 – 0.18 (13)	13.34 – 0.18 (17)	Treatment: $F_{2,56} = 0.00$, $P = 1.0$, Body mass before implantation: $F_{1,43} = 411.86$, $P < 0.0001$
Onset of egg-laying (march)[1]	46.68 – 0.87 (19)	47.56 – 0.72 (16)	48.05 – 0.65 (19)	$F_{2,51} = 0.88$, $P = 0.42$
Clutch size	11.92 – 0.45 (13)	11.42 – 0.34 (12)	11.25 – 0.42 (16)	$F_{2,38} = 0.73$, $P = 0.49$
Hatching success	0.65 – 0.10 (13)	0.62 – 0.10 (12)	0.75 – 0.06 (15)	$\chi^2 = 1.96$, df = 2, $P = 0.375$
Number of hatchlings	8.0 (5-10; 13)	7.5 (3-10; 12)	8.0 (7-10; 16)	$H_{2,41} = 0.48$, $P = 0.78$
Nestling tarsus length (mm) day 15	16.48 – 0.07 (90)	16.43 – 0.07 (71)	16.45 – 0.06 (112)	Treatment: $F_{2, 267} = 0.29$, $P = 0.75$, correcting for date ($F_{1, 267} = 0.63$, $P = 0.43$), time ($F_{1, 267} = 2.06$, $P = 0.15$) and nest size ($F_{1, 267} = 1.34$, $P = 0.25$).
Nestling body mass (g) day 15	11.22 – 0.09 (90)	11.35 – 0.11 (71)	11.25 – 0.08 (112)	Treatment:$F_{2, 216} = 1.72$, $P = 0.18$, correcting for date ($F_{1, 219} = 0.98$, $P = 0.32$), time ($F_{1, 226} = 4.32$, $P = 0.04$) and nest size ($F_{1, 224} = 7.10$, $P = 0.008$)
Nestling feather length (mm) day 15	28.68 – 0.33 (90)	27.81 – 0.37 (71)	29.40 – 0.28 (112)	Treatment:$F_{2, 267} = 7.40$, $P = 0.0007$, correcting for date ($F_{1, 267} = 31.28$, $P < 0.0001$), time ($F_{1, 267} = 6.01$, $P = 0.015$) and nest size ($F_{1, 267} = 0.02$, $P = 0.89$)
Number of fledglings	6.54 – 1.18 (13)	5.25 – 1.11 (12)	6.67 0.73 (15)	$F_{2,37} = 0.59$, $P = 0.56$
Fledging success	0.71 – 0.12 (13)	0.69 – 0.09 (12)	0.79 – 0.07 (15)	$\chi^2 = 0.73$, df = 2, $P = 0.694$

Note. Data are presented as mean – SEM (sample size) or as median (quartile range; sample size).
[1] The onset of egg-laying was scored in March days, where 1st of March is 1.

Territorial response to a female decoy

There was a significant interaction effect between treatment and trial on the aggression score ($F_{2,33} = 4.19$, $P = 0.02$, figure 2.2). This score increased from the first to the second trial for the T females and decreased for the Flu and Cfemales. This interaction was significantly different when comparing T and C ($F_{1,20} = 7.77$, $P = 0.01$) and T and Flu only ($F_{1,22} = 5.49$, $P = 0.03$), but not when comparing C and Flu ($F_{1,21} = 0.15$, $P = 0.70$).

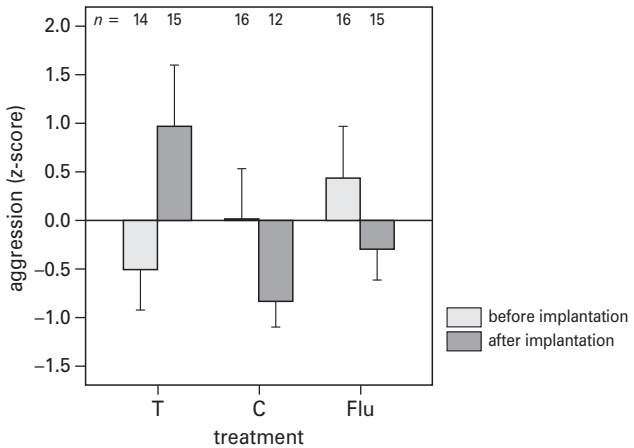


Figure 2.2. Aggression score of female blue tits towards a taxidermic mount of a conspecific female plotted against treatment, before and after implantation. Bars represent the means ± SEM per treatment for all the females that were tested. The number of females is presented above the bars.

Nest-building behaviour

There was an overall effect of the treatments on how quickly the females restarted nest-building after their nest material was removed ($F_{2,51} = 10{,}53$, $P < 0.001$, figure 2.3A). The mean numbers of days between implantation date (when nests were removed) and restart of nest-building for T- females (2.37 ± 0.27 days) was significantly less than the mean for C treated females (5.19 ± 0.56 days; Tukey test; $P < 0.001$) and Flu birds (3.84 ± 0.54 days; Tukey test; $P = 0.05$). The difference between Flu and C females (Tukey test; $P = 0.06$) almost reached significance, with relatively low value for Flu birds.

In addition, our hormone treatment affected the time females took to build a nest, correcting for the date when the females restarted with their nest-building (treatment: $F_{2,47} = 1.07$, $P = 0.001$; date restart nest-building: $F_{1,47} = 53.08$, $P < 0.001$, figure 2.3B). T females (3.56 ± 0.76 days) completed their nest faster than C females (5.07 ± 0.87 days; Tukey test: $P = 0.04$), but not faster than Flu females (Tukey test:

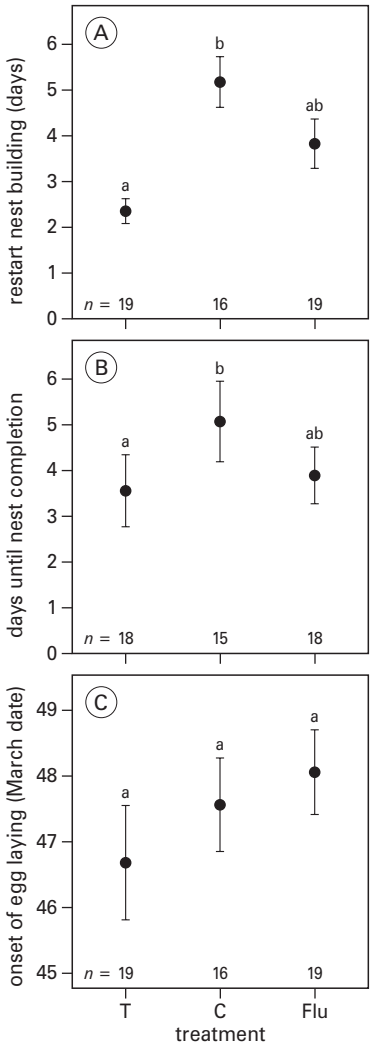


Figure 2.3. A. Effects of testosterone (T) and flutamide (Flu) treatments on the mean number of days female blue tits took to restart nest-building compared to control (C) females. **B.** Effects of testosterone (T) and flutamide (Flu) treatments on the mean number of days females took to complete their nest compared to control (C) females. **C.** Effects of testosterone (T) and flutamide (Flu) treatments on the mean date of onset of egg-laying compared to control (C) females. March date is calculated from the first day of March onwards.
In all graphs squares with error bars represent means with standard errors per treatment. The number of females is presented below the error bars. Letters in graph represent significant differences.

$P = 0.47$). Flu females (3.89 ± 0.62 days) did not significantly differ from C females (Tukey test: $P = 0.36$). We found no significant treatment effect on nest mass, correcting for date when the females restarted nest-building ($F_{2,47} = 0.20$, $P = 0.82$; C = 26.53 ± 2.52 g; Flu = 28 ± 1.91 g; T = 28.50 ± 1.83 g).

Onset of egg-laying

Our treatments did not affect the onset of egg-laying (table 2.1 and figure 2.3C). Also, the number of days between when females finished their nest and the onset of egg-laying did not differ among the treatment groups ($F_{2,50} = 2.07$, $P = 0.14$), but was negatively related to date of nest completion ($F_{1,50} = 26.50$, $P < 0.0001$). Hence, females that finished their nest later in the season, started faster with egg-laying after their nest was complete. Our treatment did not affect clutch size (table 2.1).

Nestling characteristics

The length of the third primary of nestlings was significantly affected by our treatment (table 2.1), correcting for date, time, brood size and body mass at day 15. Nestlings of Flu and T females had longer third primaries than nestlings of C females (Flu vs C, Tukey test: $P = 0.0004$; T vs. C, Tukey test: $P = 0.0015$). Nestlings of T females did not differ from nestlings of flu females in third primary length (Tukey test $P = 0.96$). There was no overall effect of the experimental treatment on all the other measured nestling characteristics (table 2,1).

Reproductive success

There was no significant difference in the number of hatchlings and fledglings produced among the treatment groups (table 2.1). Also, hatching and fledging success did not significantly differ among treatments (table 2.1).

Discussion

The aim of this study was to test whether the slight elevation of testosterone (T) levels in spring in female blue tits, as confirmed in this study, is the result of sexually antagonistic selection, the result of direct selection on T levels in females or is selectively neutral. This was tested by investigating the cost and benefit of increasing and decreasing exposure to T in females on different breeding parameters. As our T manipulation affected several female behaviours, it is unlikely that T levels are selectively neutral. Since the effects are unlikely to be detrimental, but rather beneficial, we found no support for the sexual antagonistic selection hypothesis.

Effectiveness of the treatment

We found that our hormone manipulation in 2011 was successful in that T-treatment

elevated female circulating T levels within the physiological range. Since the implantation procedure in 2010 was identical to 2011, we are confident that plasma T levels in 2011 were similar to those in 2010. Although in our experiment plasma T levels were still elevated when natural levels had returned to baseline values, the period of elevated T used in our study was still shorter and more similar to the natural variation in T levels than compared to other studies. The effectiveness of the Flu treatment is more difficult to demonstrate independently from our behavioural measurements. This is extensively discussed below.

Aggressive behaviour

The increase in aggressive behaviour of Tfemales is similar to what other studies have found (Sandell, 2007; Searcy, 1988; Zysling et al., 2006) and might be a beneficial effect rather than detrimental effect of elevated T (Cain and Ketterson, 2012). In blue tits, female-female competition is high early in the breeding season (Kempenaers, 1994); coinciding with the natural peak in T levels. This intra-sexual aggression may be important for obtaining nest sites (Kral et al., 1996; Rosvall, 2008), increasing reproductive success (Cain and Ketterson, 2012), and reducing the chance of secondary females pairing with their social male ((Jawor et al., 2006b; Kempenaers, 1994; Sandell, 2007; Slagsvold, 1993). The later may be of importance in our population since polygyny was relatively frequent (occurring on average in 13.4% of the territorial males; (Vedder, 2011). By preventing the settlement of other females as secondary females, female blue tits may secure more parental care for their offspring, leading to a higher reproductive success (Kempenaers, 1994).

In contrast to what we expected Flu did not significantly reduce aggression in female blue tits. Blocking T with Flu in males reduces aggressive behaviour in some species (Searcy and Wingfield, 1980; Sperry et al., 2010), but not in others (Heilman et al., 1976; Tokarz, 1987). Similar to our study, the only other study so far that used Flu to investigate the role of T on aggressive behaviour in females did not find an inhibitory effect on territorial aggression either (robins, *Erithacus rubecula*, Kriner and Schwabl, 1991). However, in this study female robins were implanted during their non-reproductive wintering phase and the hormonal regulation of aggression may be different between seasons (Soma et al., 2008).

So far, the potential benefits of increased aggression caused by elevated T levels undermine the sexually antagonistic selection hypothesis. But, future studies should investigate if aggressive females also have a higher reproductive success as has been found in female dark-eyed junco (Cain and Ketterson 2012).

Nest building

Experimentally elevated T levels in females positively affected nest-building behaviour; T females restarted nest-building earlier and completed their nest within a shorter time frame compared to C and Flu females. Nest completion early in the

breeding season might have an advantage for females because these females may have a wider time window to adjust the timing of reproduction to the period when food sources are most abundant (Perrins and McCleery, 1989), such as the caterpillar peak which is very important for reproductive success in blue tits (Perrins, 1991).

In male Northern mockingbirds (*Mimus polyglottos*) it has been shown that a natural increase in T at the beginning of their breeding phase is positively correlated with their nest-building behaviour (Logan and Wingfield, 1995), and experimentally elevated T levels resulted in an increase in nest building activities in zebra finches (*Taeniopygia guttata*; Hill et al., 2005). Hence, the increase in nest-building behaviour in female blue tits might be a direct result of elevated T levels. Alternatively, it might be an indirect effect via the conversion of T into oestradiol (E2) by aromatase, as E2 is known to stimulate nest-building in female canaries (Warren and Hinde, 1959).

Egg laying

T females did not start egg-laying later than control birds, indicating that experimentally elevated T did not negatively influence the timing of reproduction. This is in contrast to other studies which found that experimentally elevated T delayed the onset of egg-laying (Searcy, 1988; Clotfelter et al., 2004; Rutkowska et al., 2005). This discrepancy might be caused by the difference in experimental design compared to our study. These studies elevated T levels outside the physiological range of the species and/or for a longer period than occurs naturally, resulting in an unnatural T profile, which makes it difficult to interpret the functional consequences of their treatment. This shows the importance of scaling the dose of the implants and the period of the implantation close to the natural variation in T levels to measure the effects of T on fitness parameters.

Reproductive success

Having elevated or blocked T levels only at the beginning of the breeding season might have carry-over effects on reproductive success later in the season (Roberts et al., 2009). In our study we did not find clear carry-over effects on hatching and fledgling success. This might have been due to low sample size. However, the number of females we used in our experiment was not lower than in other studies that have found effects of T on reproductive success (Lopez-Rull and Gil, 2009; O'Neal et al., 2008). Therefore it is more likely that we did not find an effect on reproductive success because females were no longer implanted during the nestling phase, in contrast to the other studies. Interestingly, this finding is similar to what has been found in male blue tits. In a study by (Foerster and Kempenaers, 2004), where they also used a more natural set-up by only increasing T levels in male blue tits when T levels were natural high; they too found no effect on reproductive success. Thus an increase in T levels at the beginning of the breeding season only does not seem to have an effect on reproductive success later in the season in this species. We did,

however, find one effect of our manipulation later in the season. The length of the third primary feather of Flu and T nestlings was longer compared to the nestlings of control females at day 15 of the nestling phase. This might be a positive effect as it may improve early flight performance of the offspring. The cause for this effect is unclear, especially since both T and Flu treatment had this effect while having differential effects on circulating T levels in the mothers. Alternatively, since we used a large sample size to test the effect of the treatment of the mother on the wing length of the offspring it is more likely that the difference in nestling wing length is the result of chance (a type I error) rather than an effect of treatment *per se*.

The flutamide enigma

We did not find a significant direct effect of the androgen receptor blocker Flu on any of the measured parameters, with the exception of a positive effect of Flu on the feather length of nestlings. Also, we did not find any difference in T levels in the Flu group compared to the C females. These results question whether Flu was an effective androgen receptor blocker? Flu has been used as an androgen receptor blocker for at least 30 years, but, to our knowledge, it has only been used once before in adult female birds, which also did not support a significant effect of Flu (Kriner and Schwable, 1991). So far, Flu has been used more often in males. In mammals, Flu can increase T levels (in rats: Gomez et al., 2004), but in birds this effect of Flu is not observed (Alonso-Alvarez et al., 2007; Hegner and Wingfield, 1987; Kriner and Schwabl, 1991; Searcy and Wingfield, 1980; Sperry et al., 2010; Van Roo, 2004). In contrast to mammals, Flu administration in birds does not appear to disrupt the negative feedback of T (Sperry et al., 2010) and thus not altering circulating T levels. This is probably due to the fact that in birds, oestrogen rather than androgens, controls androgen levels via negative feedback acting on oestrogen receptors in the hypothalamus and pituitary (Scanes, 2000). Although Flu does not increase T levels in birds like in mammals, blocking the androgen receptors does affect behaviour in male birds. For example, Flu reduced copulation behaviour (Adkins-Regan and Garcia, 1986), dominance (Searcy and Wingfield, 1980), and aggression (Sperry et al., 2010; Vleck and Dobrott, 1993). Therefore, it is not unlikely that Flu did work as an androgen receptor blocker in our study. The lack of significant effects of Flu on behaviour in female blue tits might be because these behaviours are regulated by E2, a metabolite from testosterone. These results warrant further studies, in which not only testosterone is manipulated, but in which also the conversion of this hormone to E2 or E2 receptors are blocked.

Conclusion

By experimentally manipulating T levels within the natural range of free-living female blue tits, we found a clear effect on nest building behaviour and on female

aggression. Hence it is unlikely that current levels of T in females are selectively neutral. Moreover, our data also undermine the sexually antagonistic selection hypothesis because the effects of T on female behaviour can be interpreted as beneficial rather than detrimental for female fitness and T females did not do worse in their breeding behaviour and reproductive success compared to control females. Further research, using our design of hormone manipulation, but with additional validation of the flutamide treatment and manipulation of E2 exposure, and including additional fitness estimates such as immune system, nestling feeding rate, moult, female survival and reproductive success in the following breeding seasons is therefore needed to disentangle the three hypothesis explaining female T levels in seasonally breeding species.

Acknowledgement

We thank Peter Wolfs for field assistance, Bonnie de Vries and Ilse Weites for lab assistance and Martina Muller for her comments on the manuscript. Funding from the institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen) was provided to BdJ.

CHAPTER 3

Testosterone reduces promiscuity but not attractiveness in female blue tits (*Cyanistes caeruleus*); an experimental study

Berber de Jong, Luc Lens, Marco van der Velde, Ton Groothuis & Jan Komdeur

Abstract

In many animal species extra-pair copulations (EPC) are common and can increase fitness of males and females. In males, EPCs can result in an increase of their reproductive success, whereas in females the benefits of extra-pair copulations can be more indirect through improving the quality of the offspring. In many male vertebrate species the steroid hormone testosterone (T) plays an important role in mating behaviour by increasing, for example, their EPC rate. Although much is known about the role of T in male mating behaviour, the role of T in female mating behaviour remains unclear. To study the role of T in female mating behaviour, we created three groups of female blue tits (*Cyanistes caeruleus*): implanted with T, flutamide (androgen receptor blocker) and empty implants before egg laying under field and laboratory conditions and scored subsequent number of extra pair offspring (EPO) among three-day old nestlings (field) and female attractiveness (laboratory). The number of nests with EPO was lower for T implanted group, whereas flutamide had no effect relative to controls. The effect of T implants was not due to lower female attractiveness, because males did not show a clear preference for flutamide females when simultaneously presented with a flutamide and T female during a mate choice test. Alternatively, elevated T levels might have negatively influenced EPC rate by, for example, affecting female mating behaviour. Future studies should investigate how elevated T levels reduce the number of EPO.

Introduction

In many animal species extra-pair copulations (EPCs; copulations outside the pair bond) resulting in extra-pair offspring (EPO) are common (Griffith et al., 2002; Kleiman, 1977). In more than 85% of the passerine bird species at least some offspring are not sired by the social father, but by another male (Griffith & Immler, 2009). For males, EPCs can be beneficial because they have the potential to increase their reproductive success by increasing the number of offspring they sire (Moller and Ninni, 1998). The role of EPCs is less clear in females, but there is evidence that EPC can yield direct or indirect benefits (Griffith et al., 2002; Jennions and Petrie, 2000), which can increase female fitness (Gerlach et al., 2012). Direct benefits may be gained through extra-pair males providing greater access to breeding recourses (Birkhead and Møller, 1992) or paternal care to the offspring (Nakamura, 1998). Indirect benefits may concern fertility insurance (Sheldon, 1994) or the increase of the genetic quality of the offspring (e.g. the attractiveness ('good genes'; Griffith et al., 2002) or heterogeneity of their offspring ('compatible genes'; Griffith and Immler, 2009; Jennions and Petrie, 2000).

The ability for males to obtain EPC has been shown to be influenced directly or indirectly by the steroid hormone testosterone (T). T plays an important role in male reproduction and mating behaviour (Hau, 2007; Wingfield et al., 1990). In many male vertebrate species, T levels increase at the beginning of their breeding season when their mates are sexually receptive and territorial aggression frequently occurs, and decrease at the end of the breeding season or during the period of paternal care (Adkins-Regan, 2005; Moore, 1984, 1982; Wingfield et al., 1990). Several studies have shown that natural plasma T concentration in males are positively correlated with mating success (Alatalo et al., 1996), mate guarding and extra-pair paternity (Garamszegi et al., 2005). Moreover, experimental studies in which T levels were elevated have shown that T increases mating behaviours, such as enhanced song frequency and singing rate (De Ridder et al., 2000; Ketterson et al., 1992), attractiveness (Enstrom et al., 1997), courtship display (Alonso-Alvarez, 2001a; De Ridder et al., 2000; Edler et al., 2011; Enstrom et al., 1997), as well as the probability of EPC (Raouf et al., 1997), but see (Foerster and Kempenaers, 2004). Overall, high T levels might increase male fitness by positively affecting mating behaviour and thereby increasing reproductive success.

Females also have significant T levels (Staub and DeBeer, 1997) which show the same seasonal variation as in males (with the highest peak coinciding with their mating period, Ketterson et al., 2005), although in general T levels in females are considerably lower than in males (Ketterson et al., 2005; Møller et al., 2005). In contrast to the role of T in males, its role in female mating behaviour is not clear. Previous studies have shown that experimentally elevated T levels with hormone implants in females reduce attractiveness (Ketterson et al., 2005), choosiness in

selecting a mate (McGlothlin et al., 2004), and induce male like mating behaviour, such as establishing a territory (Lank et al., 1999), courtship feeding and mounting (Lahaye et al., 2012; Nespor et al., 1996). Recently it has been shown that experimentally elevated T resulted in a decline in extra-pair copulation rate, measured as a decrease in the number of extra-pair offspring produced in female spotless starlings (*Sturnus unicolor*; Garcia-Vigon et al., 2008), but not in female dark-eyed junco's (*Junco hyemalis*, Gerlach and Ketterson, 2013). There are, however, several problems with some of these studies mentioned above making it difficult to interpret the negative effects of elevated T in females on (EPC) mating behaviour. First, T manipulation often produces T levels outside the physiological range for females of that species causing abnormal female behaviour, e.g. male mating behaviour (Lahaye et al., 2012). Second, some of these studies do not remove the implants, leading to prolonged periods of elevated T (beyond the mating season) than would occur under natural situations (Garcia-Vigon et al., 2008; Gerlach and Ketterson, 2013), making it difficult to interpret possible carry-over effects of elevated T on copulation behaviour into the following breeding season. Third, none of these studies have investigated simultaneously both the effect of an experimental elevation as well as an experimental decrease of T levels. Therefore, they cannot make a distinction between effects of deviations from normal T levels on mating behaviour and of an increase in T levels per se (de Jong et al. **chapter 2**).

In this study we investigated experimentally whether T influences extra-pair copulation behaviour (taken as the number of EPO) in free-living female blue tits (*Cyanistes caeruleus*). Blue tits are ideally suited to study this question because extra-pair copulations frequently occur (in our population 12.8% of the young was extra-pair and 46.7% of the broods contained at least one EPO, Magrath et al., 2009). Additionally, EPO had a higher survival probability compared to within-pair offspring (Magrath et al., 2009; Schlicht et al., 2012). Furthermore, in blue tits females actively search for EPCs (Kempenaers et al., 1992) and paternity seems to be largely under female control (Kempenaers, 1995). Therefore, if T plays a role in regulating mating behaviour in female blue tits it is likely that it will also affect female EPC rate. In contrast to other studies, we used a more natural set-up in which we increased T levels within the physiological range only during the mating period until the onset of egg laying. Additionally, we decreased T effectiveness with an androgen receptor blocker (Flutamide, Flu). In total we created three experimental groups: one group received T implants; one group received Flu implants and a third group received empty implants as control (C). We predict that if even an increase in T levels within the physiological range would negatively affect female mating behaviour, then females with increased T levels would have less EPO. Alternatively, we expect that if T plays an important role in female mating behaviour, then blocking T receptors with Flu might decrease the number of EPO in females. . Furthermore, since an effect on EPC might be mediated by male choice, we also tested whether possible differences

in EPO number are related to differences in female attractiveness due to the hormone treatment, by performing mate choice tests in outdoor cages.

Materials and methods

Study area and species

Data were collected during the breeding seasons (March-June) of 2010 and 2011 at "De Vosbergen" estate in the north east of the Netherlands (53° 08' N, 06° 35' E). The study area contains 188 blue tit nest-boxes which are distributed over 54 ha forest. The forest is a mix of deciduous and coniferous trees. Blue tits have bi-parental care with both sexes defending the nest and feeding nestlings, but only the female builds the nest and incubates the eggs (Cramp and Perrins, 1993a). The blue tit population is part of a long term study, which has been studied since 2001 (Korsten, 2006).

Hormone implants and monitoring; a field experiment

In 2010 and 2011 at the beginning of the breeding season (25 March – 13 April in 2010, 23 March – 13 April in 2011), all nest-boxes were checked daily for nest building activities. Once nest material was present, females were caught and randomly assigned to either a T (2010: $n = 21$; 2011: $n = 20$), Flu (2010: $n = 20$; 2011: $n = 21$) or C treatment (2010: $n = 18$; 2011: $n = 17$). On average females were caught 14 days (range 7-23 days) before the onset of egg laying in 2010 and 13 days (7-26 days) in 2011. Females were caught inside their nest box during the day (2010: $n = 45$ (T = 17 Flu = 15, C = 13); 2011: $n = 58$) or at night when roosting in their nest box (2010: $n = 14$ (T = 4, C = 5, Flu = 5). For implantation females were transported and arrived at the field station within 10 min. after capture. Before implantation a blood sample was taken from the wing vein for molecular parentage analyses. The wing vein was punctured with a sterile needle (Terumo, 27 g × 0.75; 0.4 × 20 mm) and approximately 100–120 μl of blood was collected with heparinized microhematocrit capillaries. The blood was transferred into 90% ethanol for storage until DNA-analyses. After blood sampling, each female received one implant which was placed subcutaneously along the left flank under local anesthesia (Xylocaine 10% spray). The small wound was sutured with tissue glue (1 × 0.5 ml Histoacryl, Braun, Germany). The implant consisted of a silicone tube that was sealed at both ends with 1mm glue. The T and control-implants were 4.4 mm long (id 0.5; od. 1.0) and filled with crystalline T or left empty, respectively. The Flu implants were 7.2 mm long (id 1.47; od 1.96) and filled with crystalized flutamide (Sigma©). This dose of T produced levels that were within the physiological range (de Jong et al. **chapter 2**). After implantation nest-boxes were checked daily until the onset of egg laying. Females were recaptured after the second egg was laid to remove the implant. In 2010 the second egg was collected and replaced with a dummy egg for a different

experiment. The females were either recaptured with a mist net (2010: n = 30; 2011: n = 11), at night (2010: n = 2) or with a hand net (De Heij et al., 2008) when leaving their nest box in the morning (2010: n = 15; 2011: n = 37). After implant removal, females were returned to their territory or nest box (when captured at night) within 30 min after (re)capture. In 2010 none of the females deserted her clutch. In 2011 three females deserted their clutch after recapture (C = 1, T = 1, Flu = 1).

Nest checks were continued near the end of the incubation period, around three days before the expected hatch date (9 days after laying last egg), to determine the date of hatching per nest. When the nestlings were three days old (day of hatching was taken as day 0) a blood sample (5–25 μl) was taken for molecular parentage analysis. Additionally, nestlings were individually marked by clipping the tip of one or two toenails. On day 8 all nestling received a metal ring with a unique number. Social partners of the females were caught in the nest-box with flap traps for blood sampling when broods were 9–14 days old. Furthermore, 25 females of 2010 (T = 9, C = 9 and Flu = 7) were breeding in the population in 2011, and 16 females of 2011 were breeding in the population in 2012 (T = 8, C = 5 and Flu = 3). The number of EPO of these females was also included in the analyses to measure the long-term effects of the hormone treatment.

Molecular parentage

DNA was extracted from the blood samples of both parents and nestlings using a chelex extraction method (for blood samples; Walsh et al., 1991). To assign parentage, parents and nestlings were genotyped for eight microsatellite loci, Ase18 (Richardson et al., 2001), Pca3, Pca7 and Pca8 (Dawson et al., 2000), PmaGAn27 and PmaTGAn45 (Saladin et al., 2003), Pocc6 (Bensch et al., 1997) and Pdo5 (Griffith et al., 1999). PCR reactions were carried out in 10 μl volume using 20–50 ng of template DNA, a QIAGEN Multiplex PCR Kit and manufacturer's protocol. The microsatellites were amplified in two separate multiplex panels (panel 1: Ase18, Pca8, PmaGAn27, Pdo5; panel 2: Pca3, Pca7, PmaTGAn45, Pocc6) using the following PCR program: 15 min. 95°C, 35 cycles of 94°C for 30s, 55°C for 90s and 72°C for 60s, followed by 60°C for 30 min. Fluorescently labelled PCR products were separated on an AB3730 DNA analyser. Subsequently allele-lengths were determined using Gene-mapper 4.0 software. Of the blood samples whose loci were not visible, analyses were carried out again. In the case of too little blood, loci were not visible during the second analysis. From these blood samples we could not identify the loci. Using Cervus 3.0 (Kalinowski et al., 2007), mean exclusion probability of the 8 markers was calculated to be 0.999951 for the first (female) parent and 0.999999 for the second (male) parent (given the genotype of the first parent). Maternity of the social female was confirmed by the microsatellite data for all nestlings. Paternity of the social male was excluded if there were at least two mismatches between the

social father's and offspring's genotype, and those nestlings were regarded as extra pair young (Magrath et al., 2009).

Mate choice experiment using captive birds

For the mate choice experiment conducted in 2011 eight female and ten male captive blue tits were used. The birds were hand raised in 2007 and 2009 (for details see Vedder et al., 2010) and males and females were always separately housed in aviaries. One month before the experiment all birds were housed in individual cages (80 × 40 × 40 cm) placed within a large outdoor aviary with ad libitum food and water. Early April 2011, four females received a T implant and four females a Flu implant (for details on implantation size, see above). Due to a limit in sample size, a control group was not included in the experiment. Upon capture just prior to the implantation a blood sample was taken to determine basal testosterone levels. Seven days after implantation a second sample was taken to measure the effect of the implants on plasma T concentrations. After 18 days the implants were removed. Subsequently the birds were allowed to recover for 16 days after the implantation experiment. This recover interval was chosen because after this period plasma T levels would have been back to basal level at least two weeks (Tell, 1997). Thereafter the treatments were switched such that the T females from the first part of the experiment received Flu implant and vice versa. The blood sampling scheme of the second experiment was equal to the first experiment. The method of blood sampling was equal to the field experiment (see above). Within 2 hours after sampling the blood was centrifuged for 10 min at 600 rpm. After centrifuging plasma was removed and stored in a –20°C freezer until analyses.

The mate choice experiment started 6 days after implantation (11th and 12th of April and the 21th and 22nd of May). For each test two females and one male were placed into the mate choice set-up in the afternoon before the test, to let them habituate to the set-up (Figure 3.1). During habituation the compartments were separated by opaque partitions so that the birds could not see each other. The females were unfamiliar to the males. Both females were put in small cages (25 × 25 × 40 cm) which were placed along both sides of a larger cage (80 × 40 × 40 cm), where the male was housed. The male had a long perch over the whole length of his cage. The perch was divided into five equal parts of 16 cm each to enable scoring the position of the male. The females also had a perch that was placed at the same height as the male perch. At both sides of the male cage there was a small open window through which the male could see the female. The preference tests were all conducted at the same time between 9:00 and 10:00 am. The tests were conducted in the morning because in free living blue tits most extra-pair copulations take place early in the morning (Poesel et al., 2006). Therefore it is likely that mate choice decisions also take place early in the morning (see for example (Drevon and Slagsvold, 2005). The test started by removing the opaque partition between the male and the females and

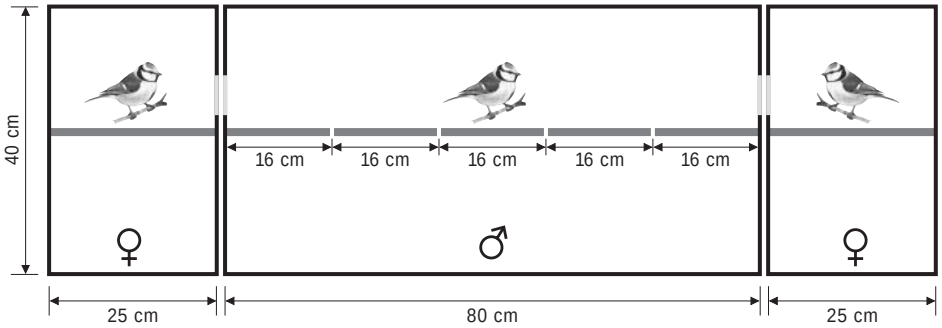


Figure 3.1. The experimental set-up of the mate choice test. The male could see the females through a small window covered with mesh wire.

lasted for two times 15 min. After the first 15 min. females were switched to the other side of the male cage (by switching their cage around), to account for a potential male-side bias, and the preference test was continued for 15 min. At the end of the test all birds were returned to their own cage. All tests were monitored with a digital video camera (Sony JVC Everio) and analysed in the lab by one observer who was unfamiliar with the treatment of the females. Eight days after the second implantation, the females and males underwent a second preference test. For this test a new set of four males was used. Thus in total eight male preference scores were collected for the analyses.

When analysing the video the amount of time a male spent in each of the five perch areas was scored. The overall male preference score was calculated as the amount of time a male spent in close proximity to the Flu female minus the time spent in close proximity to the T-female (i.e. the time a male spent in the area at the two extremes of the perch). Thus a preference score above zero represented a preference for Flu females, whereas a preference score below zero represented a preference for T females. The maximum possible preference score for one female equals 1800 sec (30×60 sec.), while a preference score of zero reflects equal time spent with each female.

Hormone analyses

Plasma samples were performed in one assay using a commercial kit (Orion Diagnostica, Spectria Testosterone RIA kit, Espoo, Finland) with a sensitivity of 0.04 ng/ml testosterone and cross-reactivities of 4.5% with DHT and 0.01% with A4 as described in de Jong et al. (**chapter 2**). Briefly, plasma samples were thawed, their volume was measured and 50 μl radio-actively labelled testosterone (Perkin Elmer Life and Analytical Science BV) was added to all samples to measure the accuracy of the extraction process (recovery). After an incubation time of 1 hour, 2.5 ml diethyl

ether/petroleum benzine (70:30) was added and samples were vortexed and centrifuged. Samples were snap frozen by a mixture of Ethanol and dry ice and decanted. The supernatant was dried under streaming nitrogen, the remaining pellet was again dissolved in 1 ml 70% Methanol and samples were stored over night at –20 ºC. The next day, samples were centrifuged, the Methanol phase was decanted and the samples dried again under streaming nitrogen. The pellet was re-suspended in 200 μl PBS buffer. 30 μl of this mixture was used for measuring recoveries (average recovery rate for testosterone: 92.96 ± 0.89 %). Hormone concentrations were measured using radio immuno assays (RIAs). Based on the standard curve values below the detection limit were calculated as being 0.10 ng/ml. All the baseline T levels were below the detection limit. The dilution curve ran parallel to the standard curve. The intra assay variation was 4.7 %.

To analyse the effects of the hormone implants on plasma T levels only the T plasma concentration after implantation was included, because all the baseline T levels were below the detection limit. Also, the order of treatment (first T or first Flu) did not have an effect on plasma T levels (two-way t-test: t = 1.93, DF = 6, P = 0.10) when the females were implanted with T. All the females that were implanted with Flu had T levels of 0.10 ng/ml, except one female of the first experiment that had a T level of 2.74 ng/ml. Because there was no order effect of treatment on plasma T levels, it was excluded from the analyses. The mean plasma T levels (2.66 ± 0.18 ng/ml) in captive females treated with testosterone were significantly higher than in females treated with Flu (0.30 ± 0.16 ng/ml, Mann- Whitney U test: U = 98, P = 0.001). The elevation of T was within the physiological range for females of this species (de Jong et al. chapter 2).

Data and statistical analyses

For the analyses of the short-term effects of the hormone manipulations on number of broods with EPO, only females that were manipulated in that year were included (thus excluding females were the implant was removed the year before). Four of the 91 broods were excluded (2010: T = 2; 2011: T = 1, Flu = 1) because the DNA was not collected from both parents (n = 2), or the female was recaptured at night, and since this is known to be severely disturbing it may have negatively affected her egg laying and (extra pair) copulation behaviour (n = 2). Hence this left us with 13 C, 15 Flu and 16 T broods in 2010, 13 C, 15 Flu and 15 T broods in 2011.

For the analyses of the long-term effects of the hormone manipulations on number of broods with EPO, nine of the 39 broods of the females that were treated in 2010 or 2011 and were breeding in the population the following year were excluded (2011: C = 2, T = 3; 2012: C = 2, T = 1, Flu = 1) because either their implant was not removed (n = 8) or lost her implant (n = 1). In total, ten C, nine Flu and 11 T females, were used to test if there was a long-term effects of the hormone treatment on the number of broods with EPO.

In total parentage was determined of 95% of the 733 nestlings. The differences in the number of broods with EPO among treatments were analysed with a generalized model (PROC GENMOD in SAS) with a binary distribution and a logit function. A nestling was scored as 1 when it was an extra pair nestling and as 0 when it was a within-pair nestling. A nest was scored as 1 if it contained at least one extra pair offspring and a 0 when the nest contained no extra pair offspring. As fixed factors, treatment and year and the interaction between these factors were included and the number of nestlings per nest (of which blood samples had been taken) was used as a covariate. To test if our treatment had an effect on the number of EPO per nest in 2010 and 2011 a GENMOD was used with a Poisson distribution, including the same predictors as above. In this analysis only the nests that had at least one extra EPO were included. To examine if the treatment effects on the number of EPO per brood had carried over to the next season a GENMOD was used. The number of EPO per broods that had at least one EPO in 2011 of the females that were implanted in 2010 was included as depended factor. Treatment was included as fixed factor and number of nestlings per nest as covariate. To test if males preferred Flu or T females during the mate choice test a one-sample t-test was used. Descriptive statistics are presented as mean ± SEM, or percentage; and all statistical tests are two tailed. The level for significance was $P < 0.05$). All data were analysed in SAS 9.2

Results

Hormone implants and extra-pair offspring

In 2010 23% (10/44) and in 2011 37% (16/43) of the broods of the manipulated females, contained at least one EPO (including only the females that had been manipulated in that year). There was an overall treatment effect on the number of broods that contained at least one EPO ($\chi^2 = 7.25$, df = 2, $P = 0.027$; table 3.1, figure 3.2). The T group (13%; 4/31) had significantly less broods with EPO compared to the C group (38%; 10/26, Fisher's exact test: $P = 0.03$) and the Flu group (40%; 12/30, Fisher's exact test: $P = 0.02$). The C and Flu groups had similar percentage of broods containing at least one extra-pair offspring (Fisher's exact test: $P = 1.0$). Brood size and year had no effect (table 3.1). Of the broods that had at least one EPO, the number of EPO per brood did not differ among treatments, but was significantly higher in 2011 than in 2010 (table 3.2, figure 3,3). In 2010 the average number of EPO per brood that contained at least one EPO was 1.2 ± 0.13 and in 2011 2.8 ± 0.61. Also brood size was positively associated with the number of EPO per nest (table 3.2).

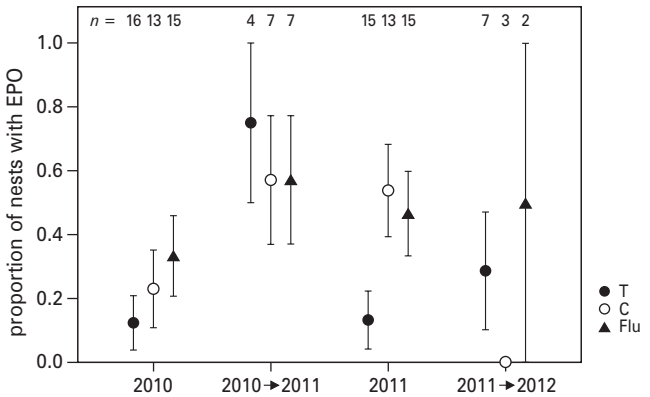


Figure 3.2. Proportion of broods that contained at least one extra -pair offspring (EPO). n is the number of broods included in the analyses. Mean ± SEM are given.

Table 3.1. Model for analysing the effect of the three treatments (testosterone, control, flutamide) on the number of broods that contained at least one extra-pair offspring.

	χ^2	Df	*P*
Full Model			
Treatment	6.17	2	**0.046**
Year	1.26	1	0.261
Brood size	0.74	1	0.389
Treatment* Year	1.34	2	0.601
Final Model			
Treatment	7.25	2	**0.027**

Table 3.2. Model for analysing the effect of the three treatments (testosterone, control, flutamide) on the number of extra-pair offspring per brood that contained at least one EPO.

	χ^2	Df	*P*
Full Model			
Treatment	0.24	2	0.889
Year	9.57	1	**0.002**
Brood size	9.57	1	**0.002**
Treatment* Year	3.89	2	0.143
Final Model			
Treatment	2.26	2	0.322
Year	7.34	1	**0.006**
Brood size	5.04	1	**0.010**

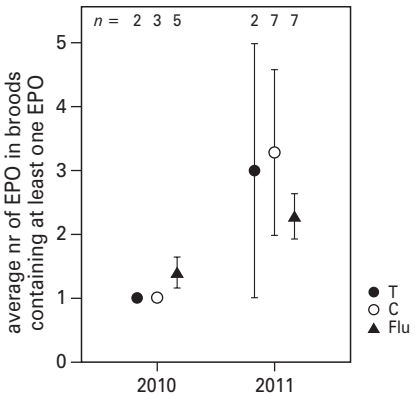


Figure 3.3. The average number of extra-pair offspring (EPO) for broods that contained at least one extra-pair offspring. n is the number of broods per treatment that contained at least one extra-pair offspring. Mean ± SEM are given.

Long-term effects of hormone implants

Of the females that were implanted in 2010 and 2011 and were breeding in the population in 2011 and 2012, respectively, 47% (14/30) of the nests contained at least one EPO. There was no long-term effect of the treatments on the number of nests with EPO (table 3.2 and figure 3.2) In short, neither increased levels of testosterone nor a decrease in T effectiveness significantly affected EPO rate in the following breeding season, suggesting that there were no carry-over effects of the manipulations on EPC behaviour.

Table 3.3. Model for analysing the effect of the long term effects (one year after the manipulation) of the three treatments (testosterone, control, flutamide) on the number of broods that contained at least one extra-pair offspring.

	χ^2	Df	*P*
Full Model			
Treatment	1.65	2	0.438
Year	6.37	1	**0.012**
Brood size	3.12	1	0.077
Treatment* Year	2.01	2	0.367
Final Model			
Treatment	0.86	2	0.651
Year	3.90	1	**0.038**

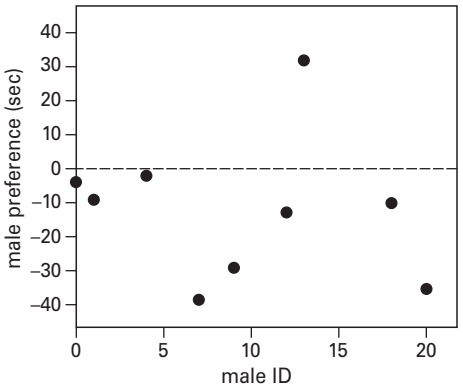


Figure 3.4. Male preference score taken as the total time a male spent in close proximity to the Flu female minus the time spent in close proximity to the T female in seconds. A preference score of zero means an equal time spent with one of the females. The maximum time a male could spend with one of the females was 1800 (30 min × 60 sec).

Preference test

During the mate choice tests the overall male preference score was -13 (± 7.95) sec. While males hence spent a fraction more time near the T females, this preference score did not significantly differ from zero (one sample *t*-test (7) = –1.64, $P = 0.15$, figure 3.4). Hence, males did not show a preference for either Flu or T females.

Discussion

Female blue tits that were treated with T produced nests with fewer EPO compared to control females, although the number of EPO per brood that had at least one EPO did not differ among the two groups. The Flu treated females had similar number of nests with EPO's and number of EPO's per nest as control females. Furthermore, T treatment only affected the chance of having a nest with an EPO during the period when T levels were experimentally elevated. The effect of T disappeared the following breeding season. In addition, also the Flu and the control implants had no effect on the number of nests with EPO in the long-term. As males did not prefer Flu females above T females, it was unlikely that the short-term decline in EPO in the latter was caused by a lower female attractiveness.

To our knowledge, so far only two other studies have investigated the effect of T in females on the number of EPO produced and found contradictory results. Elevated T had no effect on the number EPO in the dark-eyed junco (Gerlach and Ketterson, 2013), whereas elevated T did reduce the number of nests with EPO during the

period of treatment in the spotless starling (Garcia-Vigon et al., 2008). The results of the later study are in concurrence to our findings. However, in contrast to our study, T female starlings also had fewer nests with EPO in the subsequent years (Garcia-Vigon et al., 2008), whereas in our study we did not find any carry-over effects of our hormone manipulation to the following year. This difference could be due to the fact that in the spotless starling study the implants were not removed. Therefore, T levels were kept elevated for a much longer period then natural, whereas in our study the females were only implanted for the short period when T levels were naturally elevated. Under natural conditions, circulating testosterone levels decrease when females start egg laying and remain low until the next breeding season (Ketterson et al., 2005). The long-term effect of elevated T levels on the number of EPO might have been the result of irreversible phenotypic changes (Abitbol et al., 1999; Roberts et al., 2009; Staub and DeBeer, 1997). For example, the voice of women injected with testosterone develops masculine characteristics and these changes are irreversible (Abitnol et al., 1999). In our study, the experimentally elevation of T was likely too short to cause any irreversible physiological changes.

In contrast to elevated T, we did not find an effect of Flu on the number of broods with EPO. The Flu group had similar number of broods with EPO as the control group. Thus, reducing the effectiveness of T (see de Jong et al. **chapter 2**) does not seem to influence female EPC's behaviour. This may be due to the fact that T can be aromatized into oestradiol (Nelson, 2011). In females oestrogens and/or progesterone were shown to exert stronger effects on female mating behaviour than testosterone (Adkins-Regan, 2005). For example, experimentally elevated oestrogens induced mating behaviour in many female vertebrate species (Moore, 1982; Takahashi, 1990; Tokarz and Crews, 1980). Thus, the aromatization of T into oestradiol, may explain why the number of nests with at least one EPO was not affected in Flu females.

There are at least two possible hypotheses to explain why females treated with testosterone have fewer EPO. First, T might reduce female attractiveness to males and therefore females acquire fewer EPC's. Male dark-eyed Juncos (*Junco hyemalis*) were less attracted to females treated with T than Cfemales (Ketterson et al., 2005). This reduction in attractiveness might be the result of females with elevated T levels showing male like mating behaviour (Lahaye et al., 2012; Lank et al., 1999; Nespor et al., 1996), or a more male like plumage (Peters, 2007). In our mate choice experiment, however, there was no clear male preference for either the Flu or T treated females. Thus, in contrast to other studies, in blue tits experimental elevated T levels do not seem to reduce female attractiveness. Our study used elevation of T levels within the physiological range of the females of this species, while other studies induced male like concentrations (Lank et al., 1999; Ketterson et al., 2005; Lahaye et al., 2012). Therefore, it is less likely that our T manipulation caused masculinisation of the females. However, some caution should be taken when interpreting our results:

(i) First, the sample size used in our mate choice experiment was rather small. (ii) The experiment was conducted in an unnatural setting, thus making it difficult to link these results to the findings of our field experiment.

Second, a more parsimonious hypothesis explaining why females treated with T might have fewer EPO is that T might inhibit EPC- seeking behaviour in females. In blue tits, females actively go out to seek EPC's (Kempenaers et al., 1992). Therefore it is more likely that the effect of experimentally elevated T on the number of EPO was the result of a reduction in females mate seeking behaviour rather than a change in female attractiveness. This potential change in mate seeking behaviour might be a direct effect of elevated T levels, for example by reducing female choosiness in selecting a mate (McGlothlin et al., 2004), or an indirect effect by causing a shift in behaviour from mate seeking behaviour towards behaviours like aggression and nest building. In female birds it is known that experimentally elevated T increases female-female aggression (Sandell, 2007; de Jong et al. **chapter 2**) and nest building (de Jong et al. **chapter 2**).

Concluding remarks

We found that T levels in females play a role in female EPCs behaviour as it reduces the number of nests with EPO. This reduction in nests with EPO was only short term and was probably not caused by a reduction in attractiveness. Although the functional significance of female EPC is not completely clear, there is evidence that having EPC can indirectly increase female fitness (Gerlach et al., 2012). Thus a reduction of EPO by experimentally elevating T might lower female fitness. Future studies should investigate how T reduces the number of EPCs and if an elevation in T decreases female fitness by reducing her number of EPO.

Acknowledgment

We thank P. Wolfs, C. Kuipers, O. Vedder and T. Egberts for field assistance, C. Kuijpers, B. de Vries and I. Weites for lab assistance and E. Schut and V. Hulst for assisting with the mate choice experiment. Funding from the institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen) and from the Dr. J.L. Dobberke foundation was provided to BdJ.

CHAPTER 4

Testosterone in female blue tits (*Cyanistes caeruleus*); adaptive or a by-product of selection on males?

Berber de Jong, Luc Lens, Ton Groothuis & Jan Komdeur

Abstract

Testosterone (T) is an important mediator of between-sex variation in morphology, physiology and behaviour. In seasonal breeding birds, T peaks during the period of intense territorial and sexual behaviour in both sexes. In males, T plays a key role in regulating reproductive behaviour, and experimental elevation of T has been shown to increase male reproductive success. The role of testosterone (T) in females and the question whether circulating T levels result from sexual conflict or can be considered adaptive, however, remains largely unanswered. Former studies that experimentally elevated female T revealed adverse effects on female reproductive success, supporting the hypothesis that present day levels result from sexually antagonistic selection. Most of these studies, however, elevated T beyond the physiological range of females and/or during longer periods than would naturally occur. Here, we experimentally elevated T within the physiological range of free-living female blue tits (*Cyanistes caeruleus*) during a short period in spring when natural female T levels are also increased. Two other groups of females either underwent a reduction of T effectiveness by blocking androgen receptors with Flutamide, or a sham treatment that acted as control. We found that neither elevated T levels nor reduced T effectiveness significantly affected female reproductive success or survival, despite the fact that Flu females tended to show a lower survival rate than the long-term average in our population. As opposed to other studies, our results therefore do not support the hypothesis that present day T levels in females are the result of sexual conflict, possibly reflecting a more natural experimental set-up.

Introduction

In many species, males and females differ substantially in their morphology, physiology, and/or behaviour (Cox and Calsbeek, 2009; Fairbairn and Blanckenhorn, 2007). While such sexual dimorphism is commonly assumed to result from divergent selective pressures between the sexes, the fact that both sexes largely share the same underlying genome may substantially constrain such divergent selection (Lande, 1980; Rice and Chippindale, 2001). Indeed, the absence of sex-limited expression may lead to intralocus sexual conflict, in which evolution towards a selective optimum in one sex may cause a trait to diverge from its optimum in the other sex too (Bonduriansky and Chenoweth, 2009; Van Doorn, 2009).

Steroid hormones have been found to be important mediators of sexual dimorphism (e.g., Mank, 2007; Mills et al., 2012; Møller et al., 2005; Williams and Carroll, 2009). Testosterone (T) is a steroid hormone that is involved in multiple reproductive behaviours in males. At the start of the breeding season, endogenous T levels increase in many seasonal breeding birds (reviewed in Ketterson et al., 2005; Wingfield et al., 1990), which coincides with increased territorial aggression (Wingfield et al., 1990) and mating behaviour (Garamszegi et al., 2005). When T is experimentally elevated in males, they show increased aggression (Wingfield et al., 1990), increased attractivity to females (Enstrom et al., 1997), and increased numbers of extra pair fertilizations (Raouf et al., 1997). In contrast, parental care (Hegner and Wingfield, 1987), immunocompetence (Boonekamp et al., 2008; Duffy et al., 2000; Roberts et al., 2004), and survival (Dufty, 1989; Moss et al., 1994; Reed et al., 2006) have been shown to be reduced after experimentally elevating T levels.

In comparison to males, female vertebrates typically have much lower levels of T (Ketterson et al., 2005; Mank, 2007; Wingfield et al., 2001). Despite these lower levels, however, many females endure a similar shift in T at the onset of the breeding season as in males (Ketterson et al., 2005). So far, it remains unclear why females show increased levels of T near the start of the breeding season. Intralocus sexual conflict may be one potential explanation, where the currently observed T levels in females reflect a non-adaptive by-product of selection for high T levels in males (Clotfelter et al., 2004; Ketterson et al., 2005; Møller et al., 2005). Alternatively, T levels in females might be adaptive and therefore result from direct natural selection on females (Cain and Ketterson, 2012). For example, T might increase aggressive behaviour in females towards expected rivals, and, as a consequence, result into more paternal care from her mate. One method to discriminate between these two possible explanations for seasonal shifts in female T levels is by experimentally manipulating individual T hormone levels (phenotypic engineering), and subsequently comparing the fitness of altered phenotypes with that of unmanipulated (control) individuals. If experimental individuals show a higher fitness than control individuals, then one might assume that current T levels in females are not adaptive. However, if both

groups show similar fitness, then it is unlikely that selection is currently acting on the altered phenotypes (Ketterson et al., 1996).

Several studies have experimentally manipulated T levels in female songbirds. They showed that an increase in T enhances territorial aggression (Sandell, 2007; Searcy, 1988) while T decreases incubation behaviour (de Jong et al. **Chapter 5**), clutch size (Lopez-Rull and Gil, 2009; Rutkowska et al., 2005), nestling brooding behaviour (O'Neal et al., 2008), and lower hatching and fledging success (Gerlach and Ketterson, 2013; Lopez-Rull and Gil, 2009; Veiga and Polo, 2008, de Jong et al. **Chapter 5**). While the above studies are informative about putative effects of T on behaviour, it is less clear whether they allow to draw conclusions on extant selection pressures in current populations. This is because many of these studies increased T for longer periods than occur naturally, i.e. levels of T in females typically decrease after the onset of egg laying and tend to remain low throughout the rest of the year (Gerlach and Ketterson, 2013; O'Neal et al., 2008; Searcy, 1988; Veiga and Polo, 2008). Second, implants often resulted in T levels beyond the typical physiological range found in free-ranging females of the species under study (Rutkowska et al., 2005; Searcy, 1988), which hampers straightforward evolutionary conclusions. Third, to our knowledge, no study simultaneously assessed effects of experimental increases and decreases of T, which is required when aiming to discriminate between effects of deviations from natural levels of T (to test the adaptive hypothesis) and effects of increased T levels *per se*.

In this study, we describe the results of an experiment in which we both increased and decreased T in free-living female blue tits (*Cyanistes caeruleus*) between the start of nest-building and the onset of egg-laying, i.e. during the period when T is naturally increased in free-ranging individuals. Our experimental design comprised three treatment groups. (i) females implanted with crystalline T that resulted in increased testosterone levels within the physiological range of the species (further referred to as T females; see de Jong et al. **Chapter 2**), (ii) females implanted with the androgen receptor blocker Flutamide that is assumed to decrease the effectiveness of T (Flu females), and (iii) females that received empty control implants (C females). We expected the fitness of C females to be higher than of Flu and T females if current T levels are the result of direct and continuing stabilizing selection on females (figure 4.1A). Alternatively, we expected the fitness of T and C females to be lower than of Flu females if current T levels in females are constrained by a genetic correlation between the sexes (figure 4.1B). To test these predictions, we compared reproductive effort and survival during two consecutive years among females from the three experimental groups.

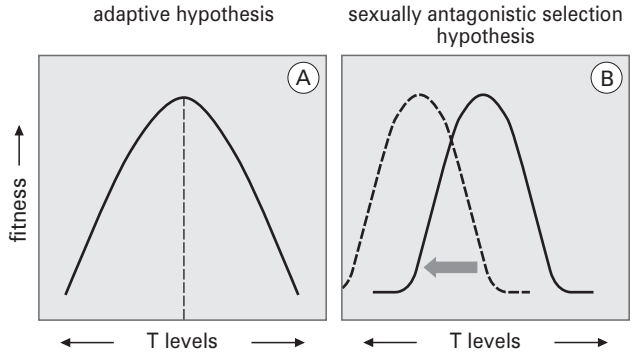


Figure 4.1. Two potential explanations for the effects of different testosterone levels on female fitness A) Under the "adaptive hypothesis" the optimum level of testosterone (dashed line) is the current testosterone level found in females. Any deviation from this optimum will lead to a decrease in fitness. B) Under the sexually antagonistic selection hypothesis, females with lower testosterone levels than under natural conditions will have a higher fitness.

Materials and Methods

Study area and population

The blue tit is a socially monogamous songbird that breeds throughout subarctic Europe and western Asia (Cramp and Perrins, 1993a). Only females build a nest, incubate the eggs, and brood the young, but both sexes defend the territory and feed the young. This study took place at "De Vosbergen" estate in the north east of the Netherlands (53° 08' N, 06° 35' E) between mid-March and mid June 2010, 2011 and 2012. The study area consists of a 54 ha mixed forest which comprises a total of 188 blue tit nest-boxes. The blue tit population has been monitored since 2001 (Korsten, 2006) and since then all nestlings were individually marked with metal rings with a unique code. In addition, adult birds received three colour rings for visual identification. All procedures used in this study were approved by the animal welfare committee of the University of Groningen.

Experimental treatment

In 2010 and 2011 females were captured at the beginning of the breeding season during nest building (in 2010: 25th of March – 13th of April; in 2011: 23rd of March - 13th of April). The capture and implantation procedure have been described elsewhere (de Jong et al., **Chapter 2**). In brief, a total of 117 females (59 in 2010 and 58 in 2011) were either caught during the day in their nest box with a flap trap or at night while roosting in their nest box. After capture, each female was transported to the field station where she was weighted, and tarsus and third primary feather (P3)

lengths were measured. Age of the birds was determined based on the colour of the wing feathers for first year birds (Svensson, 1992), or from ringing data for birds older than one year. Age was determined for 108 out of 117 individuals (average age 2010: mean ± SEM: 1.44 ± 0.09; average age 2011: 1.45 ± 0.16). Subsequently, a blood sample was taken from the wing vein to measure baseline T concentrations. Subsequent to blood sampling, each female received one implant which was placed subcutaneously along the left flank under local anesthesia (Xylocaine 10% spray). The T and control-implants were 4.4 mm long (inner diameter (id) 0.5; outer diameter (od) 1.0) and filled with crystalline T or left empty, respectively. The flutamide implants were 7.2 mm long (id 1.47; od 1.96) and filled with crystalized flutamide (Sigma©). Next, the small wound was sutured with tissue glue (Histoacryl, Braun, Germany). A total of 41 females received a testosterone implant (2010: $n = 21$; 2011, $n = 20$), 35 females received a control implant (2010, $n = 18$; 2011, $n = 17$) and 41 females received a flutamide implant (2010: $n = 20$; 2011: $n = 21$). All females were released in their territory within 30 minutes. Females were recaptured after the second egg was laid to remove the implant, and a second blood sample was taken to measure effects of implants on plasma T concentrations. A previous study showed that the T implants increased T levels within the physiological range of the species, and extended the peak testosterone levels (de Jong et al. **Chapter 2**). Briefly, the observed natural physiological range of T lies between 0.2–7.6 ng/ml. T implantation resulted in an increase of T plasma levels between 1.3–5.9 ng/ml. While our flutamide treatment did not affect T plasma levels (de Jong et al. **Chapter 2**), it may still have a biological effect through inhibit androgen uptake by binding to testosterone and dihydrotestosterone intracellular receptors, thereby blocking the effects these androgens have on behaviour and physiology (Nelson, 2011; Scanes, 2000; Sperry et al., 2010). Other studies on flutamide in birds also did not find an effect of Flu on T levels (Kriner and Schwabl, 1991; Searcy and Wingfield, 1980; Sperry et al., 2010).

Nest monitoring, reproductive success and survival

After the females were captured for implantation, all nest boxes were checked on a daily basis to determine onset of egg laying. After females started egg laying and were recaptured, nest checks were halted until the end of the incubation phase to determine clutch size, hatching date and brood size. To measure future reproductive success, nestling weight was determined to the nearest 0.1 g using a spring balance, and nestling tarsus length was measured to the nearest 0.1 mm 15 days after hatching. Previous studies have shown that nestling weight in great and blue tits is positively correlated with survival (Nur, 1984; Tinbergen and Boerlijst, 1990). Near the end of the nestling phase, daily nest checks were recommenced to determine total numbers of fledged young. Female survival to the next breeding season was determined to assess long-term effects of our experimental manipulation.

Table 4.1. Breeding parameters and nestling characteristics of testosterone (T) and flutamide (Flu) treatments compared to control (C) females. Sample sizes are between brackets.

	2010						2011					
	T nests		C nests		Flu nests		T nests		C nests		Flu nests	
	Mean/ median	SEM / Quartile range	Mean/ median	SEM / Quartile range	Mean/ median	SEM / Quartile range	Mean/ median	SEM / Quartile range	Mean/ median	SEM / Quartile range	Mean/ median	SEM / Quartile range
Clutch size	12.0 (13)	11-13	12 (12)	10-12	11.0 (16)	10.5-12.5	10.0 (15)	8-11	9.0 (11)	8-11	11.0 (17)	10-12
Brood size	8.0 (13)	5-10	7.5 (12)	(3-10)	8.0 (16)	7-10	9.0 (15)	6-9	9.0 (11)	6-10	9.0 (17)	9-11
Fledgling number	6.54 (13)	– 1.18	5.25 (12)	– 1.11	6.67 (15)	– 0.73	8.0 (15)	5-9	8.0 (11)	7-10	9.0 (17)	6-10
Body mass nestlings	16.48 (90)	– 0.07	16.43 (71)	– 0.07	16.45 (112)	– 0.06	16.67 (107)	– 0.05	16.76 (101)	– 0.06	16.71 (142)	– 0.04
Tarsus nestlings	11.22 (90)	– 0.09	11.35 (71)	– 0.11	11.25 (112)	– 0.08	11.39 (107)	– 0.07	11.42 (101)	– 0.09	11.47 (142)	– 0.06

Sample sizes

Only individuals that had been recaptured to remove implants were included in further analysis. Additionally, we excluded two females who died during recapture (one in 2010 and one in 2011) and one female who was injured in 2011. Resulting sample sizes per treatment for survival analysis during the subsequent year were as follows: 2010: T females $n = 13$, Flu females $n = 16$, C females $n = 13$; 2011: T females $n = 16$, Flu females $n = 18$, C females $n = 11$. For analysis of breeding parameters, one Flu female from 2010 was excluded because two of her nestling died by unnatural causes, while one T female and one Flu female from 2011 were excluded because they did not continue with egg laying after recapture.

Statistical analyses

A general linear model (GLM) was used to analyse variation in clutch size, brood size and fledgling number. These breeding parameters were not normally distributed, however, the residuals of the model showed a normal distribution. The linear model included treatment, year and treatment*year as explanatory variables. When analysing variation in clutch size, the date of onset of egg laying was included as covariate to correct for a decline in clutch size with laying date regardless of treatment (de Jong et al. **Chapter 2**). When analysing variation in brood size and fledgling number, hatching date was included, since brood size and fledgling number decline with the progress of the season (brood size: $r^2 = 0.08$, $P = 0.01$; fledgling number: $r^2 = 0.11$, $P = 0.003$). To test whether nestling characteristics (body mass, tarsus length) were affected by maternal hormone treatment, a linear mixed model (LMM) was used. Since body mass and tarsus length were not normally distributed they were transformed with a square-root transformation ((maximum value + 1) – x). Factor nest was included as a random effect to account for non-independence of nestlings within nests. To correct for seasonal effects, clutch size, date and time of measurement, and number of nestlings per nest were included as covariates in all analyses on nestling characteristics. To analyse variation in female survival, a generalized linear mixed model (GLIMMIX) with a binary distribution (died 0, survived 1) and a logit link function was fitted, including treatment, year and treatment*year as fixed factors. In addition, age was included as covariate. To estimate the minimal sample size per group required to find a significant difference in survival, a χ^2-test for proportions was used in the program G*Power (version 3.1.6). The effect size (ϖ) was calculated from the difference in observed number of survived females and the expected number of survived females. We expected the survival to be equal for all groups. The effect size (ϖ) used was 0.17, with $\alpha = 0.05$, $\beta = 0.80$ and $df = 2$. All other analyses were performed in SAS (SAS ® 9.2). Means are presented ± SEM or median ± quartile range. Tests were two tailed and differences were considered to be significant with a P-value < 0.05.

Results

Reproductive success

There was no effect of hormone treatment on mean clutch size, although mean clutch size differed between years (treatment: $F_{2,82} = 0.59$, $P = 0.56$; year: $F_{1,82} = 9.91$, $P = 0.002$; Table 4.1). Overall, females laid fewer eggs in 2011 compared to 2010 (see table 4.1) and this decrease in clutch size was the same for all females (i.e., no significant treatment*year interaction, $F_{2,82} = 1.52$, $P = 0.23$). Additionally, females that started egg laying later in the season had smaller clutches ($F_{1,78} = 10.06$, $P = 0.002$). There was no significant effect of treatment on brood size (treatment: $F_{2,79} = 1.02$, $P = 0.36$; correcting for hatching date $F_{1,79} = 4.77$, $P = 0.03$), average brood size did not differ between years ($F_{1,79} = 0.67$, $P = 0.42$; table 4.1), and there was no interaction between treatment and year ($F_{2,79} = 0.21$, $P = 0.81$). The number of fledglings did not significantly vary among treatments or year, nor was there a significant treatment*year interaction (treatment: $F_{2,78} = 0.22$, $P = 0.80$; year: $F_{1,78} = 1.19$, $P = 0.28$; treatment*year: $F_{2,78} = 0.46$, $P = 0.63$, correcting for hatching date: $F_{1,78} = 8.09$, $P = 0.006$; table 4.1). Nestling weight and tarsus length was not affected by the treatment of the mother (see table 4.2). In summary, neither increased levels of testosterone nor a decrease in T effectiveness significantly affected any measured parameter of reproductive success.

Table 4.2. Effects of the treatment of the mother (testosterone, flutamide or control) on several characteristics of their nestlings. The analyses used backwards elimination and values of non-significant predictors are given before they were removed from the model.

	2010			2011		
	F	DF	*P*	F	DF	*P*
Body mass						
Treatment	1.72	2,216	0.18	1.52	2,342	0.22
Date	0.98	1,219	0.32	1.27	1,341	0.26
Time	4.32	1,226	0.04	30.69	1,342	<0.0001
Brood size	7.10	1,224	0.008	18.74	1,342	<0.0001
Tarsus						
Treatment	0.29	2,267	0.75	1.38	2,343	0.25
Date	0.63	1,267	0.43	0.09	1,342	0.77
Time	2.06	1,267	0.15	0.00	1,341	0.96
Brood size	1.34	1,267	0.25	5.32	1,343	0.02

Survival

Survival of breeding females did not differ between treatments ($F_{2,74} = 1.29$, $P = 0.28$) or years ($F_{1,74} = 1.62$, $P = 0.21$), and the two-factor interaction was also not significant ($F_{2,72} = 1.95$, $P = 0.15$, see table 4.3). Survival of females was also not related to their age ($F_{1,74} = 0.16$, $P = 0.69$). When pooling both years, there was also no effect of treatment on survival ($F_{2,84} = 1.20$, $P = 0.31$). Overall, the survival of Flu females tended to be lower compared to T and C females, however this trend did not reach statistical significance (see table 4.3). Long term survival of females implanted in 2010 did not differ between treatments ($F_{2,38} = 0.26$, $P = 0.77$, see table 4.4), however, older females survival less well ($F_{1,38} = 5.07$, $P = 0.03$). Power analyses revealed that a sample size of at least 345 individuals would be required to detect a significant difference in survival among treatment groups (critical $\chi^2 = 5.99$, total sample size = 345, actual power = 0.80). In short, neither increased levels of testosterone nor a decrease in T effectiveness significantly affected survival, which might have been caused by low sample sizes.

Table 4.3. Absolute numbers of resighted females one year after implantation. A female was counted as resighted when she was breeding in the population the next year.

	Implanted 2010	Breeding in 2011	Implanted 2011	Breeding in 2012	Total implanted	Total resighted
Testosterone	13	6 (46%)	16	8 (50%)	29	14 (48%)
Control	13	7 (54%)	11	3 (27%)	24	10 (42%)
Flutamide	16	7 (44%)	18	3 (17%)	34	10 (29%)
Total	42	20 (48%)	45	14 (31%)	87	34 (39%)

Note. Excluding all the birds that had not been recaptured, of whom implants were not removed, died of unnatural cause or were injured during recapture.

Table 4.4. Absolute numbers of surviving females that were implanted in 2010 and survived to 2011 and 2012.

	Implanted in 2010*	Breeding in 2011	Breeding in 2012	Resighted in 2012 of females implanted in 2010
Testosterone	13	6	3 (50%)	23%
Control	13	7	3 (43%)	23%
Flutamide	16	7	3 (43%)	19%

Note. Excluding all the birds that had not been: recaptured, implant was not removed, or died of unnatural cause.

Discussion

In this study we addressed the question whether temporal fluctuations in T levels in female blue tits are the result of direct selection or of sexually antagonistic coevolution. We show that nor temporally elevated T levels within the physiological range of the study species, neither reduced T effectiveness, affected current and future reproductive success compared to control females. Survival of blue tit females did not differ between treatments either. To our knowledge, this the first study that simultaneously investigated the effect of short term elevated T levels within the physiological range, and reduced T effectiveness, on female reproductive success and survival.

In absence of any significant effect of testosterone on reproductive success, we conclude that neither direct selection, nor intralocus sexual conflict was prominently present in our study population. In contrast, other studies did find an effect of experimentally elevated T in females on several components of reproductive success (see introduction). There are several alternative explanations why our treatment did not affect reproductive success and survival. Firstly, the sample size used in our study might have been too small. This was supported by a formal power analysis and may have hampered the detectability of any treatment effects on survival. Nevertheless, the number of females used in our study was comparable to other studies that did report significant effects of T on survival in males (Dufty, 1989; Moss et al., 1994). Secondly, females were only treated with flutamide or testosterone for a short period of time. Two studies that experimentally elevated T levels for a longer period showed a negative effect of T on reproductive success during subsequent years after the manipulation, but not on survival (Gerlach and Ketterson, 2013; Veiga and Polo, 2008). In males, prolonged experimentally elevated T levels did reduce winter survival (Dufty, 1989; Moss et al., 1994; Reed et al., 2006). Yet, winter mortality was not affected in male blue tits that were exposed to experimentally elevated T within their natural time frame (Foerster and Kempenaers, 2005). Thus, the length of the experimental increase in T appears to have a strong influence on potential survival effects, at least in males. Thirdly, there was a non-significant trend in differential survival between both study years that may have masked effects of treatment on survival.

Intralocus sexual conflict or direct selection

Although we did not find a statistical difference in survival among the treatment groups, survival tended to be lower in Flu females compared to the other two groups, especially between 2011 and 2012. Since the survival of Flue females was also lower than the long-term average survival in our population (30–50%, Korsten, 2006), it appears that blocking the androgen receptors for a short period when T is naturally elevated might have long term effects on survival. If such pattern might indeed emerge when applying larger sample sizes (see earlier), then it is unlikely that current

T levels in females would result from intralocus sexual conflict as Flu females did not have a higher fitness than T or C females. However, since C females did not show a higher survival than T or Flu females in our study, we cannot conclude that current T levels in female blue tits are adaptive and any conclusion on underlying evolutionary drivers hence remains highly speculative. As such, there is a strong need for further studies on the effects of short term elevated T during periods when such levels are also increased in unmanipulated individuals, and of reduced T effectiveness on reproductive success and survival of females over the course of multiple years. Clearly, possible mechanism underlying direct or indirect fitness effects of T and flutamide need to be understood before strong conclusion on the evolutionary drivers of T in female birds can be drawn.

Acknowledgements

We thank P. Korsten, O. Vedder, E. Schut, P. Wolfs, C. Kuipers and T. Egberts for field assistance. We thank Bram Kuijper for commenting on earlier versions of this paper. Funding from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen) was provided to BdJ.

CHAPTER 5

Effects of experimentally sustained elevated testosterone on incubation behaviour and reproductive success in female great tits (*Parus major*)

Berber de Jong, Luc Lens, M. Amini Nasab, Kees van Oers,
Veerle M. Darras, Marcel Eens, Rianne Pinxten, Jan Komdeur & Ton Groothuis

Abstract
In many seasonal breeding birds, female and male testosterone (T) levels peak at the start of their breeding season, coinciding with pair bonding and nesting activities. At the onset of egg laying, T levels decline to baseline levels and remain low throughout the rest of the breeding season. Nevertheless, males in some species remain sensitive to experimentally increased T during this period, whereas in other species this is not the case. It has been postulated that when bi-parental care is essential for the survival of the offspring, males become insensitive to increased T levels during periods of intensive parental care as T would otherwise inhibit paternal care. This may be the case for females too. Alternatively, females may not have evolved a mechanism to become insensitive to T during the period of maternal care, because natural T levels are not elevated during this part of the breeding season in this sex as prolonged elevated T might only be beneficial for males. We tested these alternatives in female great tits (*Parus major*) in which males have been demonstrated to become insensitive to T after egg laying. We experimentally elevated T levels from the start of the breeding season up to the nestling feeding phase and measured parental behaviour and reproductive success. T did not significantly affect nest building or egg laying behaviour, although egg laying tended to be delayed in T females. T lowered mean incubation temperature and hatching success, which were positively correlated. T also reduced brood size and fledgling number. This apparent discrepancy in sensitivity to T after egg laying between male and female great tits suggests that this trait evolved through sex specific evolutionary pathways.

Introduction

In many seasonal breeding birds, female testosterone (T) levels rise, as in males, at the start of their breeding season and decline when females start egg laying (Ketterson et al., 2005). Although circulating T concentrations in females return to low levels after the onset of egg laying, many females remain responsive to an increase in T levels later in the season (Ketterson et al., 2005). Even though the adaptive significance of elevated T at the beginning of the breeding season in females is still not completely clear, there is some evidence that the seasonal peak in T levels might be beneficial for females. For example, early peak T levels are linked to female aggression (Sandell, 2007; Searcy, 1988); de Jong et al. **Chapter 2**), that can secure male care by outcompeting rivalling females (Langmore et al., 2002; Sandell, 2007) and correlate with increased reproductive success (Cain and Ketterson, 2012).

Although peaking T levels at the onset of breeding can thus be beneficial for females, elevated levels for a longer time period have been associated with costs that could limit female reproductive success. For example, prolonged experimentally elevated T levels were shown to delay the onset of egg laying (Clotfelter et al., 2004a; Rutkowska et al., 2005a; Searcy, 1988), reduce brooding of nestlings (O'Neal et al., 2008), and decrease the number of hatchlings and fledglings (Lopez-Rull and Gil, 2009; Veiga and Polo, 2008). In other species, however, prolonged experimentally elevated T levels did not affect the onset of egg laying (de Jong et al., **Chapter 2**), and incubation behaviour (Sandell et al., unpublished manuscript cited in Ketterson et al., 2005). Thus female parental care of many, but not all, bird species appears to remain sensitive to elevated T levels after egg laying, despite having inverse effects on fitness.

In many species male birds also remain sensitive to elevated T levels later in the breeding season. But in contrast to females, males' sustained sensitivity to increased T levels might be beneficial for their reproductive success by enhancing the possibility for extra pair fertilization and territorial defence. For example, in several bird species, experimentally elevated T later in the breeding season increased male singing behaviour to attract additional mates (De Ridder et al., 2000), increased courtship behaviour (Edler et al., 2011; Hegner and Wingfield, 1987), extra pair fertilization (Raouf et al., 1997) and attractiveness to females (Enstrom et al., 1997). However, in many species it also suppresses incubation behaviour (Alonso-Alvarez, 2001; Oring et al., 1989) and nestling feeding (Hegner and Wingfield, 1987). In general, sustained elevated levels of T appear to enhance sexual behaviour but reduce parental care in male birds (Adkins-Regan, 2005). In some bird species, however, males do not react to T manipulation after egg laying with a reduction of parental behaviour, perhaps to avoid detrimental effects on parental care. The differences in male sensitivity in parental behaviour to T after egg laying may be explained by the behavioural insensitive hypothesis (Lynn et al., 2002 & Lynn, 2008). This hypotheses assumes that in

species where bi-parental care is essential for the survival of offspring, males in these species may become insensitive to T during the period of increased paternal care (Lynn et al., 2002; Van Duyse et al., 2000). As opposed to males, potential benefits of elevated T during maternal care is less clear for females, as in most species they do not strive for more fertilizations by courting and competition during this stage. Therefore, an alternative for the behavioural insensitive hypothesis, in particular for females, is that species (or females) that are sensitive for T during parental care did not evolve insensitivity to T because they normally do not elevate T production after egg laying and therefore lack evolutionary selection pressures related to the costs of elevated T on parental care.

In this study we examine the effects of sustained experimentally elevated T on incubation behaviour and reproductive success in female great tits. The great tit (*Parus major*) is one of the few species in which elevated T levels in males do not suppress paternal care measured as food provisioning rate (Van Duyse et al., 2000). In support of the behavioural insensitive hypothesis, the great tit is a socially monogamous species with bi-parental care, which is essential for the survival of the offspring (Bjorklund and Westman, 1986). Only females build the nest and incubate the eggs, but both parents provide food to their nestlings. So far it remains unknown if female great tits are, like males, sensitive to elevated T levels later in their breeding season. By comparing reproductive behaviour and reproductive success between females treated with T or with empty implants (controls), we tested to what extent great tit females remain sensitive to T late in the breeding season. Since, the effectiveness and pattern of incubation might be influenced by T and affect hatching success, we measured nest temperature during incubation too. We hypothesize that on the one hand no difference between the treatments would indicate that great tit females are insensitive to prolonged elevated T levels. On the other hand, a reduction in maternal care or reproductive success would show that great tit females did not develop a mechanism to become insensitive to prolonged T levels. Based on the low levels of T in female great tits after the start of egg laying (Rost, 1990), and the lack of reason why elevated T after egg laying would enhance female reproductive success, we expected no decline in female T sensitivity in parental care, in contrast to the situation in males.

Materials & Methods

Study area and study species

The study was conducted in a nest box population of great tits near the city of Antwerp, Belgium (51° 10'N, 4° 17'E), during the spring of 2009. The study area consists of a park area with deciduous forest containing 58 nest-boxes. Great tits in this population produced an average clutch size of nine eggs (SE ± 0.44) and one

clutch per season. Full day incubation starts after clutch completion and lasts on average thirteen days, although great tits already incubate their eggs for short periods at night before clutch completion (Gosler, 1993). From early March the nest boxes were checked every other day to determine the onset of nest building. As soon as females had started nest building they were captured at night (in nest boxes) or during the day (in food-baited potter traps). At capture, all great tits were sexed (following Svensson, 1984) and received a metal ring with a unique combination of numbers and three colour rings for individual identification. Also, the age (first year or older) of the birds was determined based on the colour of the wing feather (following Cramp and Perrins, 1993).

Once females were captured for implantation, daily nest checking was continued to determine the continuation of nest building activity, the onset of egg laying and clutch size. After females started incubating, nest were checked every second day to determine the continuation of incubation. Two days before hatching, nests were checked every day to score hatching date and brood size. The number of hatched eggs was determined on day 3 after hatching. Since hatched chicks that die soon after hatching are often removed by the female, in contrast to unhatched eggs (pers. com. J.M.Tinbergen) the total number of unhatched eggs was determined by subtracting the number of chicks present at day 3 from the total clutch size. Brood size was determined on day 6. When nestlings were 10 days old they were ringed with a metal ring and their body mass was measured to the nearest 0.1g using a digital balance. When nestlings were 15 days old, their body mass was measured again and their tarsus length was measured to the nearest 0.1mm using a calliper. Near the end of the nestling phase (when nestlings were ca. 17 days of age), daily nest checks were recommenced to determine the fledging date and number of fledged young.

Implantation procedure

Females were assigned randomly to a control or a T treatment group, thereby taking into account the nest building stage of the female (i.e. roughly equal number of females with the same nest building stage per treatment). Four nest building stages were distinguished: small parts of nesting material present (stage 1), a solid layer of nesting material present (stage 2), a nest cup present but not yet lined with hairs and feathers (stage 3), and a completed nest lined with hair and feathers (stage 4). A total of nine control birds and 12 testosterone birds were implanted (details see below). The average nest stage at the time of implantation (2; 1–2.5) was not significantly different between the treatments (Mann-Whitney U-test (1) = 48.5, Z = 0.40; $P = 0.70$). Just prior to implantation, females were weighed and their tarsus length and third primary feather (P3) were measured to the nearest 0.1 mm with a ruler. There was no difference in body mass, tarsus length or P3 length between control and T groups prior to implantation (all $P > 0.48$). All females of which the age was determined ($n = 14$) were scored as first year birds, therefore age was not included in the analyses.

The females were implanted with a 6-mm long silastic tube (Degania silicone; i.d. 0.762 mm, o.d. 1.651 mm), which was sealed at both ends with silastic glue (Dow corning). The implant was inserted subcutaneously along the left flank under local anaesthesia (Xylocaine, 10% spray). After implantation the small incision was sutured with tissue glue (1 × 0.5 ml Histoacryl, Braun, Germany). The testosterone implants were filled with 0.6mg ± $1.5*10^{-5}$ mg crystalline testosterone (Fluka) over a length of 2 mm. Control females received empty implants. Females were implanted between 16th of March and the 12th of April 2009. There was no difference in implantation date between the two treatments (independent *t*-test (19) = –1.00, $P = 0.33$). Two days after implantation one control bird was found dead. Additionally one control bird and five testosterone birds were not found breeding in one of our nest boxes, resulting in a total of seven control and seven T females breeding in our experiment. After implantation, two of the seven control females and four of the seven T females moved to a different nest box. The difference in dispersal probability between the two treatments was not significant ($\chi^2 = 1.17$, df = 1, $P = 0.28$).

Incubation measurements

As soon as a female was observed incubating, her nest attentiveness and nest temperature was measured. The onset of incubation was determined when a female was found incubating on her eggs or when the eggs were found uncovered and warm. Nest attentiveness was measured with a data logger (HOBO logger, Mulder-Hardenberg BV., The Netherlands).The data logger registered the temperature inside the nest box via a sensor that was positioned in the middle of the nest. To place the sensor the eggs were removed and a small hole was drilled in the bottom of the nest box. Through this hole a sensor was mounted in the middle of the nest cup. The logger was stored in a small green plastic box, which was taped to the lid of the nest box on the outside (picture 5.1A). After mounting the sensors, the eggs were placed back into the nest box, around the sensor (picture 5.1B). The sensor did not extend above the eggs. Drilling and placing the equipment did not take more than 10 min. The incubation temperature was registered every 15 s for an average period of 9967 ± 688 min., which did not significantly differed between the treatments (independent *t*-test (11) = –0.04, $P = 0.96$). To verify if the temperature fluctuation recorded by the data logger coincided with an on-bout or recess of the incubating females, video observations were made of five nest boxes. A camera was placed approximately 5 m away from the nest box. The entrance of the nest box was filmed to record when a female entered or left the nest box and when a male entered the nest box to feed the female. Before the start of the video recording, each nest box was checked to see if the female was on the nest. In total 264 min of video observations were made. The on-bouts and recesses recorded during the video observations were compared to graphs of the temperature data plotted against time per female. A recess in incubation during the video observations corresponded to a sharp decline in temperature in

Picture 5.1. Nest box with box attached to it. Inside the box was the data logger that recorded incubation temperature. B) Great tit clutch with a sensor placed in between the eggs (see arrow).

these graphs, and for the on-bouts the pattern was vice versa. Once the temperature data was verified a selection criteria for the on-bouts and recesses of incubation was developed on the bases of the patterns of the temperature graphs. The criteria were as followed; a sharp decline in temperature of more than 1.3 degrees/min for at least 4 min was considered as a recess of incubation. An increase in temperature with an initial slope of at least 0.2 degrees/min and a maximum slope of at least 1.0 degrees/min was considered the start of an on-bout. These criteria were used to automatically select the on-bouts and recesses of incubation in the program Rhythm 1.0 (Cooper and Mills, 2005). Since the video recordings concerned a subsample of all nests, we subsequently visually inspected with the program Raven Pro (1.4) the selections made by Rhythm and manually corrected by adjusting the onset and/or offset of incubation periods and selecting recess periods that had not been selected by Rhythm (for example recesses of incubation that were shorter than 4 min). From the selected data the duration of every on-bout was calculated (the difference between the start and end of the on-bout in min). Also, the duration of every recess and minimum temperature during a recess in °C was calculated, and the mean temperature during the whole day (combining on-bouts and recesses), and mean night temperature were measured. Incubation data from 13 females (n = 6 control and n = 7 T females) were used for subsequent analysis.

Natural hormone concentrations

To examine the natural profile of circulating T levels, female blood was collected just prior to implantation (n = 17) and from seven control birds that were recaptured during the nestling period (between the 2nd and 15th of May 2009). None of the recaptured females had lost their implant. During these captures 50 – 150 μl blood was taken within 30 min after capture by puncturing the wing vein with a sterile needle (Terumo, 27 g × 3/4; 0,4 × 20 mm) and transferred into an Eppendorf tube using heparinized microhematocrit capillaries. The blood was stored on ice and centrifuged for 10 min at 7000 rpm within six hours after sampling. The plasma fraction was removed and stored at –20°C until analysis. Testosterone was quantified in plasma extracts by radioimmunoassay (RIA) using a commercial double antibody system purchased from MP Biomedicals (Solon, Ohio). For extraction, 500 μl of a 50/50 mixture of cyclohexane/ethylacetate was added to 50 μl plasma and the tubes were incubated for 10 min with continuous shaking. After centrifugation, the tubes were placed in a mixture of dry ice and ethanol for snap freezing, followed by transfer of the organic phase to a new tube. After thawing, samples were re-extracted following the same method. The combined supernatants were dried by vacuum centrifugation and stored at –20°C until further analysis. For testosterone measurements, the dried samples were dissolved in 25 μl steroid diluent buffer and further treated following the protocol of the RIA kit. The primary antibody used in this assay does not cross-react significantly with other androgens beside T (5α-dihydrotestosterone: 3.4%; 5α-androstane-3β, 17β-diol: 2.2%; 11-oxo-testosterone: 2%; all other steroids: <1%). Testosterone standards ranged from 0.10 ng/ml to 11.75 ng/ml, but the effective detection limit could be extended to 0.05 ng/ml owing to the concentration effect of the extraction procedure. All samples were measured in a single assay and the intra-assay coefficient of variation was 4.6 – 9.1 % (medium - low/high concentrations).

Hormone implants

To thoroughly examine the effects of the implants on T plasma concentrations a lab experiment was conducted. In this experiment eight female great tits were implanted with T on the 10th of December 2012. The great tits were hand-reared and all of the same age (2 years). Before and during the first 14 days of the experiment they were housed in single-sex groups of eight individuals in free-flight, half-open aviaries (2.0 × 4.0 × 2.5 meters). After 14 days, four of the eight females were housed together with a male in separate aviaries. Birds had ad libitum food and fresh water at all times. For implantation females were captured and transported to an operation room. Before implantation a blood sample was taken, within 10 min after capture, to measure baseline T plasma concentrations. Next the females were implanted with silicone tubes filled with T. The implantation procedure and the implants used were equal to the field experiment (see above). The birds returned to their aviary within

90 min after capture. Seven and 28 days after implantation another blood sample was taken to measure the effects of the implants on T plasma concentrations. All the blood samples were taken around the same time (between 12:00 –14:00 GMT+1). Directly after sampling the blood was centrifuged for 10 min at 6000 rpm, the plasma was removed, and stored in a –20°C freezer. After the last blood sample was taken the implants were removed under local anaesthesia (Xylocaine, 10% spray) by making a small incision below the implant, and the incision was sutured with tissue glue (1 × 0.5 ml Histoacryl, Braun, Germany). During each of the captures the health of the females was checked and they were weighed to the nearest 0.1 g. There was no significant weight change over the course of the experiment (One-way repeated measures ANOVA: $F_{1, 14} = 2.26$, $P = 0.15$).

The plasma samples were analysed in one assay using a commercial kit (Orion Diagnostica, Spectria Testosterone RIA kit, Espoo, Finland) with a sensitivity of 0.04 ng/ml testosterone and cross-reactivities of 4.5 % with DHT and 0.01 % with A4 as described in de Jong et al. (**Chapter 2**). In brief; plasma samples were defrosted, their volume was measured and 50 μl radio-actively labelled testosterone (Perkin Elmer Life and Analytical Science BV) was added to all samples to measure the accuracy of the extraction process (recovery). After an incubation time of 1 hour, 2.5 ml diethyl ether/petroleum benzine (70:30) was added and samples were vortexed and centrifuged. Samples were snap frozen by a mixture of Ethanol and dry ice and decanted. The supernatant was dried under streaming nitrogen, the remaining pellet was again dissolved in 1 ml 70% Methanol and samples were stored over night at –20ºC. The following day, samples were centrifuged, the Methanol phase was decanted and the samples dried again under streaming nitrogen. The pellet was re-suspended in 200 μl PBS buffer. 30 μl of this mixture was used for measuring recoveries (average recovery rate for testosterone: 92.96 ± 0.89%). Hormone concentrations were measured using radio immuno assays (RIAs). Based on the standard curve values below the detection limit were calculated as being 0.10 ng/ml. The dilution curve ran parallel to the standard curve. The intra assay variation was 6.9 %.

Sample size

For the analyses of natural T plasma concentrations seven individuals from the early breeding period were excluded and two from the nestling period because not enough blood was collected for the hormone analyses. The hormone concentrations could be analysed from ten individuals before implantation and five control females after implantation. For the baseline T plasma concentrations analyses of the captive birds only four females were included. The other four females were not included because their blood sample was not at baseline levels anymore when captured (see figure 5.1). For the analyses of the second and third sample one female was excluded because she had lost her implant before the second sample was taken. For the analyses of the different breeding parameter seven controls and seven testosterone

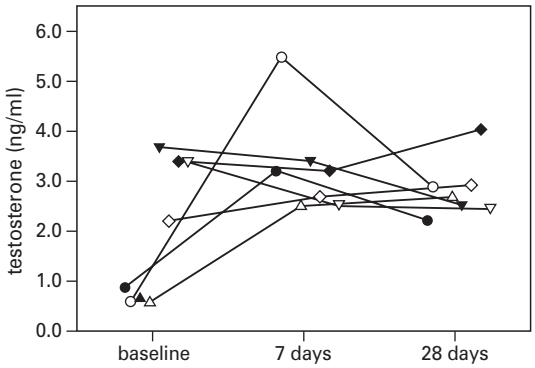


Figure 5.1. The effect of T implants on plasma testosterone of female great tits. Blood samples were taken prior to implantation (baseline sample) and seven and 28 days after implantation.

nests were included unto the onset of egg laying. During the incubation phase, one nest box of a testosterone female was lost due to vandalism. Therefore seven control and six testosterone birds were included in the analyses of the nestling phase.

Statistical analyses

All the data were checked for normality. Data that were not normally distributed (long term T plasma levels, on-bout and recess time, hatching success (%), fledging date, nestling body mass, tarsus and growth) were either transformed (see below) or, when unsuccessful for reaching normality, tested using non-parametric tests. To analyse the T plasma levels before implantation, a Mann-Whitney U-test was used. T plasma levels after implantation were transformed with a Log10 transformation and analysed for the effect of treatment with an independent *t*-test. An independent *t*-test was used to analyse the effect of treatment on female characteristics (body mass, tarsus and wing length), the onset of egg laying and incubation date, clutch size, hatching date, and the number of hatchlings and fledglings. To test whether the T implants had an effect on T plasma levels of the captive females 7 and 28 days after implantation, a one sample Wilcoxon signed rank test was used comparing these levels to the average baseline level and average elevated levels from before implantation. A Wilcoxon signed rank test was used to test if there was a difference in T plasma levels at 7 and 28 days after implantation. The housing condition of the females when the third sample was taken had no effect on the hormone levels (independent *t*-test (5) = 0.85, $P = 0.43$) and thus was not included in the analyses. For the analyses of incubation data we used: the average time (min) a female spent off and on here nest during the day, the number of recesses and the average minimum temperature during incubation recesses, the overall mean incubation temperature during the day, the mean variation in temperature during the day, and mean night

temperature inside the nest. The number of recesses, mean on-bout and recess time, mean minimum temperature, mean day temperature and mean variance in day temperature, and mean and night temperature was analysed with a general linear model (GLM). Treatment was included as a fixed factor and clutch size was included as a covariate in all models, as females with larger clutches spent more time inside their nest boxes ($F_{1, 12} = 8.37$, $P = 0.02$). The mean recess time and time spent incubating were not normally distributed and were transformed with a log10 transformation. Linear regression models were used to quantify the relationship between the average incubation temperatures during the day or during the night and the proportion of hatching success per nest, for each treatment separately (variance in hatching success was not equally distributed between treatments). Hatching success (after arcsine transformation) was calculated as the number of hatchlings divided by clutch size. To test if hatching and fledging success differed between treatment groups, Chi-square goodness of fit tests were conducted to compare the number of unhatched/hatched eggs or died/fledged nestlings, respectively. Fledging success was calculated as the number of fledglings divided by the number of hatchlings.

Nestling body masses and tarsus lengths were transformed with a square root transformation (sqrt ((maximum value +1) – x)) and Log10 transformation (Log10 ((maximum value +1) –x)), respectively. Nestling growth rates were calculated as the difference in body mass between days 10 and 15. The change in body mass was transformed with a square root transformation (sqrt (x+1)). To examine whether the treatment of the mother had an effect on nestling body mass at day 10 and day 15, growth rate (measured as the increase body mass between day 10 and 15) and tarsus length at day 15, a mixed model was used, including treatment as fixed factor, nest box as a random factor (to correct for non-independence of nestlings of the same mother), date of measurement (for body mass), or hatching date (for hatchling growth) and brood size as co-variables.

Tests were two tailed and differences were considered to be significant with a *P*-value <0.05. SAS (SAS® 9.2) was used to analyse the nestling characteristics and incubation data. All the other data were analysed using STATISTICA 7.0 (StatSoft, Inc.). Unless stated otherwise, average values are presented ± SEM while median values are presented ± range.

Results

Hormone concentrations

During the nest building phase, the average natural T plasma concentration was 0.88 ± 0.20 with a range of 0.09–2.67. During the nestling phase, the average natural T concentration was 0.54 ± 0.07 with a range of 0.31–0.67 (see Fig. 5.2). The average baseline T level of the captive female blue tits was 0.69 ± 0.06 with a range of

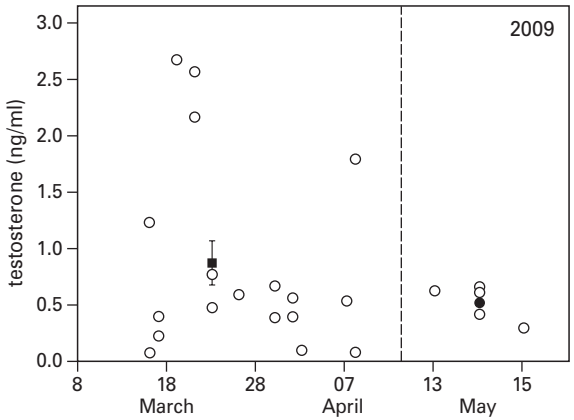


Figure 5.2. Natural testosterone levels during the breeding season in female great tits. Open circles are individual T levels, the square is the average T level pre-breeding and the closed circle is the average T level during the nestling phase, mean ± SEM.

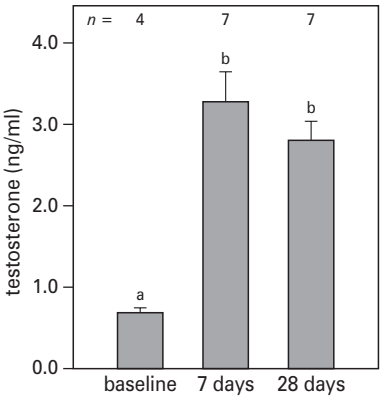


Figure 5.3. Effects of T implants on T plasma concentrations seven and 28 days after implantation compared to baseline T levels. Letters indicate significant difference with $P < 0.05$. Mean ± SEM and sample size are presented.

0.60–0.89 (see Fig. 5.3). The T plasma levels were significantly increased seven (one sample Wilcoxon signed rank (6) = 3.5, $P = 0.016$) and 28 days (one-sample Wilcoxon signed rank (6) = 3.5, $P = 0.016$) after implantation. The mean plasma T value after 7 days was 3.29 ± 0.39 and after 28 days 2.81 ± 0.22. The plasma T levels did not significantly differ between the second and third sample (Wilcoxon signed rank test: $P = 0.47$). Elevated T levels were within the physiological range since these levels did not exceed the average elevated levels from before implantation

(3.15 ± 0.32;Indeed, there was no significant difference between the elevated natural T levels before implantation and seven days (one sample Wilcoxon signed rank (6) = 0.5, P = 1.0; 28 days) and 28 days after implantation(one sample Wilcoxon signed rank (6) = -2.5, P = 0.12, see Fig. 5.1).

Breeding parameters

After females were implanted it took individuals from the T treatment a similar amount of time to complete their nest compared to those from the control group (Table 5.1). T females laid their eggs on average four days later than C females; however this difference just did not reach statistical significance, probably due to a limited sample size. Clutch sizes of T and C females did not differ (Table 5.1).

Incubation behaviour

The mean on-bout duration of females did not differ between the two treatments, nor was there a relationship with clutch size (Table 5.2). There was no difference in the number of recesses between the treatments, nor was this related to clutch size (Table 5.2). Mean recess time did not significantly differ between treatments, neither was it correlated with clutch size. Treatment had a significant effect on incubation tempera-

Table 5.1. Summary of the overall treatment effects on different breeding parameters, nestling characteristics and reproductive output of female great tits. Data are presented as mean ± SEM or as median (quartile range).

	Testosterone		Control		Test	
	Mean/ *Median*	SEM/ *Quartile range*	Mean/ *Median*	SEM/ *Quartile range*	T/U	*P*
Nest building time (days)	9.83	2.24	13.86	2.31	1.24[2]	0.24
Onset of egg laying[1]	43	1.20	39.14	1.61	1.92[2]	0.08
Clutch size	8.71	0.52	10.00	0.82	1.33[2]	0.21
Hatching date[1]	66	1.34	61.00	1.41	2.46[2]	**0.03**
Number of hatchlings	5.83	1.38	9.71	0.87	2.46[2]	**0.03**
Brood size at day 6	4.17	1.42	9.57	0.75	3.44[2]	**0.005**
Number of fledglings per nest	4.00	1.37	9.57	0.75	3.72[2]	**0.003**
Fledge date[1]	*84*	*82—87*	*83*	*74—84*	-1.23[3]	0.22

[1] The onset of egg-laying, hatching date and fledge date were scored in March days, where 1st of March is 1.
[2] Independent *t*-test
[3] Mann-Whitney U test

ture, i.e. both the mean minimum temperature inside the nest box during a recess of incubation (Fig. 5.4A) and the mean day and night temperature (Fig. 5.4B, C) were significantly lower in the T than in the control group. Clutch size did not show a significant relationship with mean minimum temperature, nor was it related to day temperature inside the nest box. Likewise, clutch size was not related to mean night temperature inside the nest box (Table 5.2). Mean variance in day temperature was not affected by treatment, but clutch size did show a significant negative relationship with mean variance in day temperature. Females with large clutches showed less variation in day temperature, perhaps because eggs buffer each others temperature. Mean day and night temperatures were positively correlated with hatching success in T females whereas mean day temperature was negatively correlated with hatching success in C females (Table 5.3).

Reproductive success

The average hatching date for T offspring was significantly later than that of control offspring (Table 5.1). The number of hatchlings (Table 5.1) and hatching success

Table 5.2. Summary of the overall treatment effects on incubation behaviour of female great tits. Data are presented as mean ± SEM or as median (quartile range).

	Testosterone		Control		Test
	Mean/ *Median*	SEM/ *Quartile range*	Mean/ *Median*	SEM/ *Quartile range*	GLM
On-bout time	*20.00*	*18.31-26.45*	*21.13*	*20.62-23.43*	Treatment: $F_{1,12}$, = 0.16, P = 0.70; Clutch size: $F_{1,12}$ = 3.92, P = 0.08
Recess time	*6.44*	*5.23-10.30*	*6.39*	*4.43-19.17*	Treatment: $F_{1,12}$, = 0.12, P = 0.74; Clutch size: P > 0.45
Nr of recesses	185.14	33.52	181.33	21.46	Treatment: $F_{1,12}$, = 0.93, P = 0.36; Clutch size: P > 0.80
Minimum temperature during a recess	23.37	0.69	25.88	0.33	Treatment.: $F_{1,12}$, = 9.67, P = **0.01**; Clutch size: P > 0.20
Day time temperature	28.62	0.79	31.23	0.44	Treatment.: $F_{1,12}$, = 7.59, P = **0.02**; Clutch size: P > 0.70
Night time temperature	31.13	0.94	33.92	0.58	Treatment.: $F_{1,12}$, = 5.93, P = **0.03**; Clutch size: P > 0.90

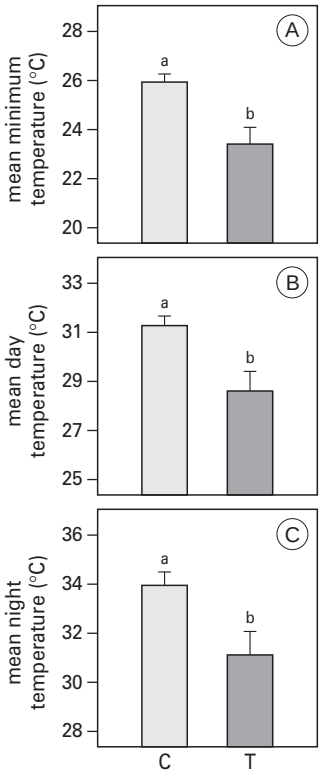


Figure 5.4. A) The mean minimum incubation temperature during the recess of incubation. B) Mean incubation temperature during the day (including progresses and recesses of incubation). C) Mean incubation temperature during the night. Light grey bars are the control females, dark grey bars are the testosterone females. Letters indicate a significant difference with $P < 0.05$. See table 5.2 for specific *P*-values. Means ± SEM are presented.

were significantly lower in the T treated group. A total of 35 out of 52 (64%) eggs of testosterone females hatched compared to 68 out of 70 (97%) control eggs (Fisher's exact test: $P < 0.0001$). After hatching, a higher proportion of nestling of T mothers did not survive until day 6 (10 out of 35) compared to those of C mothers (1 out of 68, Fisher's exact test: $P < 0.0001$). Brood size at day 6 after hatching was also significantly smaller in the treatment group compared to the control group. After day 6 nestling survival was almost equal in both groups, only one T nestling died after day

Table 5.3. Summary of the regression analyses several different measures of incubation temperature and hatching success.

	Testosterone				Control			
	Intercept	Slope	R^2	*P*	Intercept	Slope	R^2	*P*
Day temperature	-6.36	0.25	0.66	**0.048**	5.90	-0.14	0.80	**0.02**
Night temperature	-6.34	0.23	0.67	**0.046**	4.59	-0.09	0.59	0.08

15. But because the number of hatchlings and the number of nestlings surviving to day 6 was lower for T females, the overall fledging success was significantly lower in the T treatment groups (T = 77%, C = 99%; Fisher's exact test: $P < 0.0001$. A total of 24 fledglings from six T nests fledged, compared to 67 fledglings from seven control nests, being statistically significant (Table 5.1). The average fledging date of the nestlings did not differ between treatments.

Nestling characteristics

On day 10, the average body mass of T nestlings (12.76 ± 0.45) was significantly lower than that of control nestlings (13.39 ± 0.22) correcting for brood size (treatment: $F_{1,89} = 8.77$, $P = 0.004$; brood size: $F_{1,89} = 8.81$, $P = 0.004$). Nestlings of larger broods were on average lighter. The date at which 10 day old nestlings were measured did not have a significant effect on their weight (date: $F_{1,88} = 3.18$, $P = 0.08$). There was no difference in weight between 15 day old nestlings of T females (16.62 ± 0.27) and nestlings of control females (16.59 ± 0.18; treatment: $F_{1,88} = 0.26$, $P = 0.61$; date: $F_{1,88} = 4.22$, $P = 0.04$). Brood size had a negative effect on the weight of 15 day old nestlings (brood size: $F_{1,88} = 4.39$, $P = 0.04$), with nestlings of larger broods being lighter. Between day 10 and day 15, T nestlings (3.85 ± 0.28) grew significantly faster than control nestlings (3.20 ± 0.16; treatment: $F_{1,88} = 26.11$, $P < 0.0001$; hatching date: $F_{1,88} = 24.49$, $P < 0.0001$; brood size: $F_{1,88} = 9.42$ $P = 0.003$). Nestling growth was less for the nestlings that hatched later in the breeding season and/or grew up in larger broods. Mean nestling tarsus length did not differ between treatments (T nestlings: 19.61 ± 0.17; C nestlings: 19.48 ± 0.11, treatment: $F_{1,88} = 0.57$, $P = 0.45$) and was not affected by brood size or date of measurement (brood size: $F_{1,88} = 2.41$, $P = 0.12$; date: $F_{1,88} = 3.62$, $P = 0.06$).

Discussion

Since male courtship behaviour is T dependent in many species (Adkins-Regan, 2005), an elevation in T levels may increase their reproductive success even during the period of paternal care as long as other females are fertile and males can perform extra pair copulations (Lynn et al., 2002). However, since T can also suppress parental care males have been demonstrated to be insensitive to T in species were parental care is essential for the survival of the offspring. This has also been demonstrated for our study species, great tits (Van Duyse et al., 2002). In contrast to males, elevated T after the period when it is naturally increased does not seem to have clear fitness advantages for females as they do not seek new partners to perform courtship behaviour. We therefore hypothesized that experimentally elevated T late in the breeding season may affect female parental behaviour as females are generally not exposed to elevated T after egg laying and may therefore not have evolved insensi-

tivity towards it. In line with our hypothesis we found that great tit females had low circulating T levels after the temporary elevation during the pre-egg laying phase, similar to what has been found in other socially monogamous female passerine birds (Ketterson et al., 2005) and that elevation of T after egg laying did affect female parental care.

The hormone implants we used caused a significant increase in T levels, within the physiological range, within a week and levels remained elevated for at least 28 days, until the implants were removed. In the period when T levels were naturally elevated in the free-living great tits, we did not find a significant effect of our T implants on reproduction behaviour, e.g. nest building, onset of egg laying and clutch size. However, in support of our hypothesis experimentally elevated T resulted in a lower incubation temperature, delayed hatching and reduced the number of hatchlings and fledglings. Also nestlings of T females had a lower body mass early in the nestling phase, although they caught up in weight later.

We found strong negative effects of elevated T later in the breeding phase, with T females showing significantly lower incubation temperatures than control females. To our knowledge, no other study has looked at the effect of elevated T in female birds on incubation temperature itself. So far, two other studies looked at the effect of elevated T on incubation behaviour in female. Experimental elevation of T levels did not affect the total time females spent incubating in dark-eyed junco's (Clotfelter et al., 2004), but did decrease incubation temperature in the tree swallow (*Tachycineta bicolor*; Rosvall, 2013). The reduction in incubation temperature that we found may be explained by a less well developed brood patch of T females compared to that of controls, such as observed in dark-eyed junco's (Clotfelter et al., 2004). In less well developed brood patches less blood flows through the veins of the brood patch thereby reducing the maximum temperature a female can reach during incubation (Massaro et al., 2006). In our study it was not possible to measure the size of the brood patch, because we could not recapture the birds during the incubation phase. Female great tits are easily disturbed when taken from their nest when incubating and thus recapturing them would most certainly have resulted in high numbers of nest desertion. But since there was no difference in the time females spent incubating between the two treatment groups, it is likely that the lower nest temperature of T treated females was due to a reduced brood patch development. More studies should investigate whether T influences brood patch development in this and other species as the reduction of the brood patch caused by elevated T levels could be one of the functional explanations why T levels are decreased when females start incubating.

The lower incubation temperature in the nest of T females was inversely correlated with the hatchability of their eggs. It is well known that the egg temperature during incubation is important for the development of the embryo (Webb, 1987). Low incubation temperature can cause mortality of the embryos before hatching (Deeming and Ferguson, 1991), decrease nestling weight (Ardia et al., 2010),

increase nestling mortality (Evans, 1990) and decrease hatching success (Nord and Nilsson, 2011). Therefore the lower hatching success of eggs of T females compared to C females might be causally explained by the low incubation temperature in the former (Suarez et al., 1996). As a consequence, females treated with T produced fewer hatchlings and fledglings and therefore had a lower reproductive success. A reduction in hatchling and/or fledgling rates due to elevated T has been shown in other studies (Lopez-Rull and Gil, 2009; O'Neal et al., 2008; Veiga and Polo, 2008). Surprisingly, in our study control females, with a higher mean incubation temperature during the day had lower hatching success. In fowls (*Gallus gallus*), too low and too high incubation temperature have both been shown to negatively affect embryonic development, indicating that there is an optimum incubation temperature for embryos to develop normally (Romanoff et al., 1938). Since in our study the nests with the lowest and the highest incubation temperature had lower hatching success, this might have been caused by a deviation from the optimum incubation temperature in these nests.

At day 10, T nestlings had lower body masses compared to C nestlings. There are three possible (non-mutually exclusive) explanations why nestling weight was affected by the T treatment. First, eggs laid by T females may have been lighter and therefore produce lighter nestlings. Great tit nestlings that hatched from lighter eggs grow slower during the early period of the hatching phase, but do catch up before fledging (Schifferli, 1973). However, effects of T on egg mass are ambiguous; studies either showed no effect of T on egg mass (Clotfelter et al., 2004; Lopez-Rull and Gil, 2009), or an increase in egg mass when females were treated with T (Rutkowska et al., 2005). A pilot study on the effects of T on egg mass in great tits, however, did show a decrease in egg mass (unpublished manuscript Pinxten). Second, a lower brooding temperature of the mother might have affected the weight of the nestlings. A previous study in dark-eyed junco's (*Junco hyemalis*) showed that females treated with T brooded less on the young (O'Neal et al., 2008). Brooding behaviour of the mother is very important for nestlings because thermoregulation of altricial young is not yet fully developed in the early nestling stage (Dunn, 1975). When nestlings cool down, their call rate increases which induces brooding behaviour of the parents. This vocalization behaviour may be costly and therefore poorly brooded nestlings may have less energy to spend on growth and development. Also, negative effects of T on brooding may explain why the survival was lower of T nestlings during the first 6 days compared to the period of the nestling phase after day 6. After 6 days nestlings become thermoregulatory and therefore they are less dependent on the brooding of the mother. Third, nestling body mass may have been affected by differential food provision rates of T mothers. In the spotless starling (*Sturnus unicolor*) T reduced female feeding rate (Veiga and Polo, 2008), whereas in the dark-eyed junco there was no difference in feeding rate between control and T females (O'Neal et al., 2008). Feeding rate was not observed in this study, but if feeding behaviour of T females was

affected, then this most likely occurred only during the early nestling period since nestling weight did not differ anymore on day 15.

Many bird species show a peak in testosterone levels at the beginning of their breeding season, when females are building their nests. Therefore it was unexpected that we did not find a significant effect of elevated T on nest building behaviour. Only a few other studies have looked at the role of T in nest building behaviour and found inconsistent results. In the European starling, T also did not affect nest building (De Ridder et al., 2002), whereas in the blue tit T females speeded up nest building (de Jong et al. **Chapter 2**). In our study we might not have found a treatment effect on nest building because we did not measure nest building activity as accurate as the previous studies. For example, we did not observe the females while they were nest building nor measured nest weight. However, we have some indication that T did affect nest building behaviour since there was one T female in our study that barely built a nest at all, and the eggs were laid on the bottom of the nest box, which was never observed among control females in this and other experiments (personal observation BdJ).

Overall, our results suggest that great tit females are sensitive to elevated T levels during the period of maternal care when natural T levels are low and that such elevated levels are detrimental to their fitness. Thus our results do not support the behavioural insensitive hypothesis for female great tits. Instead it is more likely that great tit females did not evolve a mechanism to become insensitive to T, because T levels are generally not elevated during this time of the breeding season. Our result are in contradiction to what has been found in male great tits, where males were insensitive to elevated T levels during the period of intense parental care (Van Duyse et al., 2000 & 2002). This sex difference in response to elevated T during the late breeding season suggests that the insensitivity to T has evolved through sex specific evolutionary pathways, showing no indication for sexual antagonistic selection.

Acknowledgments

We thank Peter Scheys and Ann Geens for assisting in the field. This research has been funded by an IWT-Vlaanderen grant to Berber de Jong. The study was conducted in full compliance with Belgian laws and regulations.

CHAPTER 6

General discussion

Berber de Jong

Introduction

Males and females of many species differ substantially in their morphology (e.g. size and colouration), physiology (e.g. hormone profiles), and behaviour (e.g. singing behaviour, Andersson, 1994; Darwin, 1874; Fairbairn and Blanckenhorn, 2007). It is widely thought that this sexual dimorphism primarily reflects adaptation of the sexes to different reproductive strategies. For example, males are generally selected for high mating frequency and females for low mating frequencies (Davies et al., 2012; Trivers, 1972). This difference in reproductive strategies between the sexes can cause sexual morphological differences, for example the antlers of male deer are much larger compared to those of females. In male deer, antlers serve as a weapon in male-male competition for the possession of females (Andersson, 1994), and the male with the largest antlers might be the most powerful competitor and thus the male with the most fertilizations. Nonetheless, it is remarkable that both sexes have developed such striking phenotypic differences, as they have most of their genome in common (barring any sex chromosomes). This begs the question which mechanisms underlie the evolution of these sex differences (Williams and Carroll, 2009) and whether such mechanisms are sufficient to allow either or both sexes to evolve independently, or whether there are inherent constraints in the evolution of sex-specific traits (e.g., Björklund and Senar, 2001; Bonduriansky and Chenoweth, 2009).

In this thesis, I studied the presence of sex differences in a pivotal trait that underlies reproductive behaviour: the steroid hormone testosterone (T), which plays a key role in male reproductive behaviour in many vertebrate species (Adkins-Regan, 2005). In seasonally reproducing males T levels typically increase at the beginning of their breeding season to regulate reproductive processes, such as aggression and mating behaviour (Adkins-Regan, 2005). Females also show a significant increase in T levels at the beginning of the breeding season (Ketterson et al., 2005), and this peak in T levels is correlated with the T peak of males of the same species (Møller et al., 2005). However, the peak in T levels is generally lower in females than in males (Adkins-Regan, 2005; Ketterson et al., 2005; Mank, 2007). In addition, in seasonally breeding birds T levels in males gradually decrease during the breeding season whereas females show a more rapid decrease in T levels around the start of egg laying (Ketterson et al., 2005; Wingfield et al., 1990). Although many studies have shown possible fitness benefits of elevated T levels at the beginning of the breeding season in males (Adkins-Regan, 2005; Wingfield et al., 1990), compared to males little is known about the functional role of the T peak in females.

I studied three possible explanations for why adult females have elevated T levels during early breeding. The first hypothesis focuses on the central tenet that the genomes of both sexes largely overlap, causing many male and female characters to be strongly genetically correlated between both sexes (e.g., Cox and Calsbeek, 2009; Poissant et al., 2010). Consequently, if T levels between males and females are gene-

tically correlated, natural and/or sexual selection on T levels in one sex may result in a corresponding maladaptive response of T levels in the other sex. Hence, this "*sexually antagonistic selection hypothesis*" proposes that the evolution of T is sub-optimal for at least one of the sexes (Clotfelter et al., 2004; Ketterson et al., 2005), where either males would benefit from even higher T levels than currently observed or females from lower T levels, or both. Alternatively, the "*adaptive hypothesis*" considers that the observed profile of female T levels may well be adaptive in females and under direct natural or sexual selection (Cain and Ketterson, 2012), whilst not being constrained by selection on male T levels. Lastly, the third hypothesis considers that the T profile is selectively neutral in females, so that higher or lower levels or a different profile do not influence female fitness. I refer to this hypothesis as the "*neutral hypothesis*".

To test these hypotheses, knowledge about the costs and benefits of the levels and temporal dynamics of circulating T levels in both males and females is essential. In many seasonally reproducing bird species, T levels decline during parental care (Ketterson et al., 2005; Lynn et al., 2002). So far it remains unknown whether the observed seasonal profile in T levels is adaptive in females. In males it has been shown that a prolonged elevation of T after egg laying can increase their reproductive success, for example by increasing their number of extra-pair fertilizations (Raouf et al., 1997). However, sustained sensitivity to elevated T levels later in the season, when these levels normally decrease during nestling feeding, may entail costs, as such elevation has been shown to reduce paternal care in most birds (reviewed in (Lynn et al., 2002 & Lynn, 2008), albeit not in all species (Lynn et al., 2002; Van Duyse et al., 2000 & 2002). As opposed to males, potential fitness benefits of elevated T in females are less clear, as in most species females do not strive for more fertilizations by courting and competiting during this stage.

In this thesis, I have addressed two main questions: i) Are the observed peak testosterone levels in females the result of sexually antagonistic selection, direct selection in females, or neutral selection, and ii) whether the current seasonal profile of T levels is adaptive for females. The first question was addressed in a nest-box population of free-living blue tits (*Cyanistes caeruleus*). To this end, I manipulated T levels within the physiological range of females during the period when natural T levels are increased (i.e., between the nest building phase and the onset of egg laying) by implanting females either with T-filled implants ("T females") or reduced T effectiveness by implanting them with Flutamide-filled implants ("Flu females"). Flu does not affect circulating plasma T levels *per se* but it does inhibit androgen uptake by binding to testosterone and dihydrotestosterone (DHT) intracellular receptors, thereby blocking the effects these androgens have on behaviour and physiology (Adkins-Regan and Garcia, 1986; Hegner and Wingfield, 1987; Nelson, 2011; Neri and Peets, 1975; Peets et al., 1974; Sperry et al., 2010). As controls I provided a third group of females with empty implants (C females). I hypothesized that if T levels are

the result of sexually antagonistic selection, Flu females would perform better than C and T females. This is because it is assumed that according to the "*sexually antagonistic selection*" hypothesis females would benefit from lower levels than currently observed. Hence, I expected that if T levels in males and females are under sexually antagonistic selection pressure, then reducing the effectiveness of T by Flu in females would increase their fitness. In contrast, if current T levels are, in fact, optimal for females, I expected C females to perform better than both T and Flu females. Finally, if variation in T levels is selectively neutral to females, I expected no effects of my manipulation on female performance.

The second question about the adaptiveness of the seasonal profile in T levels in females was addressed in a nest-box population of free-living great tits (*Parus major*). In this experiment I experimentally elevated T levels in females from the nest-building phase onwards since I did not remove the implants. In this species biparental care is essential for the survival of the offspring (Björklund and Westman, 1986), thus if T suppresses maternal care and consequently reduce reproductive success, then I expected females to be insensitive to elevated T levels during the periods of parental care. Alternatively, females may not have evolved a mechanism to become insensitive to T during the period of maternal care because natural T levels in females are not elevated during this part of the breeding season and thus there would not have been selection pressure for this insensitivity.

Below, I will first discuss the results of my experiments in the light of the adaptive significance of peak T levels in females. Subsequently I will focus on the possibility that currently observed peak T levels in females are the result of sexually antagonistic selection pressures. Then, I will discuss the potential fitness consequences of the seasonal T profile in females. Finally, I will discuss the caveats and limitation of current studies on the adaptive role of T, which will include recommendations for futures studies.

The adaptive significance of T in females

In many vertebrate species, T levels increase at the beginning of their breeding season, and decrease after the onset of egg laying (Adkins-Regan, 2005; Ketterson et al., 2005; Wingfield et al., 1990). For the discussion on the adaptive significance of T in males and females, I will deal with the period when T levels are naturally increased separated from the seasonal variation in T levels.

The role of T in aggressive behaviour

T is strongly involved in male aggression during the reproductive period (Adkins-Regan, 2005). In **Chapter 2** I demonstrated that elevated T levels also increase aggression in female blue tits, by showing that females implanted with T significantly

increased their aggression towards a taxidermic mount of a female blue tit, whereas control and Flu females became less aggressive after implantation. There was no significant difference in aggression between the Flu and control females. These results are in correspondence with previous studies, which showed that experimentally elevated T levels increase female aggression in the European starling (*Sturnus vulgaris*; Sandell, 2007), red-winged blackbird (*Agelaius phoeniceus*; Searcy, 1988), and dark-eyed junco (*Junco hyemalis*; (Zysling et al., 2006). Also, Flu did not affect aggressive behaviour in female European robins (*Erithacus rubecula*, Kriner and Schwabl, 1991). Increased aggression during the pre-laying phase could potentially be adaptive for females. For example, by chasing away other females they may prevent their social mate from engaging in extra-pair copulations and help to secure parental care for her offspring (Rosvall, 2008; Sandell, 2007). In blue tits it has been shown that resident females behave aggressively towards intruding females to prevent polygyny. Polygyny is costly for females (Kempenaers, 1995), as females paired with polygamous males receive less help when feeding their offspring, resulting in reduced offspring survival (Kempenaers, 1994). Thus, an increase in aggressive behaviour caused by an elevation in T levels may be beneficial for female blue tits. The evidence that T may play a role in female aggressive behaviour is also supported by correlative studies in other species. For instance, studies that investigated the role of endogenous T found positive relationships with female aggressive behaviour (Langmore et al., 2002; Mazuc et al., 2003, but not all Elekonich and Wingfield, 2000; Jawor et al., 2006a; Schwabl et al., 2005). A recent study even showed that more aggressive female dark-eyed juncos had a higher reproductive success, and that the degree of aggression was correlated with the ability to produce T after a challenge with gonadotropin-releasing hormone (GnRH (see **box 1.1.**), Cain and Ketterson, 2012).

Although elevated T in females increased aggressive behaviour in our study and in others, we currently do not know whether T itself directly activated aggressive behaviour, or whether T does so only indirectly, by increasing levels of other steroid hormones, which in turn affect aggression. T can be aromatized into oestradiol, and aggression has been linked with aromatase activity in the brains of male birds (Schlinger and Callard, 1990; Silverin et al., 2004). Also, oestradiol mediates aggressive behaviour in female leopard geckos (*Eublepharis macularis*; Rhen et al., 1999) and female mountain spiny lizards (*Sceloporus jarrovi*; Woodley and Moore, 1999). The aromatization of T to oestradiol might also explain why I did not find any significant blocking effect of Flu on aggression (**Chapter 2**). Since Flu only blocks androgen receptors and not the production of T (Scanes, 2000; Sperry et al., 2010), treated females still had circulating T which might be aromatized into oestradiol. In female song sparrows (*Melospiza melodia*), however, experimentally elevated oestradiol did not affect aggressive behaviour (Elekonich and Wingfield, 2000). To the best of our knowledge no study has investigated the combined effect of Flu and ATD, a blocker

of aromatase, on female aggression. This is necessary to disentangle the hormonal pathways through which T affects aggression in females.

The role of T in courtship and mating

T plays an important role in regulating reproductive behaviour in male birds (Adkins-Regan, 2005; Hau, 2007), and the seasonal fluctuation in T levels reflect the mating system of the species. In polygynous species, male T levels remain high throughout the breeding season, whereas in socially monogamous species male T levels are the highest during the time when females are fertile (Goymann et al., 2007; Wingfield et al., 1990). A comparative study also showed that the level of peak T was positively correlated to the occurrence of extra-pair paternity within a species (Garamszegi et al., 2005). Studies that investigated the role of experimentally elevated T in males showed that T increased certain aspects of male mating behaviour. For example, elevated T increased attractiveness to females (Enstrom et al., 1997), the number of extra-pair offspring sired (EPO, (Raouf et al., 1997), prolonged courtship behaviour (Silverin, 1980), and even induced polygyny in a monogamous bird species (white-crowned sparrows (*Zonotrichia leucophrys pugetensis*) and song sparrows, (Wingfield, 1984). Hence, the effects of increased levels of T on male mating behaviours are relatively well established in birds.

By contrast to the vast number of studies on males, the role of T in female mating behaviour is less well understood. In **Chapter 3** I therefore manipulated levels of T in females by implanting female blue tits with either T or Flu, thereby showing that elevated T in females decreased the probability of producing a nest with at least one extra-pair offspring (EPO). In addition, blocking androgen receptors with Flu did not have any effect on the number of nests with at least one EPO.

So far, to my knowledge, two studies have also investigated the role of T on the number of EPO in females and found contradictory results. In spotless starlings (*Sturnus unicolor*) elevated T levels also decreased the number of EPO (Garcia-Vigon et al., 2008), whereas in dark-eyed juncos there was no effect of elevated T levels on the number of EPO (Gerlach and Ketterson, 2013). An open question is whether this reduction in EPO is costly for female fitness. Although there is an on-going debate about the adaptive significance of extra-pair copulation (EPC) behaviour in females (Akcay and Roughgarden, 2007; Arnqvist and Kirkpatrick, 2005; Griffith and Immler, 2009; Griffith et al., 2002; Jennions and Petrie, 2000), the fact is that EPCs frequently occur in more than 90% of bird species (Griffith et al., 2002). Also, female blue tits are known to actively search for EPCs (Kempenaers et al., 1992). The near-ubiquity of EPC in birds and the active pursuit of EPC in female blue tits could indicate that there might be some fitness benefit of EPC for females. Or put differently, if the costs of EPC are larger than the benefits for females it would have been selected against and thus this phenomenon of EPCs would have disappeared from a population.

An alternative hypothesis is that female EPC behaviour is the result of sexually antagonistic selection. In general, males can increase their fitness by acquiring EPCs, whereas EPCs might reduce female fitness by reducing the paternal investment of their partner (Arnqvist and Kirkpatrick, 2005). Thus for males it might be beneficial to increase their number of EPCs but EPCs might not be beneficial for females. If EPCs are not adaptive for females then it is likely that current EPC behaviour in females is the result of sexually antagonistic selection pressures (Arnqvist and Kirkpatrick, 2005). Indeed there is some evidence that female EPC behaviour has evolved via indirect selection on males. In zebra finches (*Taeniopygia guttata*) there was a strong genetic correlation between males and females in extra-pair mating behaviour. Hence, positive selection on male extra-pair mating behaviour would also lead to an increase in female extra pair mating behaviour (Forstmeier et al., 2011). A recent study in dark-eyed junco's, however, showed that EPCs can increase female fitness (Gerlach et al., 2012). They found that offspring produced by EPCs had a higher lifetime reproductive success, compared to offspring that were sired within a social pair. This difference in reproductive success most likely depends on the genetic contribution of the extra-pair sires (Gerlach et al., 2012). In male dark-eyed juncos EPCs also increased their fitness by increasing the number of EPO they produced (Reed et al., 2006). These results contradict the hypothesis that current female EPC behaviour is the result of sexually antagonistic selection pressures since both sexes would benefit from an increased number of EPOs. Therefore, EPC behaviour is a positively selected trait in both sexes in this species and the evolution of this trait is not constrained by sexually antagonistic selection pressures. Future studies should investigate if the sexually genetic correlation for extra-pair mating behaviour found in zebra finches is a general pattern across species. Although the fitness benefits of EPO in blue tits are less clear (reviewed by Vedder, 2011) compared to what has been found for female dark-eyed juncos, there is some evidence that EPO outperform within-pair offspring (WPO). For example, EPO were more likely to survive until fledging (Charmantier et al., 2004) and fledged earlier than WPO (Schlicht et al., 2012). Although at least part of these effects might be confounded by laying order and hatch time – the majority of EPO were positioned in the first half of the clutches and EPO hatched earlier than their within-pair half siblings. Offspring that hatched earlier had a higher survival probability, irrespective of whether they were EPO or WPO. However, EPO still tended to be heavier and have longer tarsi compared to WPO even after correcting for earlier hatching of EPO (Magrath et al., 2009). Offspring weight and tarsus length at fledging is important since it can influence survival and competitive ability later in life (weight: Tinbergen and Boerlijst, 1990); tarsus: (Garnett, 1981). Thus, if EPO might increase female fitness, then a reduction of EPO in females with experimentally elevated T levels might reduce their fitness.

In **Chapter 3** I hypothesized that a reduction in the number of EPO in clutches of T females could have been caused by a reduction in attractiveness or a change in

mating behaviour. Attractiveness did not seem to be affected since I did not find any male preference for either Flu or T treated females in a controlled mate choice test (**Chapter 3**). Therefore it is more likely that the reduction in EPO in T females resulted from a change in female mating behaviour. For example, other studies that explored the role of T in female mating behaviour showed reduced choosiness for a particular mate (McGlothlin et al., 2004) and increased male-like mating behaviour, such as establishing a territory (Lank et al., 1999), courtship feeding and mounting attempts (Lahaye et al., 2012; Nespor et al., 1996). Since I did not observe mating behaviour in our blue tit population, I cannot conclude that the fewer nests with EPO in the T group resulted from a change in female mating behaviour. But since female blue tits are known to actively search for extra-pair mattings (Kempenaers et al., 1992), it is likely that the reduced number of EPO per nest was the result of a change in female mating behaviour because of experimentally elevated T levels.

Even though T affects mating behaviour in females, other ovarian hormones, such as oestrogens and/or progesterone, have been shown to exert stronger effects on female mating behaviour (Adkins-Regan, 2005). For instance, experimentally elevated oestrogens induced mating behaviour in many female vertebrate species (Moore, 1982; Takahashi, 1990; Tokarz and Crews, 1980), and oestrogen treatment even induced sexual behaviour in ovariectomized females (Takahashi, 1990). As mentioned before, T can be aromatized into oestradiol, which may explain why the number of nests with at least one EPO was not affected in Flu females.

The role of T in nest building

At the beginning of the breeding season females not only engage in courtship behaviour and chase away intruding females, they also search for nest material and build the nest. Building a high quality nest is an essential activity for successful reproduction in birds. For example, nest size is positively associated with higher reproductive success in great tits (Alvarez and Barba, 2011). Nest completion early in the breeding season has an advantage for females, because they might have a wider time window to adjust the timing of reproduction to caterpillar abundance peaks (Perrins and McCleery, 1989), which is very important for reproductive success in blue tits (Perrins, 1991). In **Chapter 2** I showed that T females restarted nest-building earlier and completed their nest within a shorter time frame compared to C and Flu females. This is consistent with the finding that a natural increase in T in males at the beginning of the breeding phase is positively correlated with male nest-building behaviour in northern mocking birds (*Mimus polyglottos*; (Logan and Wingfield, 1995), and experimentally elevated T levels resulted in an increase in nest building activities in male zebra finches (Hill et al., 2005). However, experimentally elevated T levels in female dark-eyed juncos, which were implanted just before the start of nest building, reduced the chance that females would start to build a nest (Gerlach and Ketterson, 2013).

The increase in nest-building behaviour in female blue tits might either be a direct result of elevated T levels, or an indirect result of the conversion of T into oestradiol by aromatase. This conversion might explain why Flu females did not show a decrease in their nest building behaviour. In female canaries (*Serinus canaria domestica*), oestradiol is known to stimulate nest-building behaviour (Warren and Hinde, 1959), albeit this study did not investigate the timing of nest completion. Even in males, oestrogens had a positive effect on nest-building, as shown in zebra finches (Walters and Harding, 1988). Future studies should investigate whether nest building behaviour in female blue tits, and in females of other bird species, is regulated by androgens, oestrogens, or both.

Overall, elevated T levels during the pre-laying phase appear to be adaptive for the regulation of aggression and nest building behaviour, but might have negative effects on female mating behaviour. This indicates that there might be a trade-off between the positive effects of naturally elevated T levels in females on nest building and aggression versus reduction of EPCs. This trade-off might limit the evolution of higher peak levels of plasma T in females.

Is the evolution of T constrained by intersexual genetic correlation?

In female blue tits I did not find any significant difference among the two treatment groups (T, Flu) and the C group in reproductive success, measured as number of hatchlings and fledglings produced per female (**Chapter 2** & **4**), and in survival, (**Chapter 4**). The lack of a significant difference among the treatments might be the result of the small sample size. To be able to detect differences in survival, I would have needed a sample size of at least 345 individuals (**Chapter 4**). Such a large sample size is hard to obtain in a study population in the wild. Also, to attain a good estimate of the fitness effect of T in females, a long-term study would be necessary to monitor the possible effects of T on reproductive success and survival. Although I followed the yearly survival of the females for a maximum of two years, this time period may have been too short to detect any difference in fitness among the treatment groups. Also, the sample size of the number of females that survived these two years was too small to detect any significant difference in survival (in total, three females per treatment group survived; **Chapter 4**). Nevertheless, it is unlikely that my treatments had any carry-over effects on fitness (Harrison et al., 2011), since the effects of my treatments on reproductive success were already absent in the year the females were implanted. Also, I did not find any long-term effects of my treatments on the number of nests with at least one EPO. Furthermore, if there were strong negative selection pressures on elevated T levels in females, then even with a small sample size one would find a reduction in fitness in the T female group. Since I did not find any negative effects of elevated T levels on female fitness, it is unlikely that currently

observed T levels in females are the result of sexually antagonistic selection. On the other hand, because C females did not have a higher fitness than T or Flu females, I cannot support the hypothesis that present day T levels in females are adaptive (**see General Introduction, Figure 1.2**). Thus, although I did find effects of T on indirect measures of fitness, such as aggression and nest building, I did not find any fitness effects of my treatments. Therefore peak T levels in females might be the result of neutral selection.

These results contradict those from a series of other studies that showed lower hatching and fledging success in broods of females with experimentally elevated T (Gerlach and Ketterson, 2013; Lopez-Rull and Gil, 2009; O'Neal et al., 2008; Veiga and Polo, 2008; Veiga et al., 2004). Based on these results, it was concluded that current T levels in females are the result of sexually antagonistic selection (Gerlach and Ketterson, 2013; Ketterson et al., 2005; O'Neal et al., 2008). These studies, however, did not investigate T levels within the physiological range of the species nor the period when T is naturally increased. They also did not explore the effect of lowered T effectiveness by means of for example flutamide treatment. The latter is relevant to be able to distinguish between effects of deviations from normal female T levels (needed for testing the "*adaptive hypothesis*") and an increase in T levels *per se* (used to test the "*sexually antagonistic selection*" hypothesis). In contrast to the earlier studies just mentioned I did not find any negative effects of experimentally elevated T on reproductive success (**Chapter 1, 2** & **3**) highlighting the importance of the use of an experimental design which mimics the natural situation. Since other studies merely considered levels of T outside of the naturally occurring range, their finding that T has substantial effects on female reproductive behaviour and female fitness are unsurprising. By contrast, the lack of any obvious costs in our study demonstrates that when considering the natural range of T levels, any intralocus sexual conflict over T levels may well be resolved in natural populations.

Interestingly, our findings of the effects of elevated T on reproductive success is similar to what has been found in male blue tits, where researchers also mimicked a more natural situation when they experimentally elevated T levels (Foerster and Kempenaers, 2004). These results are important because to understand the evolutionary ecology of sexual dimorphism in T levels, both male and female patterns need to be explained. In general, the potential beneficial effects T has on male reproductive success led to the conclusion that males might benefit from higher T levels than currently observed, and thus might be constrained by sexually antagonistic selection. However, there are some concerns with the notion that sexually antagonistic selection is solely responsible for constraining male T levels. First, besides evidence for positive effects of T on male fitness, experimentally elevated T levels have also been shown to lead to negative fitness effects, for example through the suppression of immune function (Boonekamp et al., 2008; Roberts et al., 2004), or by decreasing paternal care resulting in a lower nestling survival (Hegner & Wingfield 1987), and a reduction in

annual survival (Dufty, 1989; Moss et al., 1994; Reed et al., 2006). Second, even in cases when high T levels are indeed beneficial to males, lowering T effectiveness with the androgen blocker Flu should result in a decrease in male fitness (at least, when T itself, and not oestradiol, mediates its effects on male behaviour). In support of this, it has been shown that Flu males showed reduced copulation behaviour and aggression (see table 6.1). However, Flu has also been shown to increase male parental feeding rates (see table 6.1), which can be beneficial for offspring survival, and hence, for male lifetime reproductive output. Overall, these results indicate that T is important for regulating sexual behaviour and aggression in males, but at the same time might be costly for males, for example by decreasing parental care. Thus, because elevated T levels may cause a trade-off between sexual behaviour and paternal care, it is just as likely that current levels of T in males are under direct selection only and not also subject to intersexual genetic correlation. Combining my results on female T and that of the studies on the role of T in males, it is probable that present day T levels in the sexes are not subject to sexually antagonistic selection at all. In the following paragraph I will discuss possible mechanisms that could have resolved the genetic constraint, caused by sexually antagonistic selection pressures.

Mechanisms that resolve intralocus sexual conflict

Sex linkage

In vertebrates with chromosomal sex determination, most genes are located on the autosomal chromosomes, but some genes are located on the sex chromosomes, which are called sex-linked genes. Sex-linked genes can reduce sexually antagonistic selection because the expression of genes is sex specific (Lande, 1980). In humans, for example, the sex chromosomes are XX for females and XY for males. If a certain trait, e.g. colour blindness, is linked to a recessive allele on the X-chromosome, than it is more likely to be expressed in males. This is because males only have one copy of that gene; hence they will express the trait when inheriting the recessive allele. Females, however, need two copies of that recessive allele before it is expressed. Thus, if a trait is limited to one sex, then the coding for this trait is likely to be disproportionally expressed on sex chromosomes. In birds, females are the heterogametic sex (ZW) and males are the homogametic sex (ZZ). An example of sex-linked gene expression in bird behaviour is the species-assortative mating preference in flycatchers (Sæther et al., 2007). Cross-fostered hybrid females with pied flycatcher (*Ficedula hypoleuca*) fathers predominately mated with pied flycatcher males, whereas hybrid females sired by collared flycatcher (*Ficedula albicollis*) fathers predominantly mated with collared flycatcher males, regardless of whether or not the females were cross-fostered. On the other hand, male hybrids from either parental species (pied or collared) did not discriminate against heterospecific mating. This study showed that sexually

imprinting on the social father is probably absent in females of these species. Hence, the species-assortative mating in these species has a genetic base and the genes coding for this behaviour are most likely linked to the Z-chromosome (Sæther et al., 2007).

How likely is it that genes coding for T are linked to the sex chromosomes in birds? Although the flycatcher study presents evidence of sex-biased gene expression, the majority of sex linked genes are equally expressed in males and females through sex chromosome dosage compensating mechanisms (Mank, 2009a). In general, the heterogametic sex (XY or ZW) has a lower gene dose (the number of copies present in a cell or nucleus) compared to the homogametic sex (XX or ZZ); the former has only one copy of X/Z-linked genes on their X/Z chromosome, whereas the later has two copies of X/Z-linked genes. If genes for a trait, that is essential for both sexes, are linked to the X or Z-chromosome, then the difference in gene dosage could be costly for the heterogametic sex. To compensate for this difference in gene dosage, it is hypothesised that there are regulatory mechanism that equalize the transcription of X/Z-linked genes, so that they are equally expressed in the hetero- and homogametic sex (Charlesworth, 1978). This dosage compensation has been found in the majority of organisms (Mank, 2009a). In birds, however, dosage compensation does not seem to occur, i.e. many Z-linked genes are less expressed in females compared to males (Ellegren and Parsch, 2007). This distinction might mitigate sexual dimorphism in birds. Thus, it is a possibility that the sexual dimorphism in T production is the result of sex-linked gene expression. However, since many studies show that peak T levels of males and females of the same species are highly correlated (Ketterson et al., 2005; Møller et al., 2005); it is unlikely that genes coding for T are linked to the sex chromosomes in birds.

Sex-limited trait expression of autosomal loci

As a limited number of genes are only located on the sex specific chromosomes (the W chromosome in birds; Chue and Smith, 2011), it is more likely that sexually dimorphic traits result from sex-differential expression of genes located at autosomal chromosomes that are present in both sexes (Ellegren and Parsch, 2007). Such "sex-biased genes" can be classified in two groups: (i) genes that are exclusively expressed in one sex (sex-specific expression), or (ii) genes that are expressed in both sexes but at a higher level in one sex (i.e. male- or female-biased). Genes that are equally expressed in both sexes are denoted as unbiased (Ellegren and Parsch, 2007).

Sex-limited trait expression can come about through organizational differences in males and females during early development (Adkins-Regan, 2005). Sex-specific development is initiated through the expression level of major sex determining genes located on the avian Z chromosome, for which expression is necessarily lower in females (e.g., DMRT1, Smith et al., 2009). Either testis or ovary development is then initiated via the sex-determination cascade, which comprises a series of interacting genes that govern sex-specific gene expression and development (Smith et al., 2009,

Chue and Smith 2011). Subsequently, the presence of testis or ovaries then lead to the expression of male- or female-specific sex hormone levels, including androgens, like T, and oestrogens. Particular levels of these hormones determine whether the embryo will develop into an adult male or female behavioural phenotype (Adkins-Regan, 2008). These organizational effects during early development not only cause irreversible changes in the phenotype of an individual, but are also the major cause of sexual dimorphism in behaviour (Balthazart et al., 2009). Although, the sexual dimorphism of specific cells or tissue is also influenced by the sex chromosomes, sex-specific traits may even arise in the absence of regulation from gonadal hormones during the development (Arnold, 2012). As these early organizational effects lead to the development of sex-specific behaviour, constraints on both sexes due to a genetic correlation with the other can, at least partially, be eliminated (Adkins-Regan, 2008). Indeed, in birds, ovaries produce a high ratio of oestradiol to T and early exposure to this steroid eliminates the capacity for male mounting behaviour and sexual interest in females, while blocking of oestrogen receptors or oestrogen synthesis in eggs with female embryos results in male-like behavioural profiles, regardless of the genetic sex (Adkins-Regan, 2008). Similarly, early ontogenetic exposure to oestradiol in female embryos can result in a lower sensitivity to the activational effects of T in adult females.

In summary, although males and females share most of their genes, the constraint of intersexual genetic correlation can be resolved through differential early development initiated by the presence of genes on the sex chromosomes that can induce sex specific gene expression on other chromosomes. It remains to be seen whether such early developmental effects are always the most effective means to resolve intralocus sexual conflict across a broad range of traits (e.g., Bonduriansky and Chenoweth, 2009; Mank, 2009b; Poissant et al., 2010). However my study provides no indications that T levels are genetically constrained for either sex in blue tits.

Is the seasonal T profile adaptive in females?

In many seasonal breeding birds, T concentrations in both sexes decline during socially stable periods, or after the onset of egg laying (Ketterson et al., 2005; Wingfield et al., 1990). Although T levels remain low for the rest of the breeding season, many male species remain sensitive to elevated T levels during this period. This can have negative effects on paternal care which in turn may reduce reproductive success. At the same time, sustained sensitivity to T may have positive effects on various components of sexual behaviour which can increase reproductive success. The costs and benefits of prolonged sensitivity to T causes a trade-off between sexual behaviour and paternal care. However, other studies did not show effects of experimentally elevated T on paternal care (Lynn et al., 2002; Van Duyse et al., 2002; Kazama et al., 2011).

The behavioural insensitive hypothesis (Lynn et al., 2002 & Lynn, 2008) addresses the question of why species differ in paternal sensitivity to T after egg laying. While elevated male T levels may be beneficial for obtaining fertilizations, they may come at a cost in terms of reduced paternal care and in species where males are indispensable for parental care insensitivity to T may have evolved.

In **Chapter 5** I demonstrated that female great tits remained sensitive to prolonged experimentally elevated T levels. This prolonged sensitivity had negative effects on incubation behaviour and reproductive success, in terms of reduced hatching and fledging success. Hence, these results suggest that it is adaptive for females to reduce their T levels as soon as they start laying eggs. Most studies that have investigated the effects of prolonged elevated T levels in female birds also found negative effects on maternal care. For example, experimentally elevated T reduced the time spent brooding nestlings in dark-eyed juncos (O'Neal et al., 2008), reduced nestling feeding rates and hatching success in spotless starlings (Veiga and Polo, 2008; Lopez-Rull and Gil, 2009) and reduced incubation temperature and hatching success in tree swallows (*Tachycineta bicolor*, Rosvall, 2013). But, as in males, there are exceptions where maternal care is not affected by experimentally elevated T levels. For example, elevating T levels for a prolonged period did not affect incubation and feeding behaviour in female European starlings and dark-eyed juncos (Sandell unpublished manuscript cited in Ketterson et al., 2005; Clotfelter et al., 2004; De Ridder et al., 2002; O'Neal et al., 2008), and fledgling success in spotless starlings (Lopez-Rull and Gil, 2009). These interspecific differences in behavioural sensitivity/insensitivity to prolonged T levels might be explained by the differences in ecology and life history of these species. For example, in colonially breeding species such as the starling, the level of territorial interactions is relatively high, and endogenous T levels are in general higher in colonially-breeding (Møller et al., 2005). Thus in those species, elevated T is still needed during the phase of parental care and, therefore, a disassociation between T and parental care is needed. Indeed, an increase in aggression after T levels were experimentally elevated increased the reproductive success of European starlings (Sandell, 2007).

Although the differences in ecology and life history between species may explain species specific behavioural sensitivity/insensitivity to prolonged T levels, it does not clarify why some behaviours remain sensitive to prolonged T levels and others do not within a species. For example, in dark-eyed juncos aggressive behaviour was still higher in females with elevated T later in the breeding season (Zysling et al., 2006), whereas nestling feeding rate was un-effected (O'Neal et al., 2008). Behavioural differences in sensitivity to T within a species might be explained by differences in sensitivity of specific brain regions to T. The brain is morphologically highly plastic with, for example, changes in cell number and cell size within and between seasons. Furthermore, neuroplasticity is related to changes in steroid hormone levels (Balthazart et al., 2010). For example, exogenous T in castrated males increased the

neuronal cross-sectional area of the medial preoptic nucleus (POM; Panzica et al., 1991). The POM nucleus in the brain plays a role in the activation by testosterone of male copulation behaviour (Balthazart and Ball, 2007). A different neural system, which is also steroid dependent, is the telencephalic nucleus (HVC). The HVC mediates singing behaviour and is also activated by T (Meitzen et al., 2007). Both areas vary within and/or between seasons in morphology (Balthazart et al., 2010). Thus, differences in sensitivity to T in brain areas which correspond with specific behaviours might explain why some behaviours remain sensitive to elevated T levels and others do not.

In addition, prolonged sensitivity to T has been shown to differ between sexes within species. For example, prolonged elevated T levels affect nestling feeding in male, but not female, dark-eyed juncos (O'Neal et al., 2008; Ketterson and Nolan, 1992). In my study I found opposing results between what has been found in males and females. Paternal care in male great tits seems to be insensitive to elevated T levels (Van Duyse et al., 2000 & 2002), whereas maternal care is not (**Chapter 5**). This dissimilarity between the sexes in the great tit should be interpreted with caution, since different parental behaviours were measured, i.e. incubation behaviour in females vs feeding behaviour in males.

Differences in sensitivity to T later in the season between sexes may have arisen through different selection pressures between the sexes. For example, imagine if elevated T levels reduce paternal care in males, but not their fitness. Females would likely compensate for this reduced paternal care (e.g. Whittingham et al., 1994) and there would not be negative selection for prolonged sensitivity in males. In contrast, if prolonged sensitivity to elevated T levels reduces maternal care without compensation by the male and thereby female fitness is affected, then females with prolonged sensitivity to T would be selected against. Hence, if the heritability for prolonged sensitivity to T levels is high in both sexes, then ultimately this will result in sexually antagonistic selection for sensitivity in T levels.

Caveats and limitations of current studies on the adaptive role of T

There are several caveats and limitations of current studies (including my studies) on the adaptive role of T in the sexes, which makes it difficult to conclude what selection pressures are currently acting upon T levels in males and females. I will briefly discuss these caveats below.

The heritability of T

Evolutionary changes only occur when natural or sexual selection acts on heritable phenotypic traits. This means that a phenotypic trait has heritable genetic variation and that selection will act on the genotype through the phenotype. Yet, despite our

knowledge about how T influences variation in avian phenotypic traits, not much is known about the heritable genetic variation of avian T levels. There is one study in birds that contains evidence that T levels might be heritable, although the evidence is indirect. Male great tits selected for slow exploratory behaviour had higher T levels than birds selected for fast exploratory behaviour. Suggesting that when a particular phenotypic trait (fast or slow exploration behaviour) is selected, this consistently results in different T profiles (Van Oers et al., 2011). Heritability of T levels is rarely studied in other taxa as well. In captive bank voles (*Myodes glareolus*), the heritability of T from father to son was h^2 0.52 ± 0.16 (Mills et al., 2009) and in domestic pigs this was $h^2 = 0.37 \pm 0.16$ (Lubritz et al., 1991). Studies on the heritability of testosterone in humans, based on mono- and dizygotic twins, have estimates ranging between 0.16 and 0.69 (Harris et al., 1998; Meikle et al., 1997; Ring et al., 2005).

Until now only one study investigated the heritability in a free- living animal population. In this study they found that the heritability of T levels in garter snakes (*Thamnophis sirtalis*) was close to 1 (King et al., 2004). In this study full-siblings were compared, meaning their heritability estimates may have been inflated as a result of maternal effects and other factors (Lande and Price, 1989). Overall the lack of knowledge on the heritability of T levels makes it difficult to assess if T levels in birds are genetically correlated between sexes, and if so, whether T levels in females are adaptive or not.

In addition, the phenotype of an organism is not only determined by its environment and genotype, but also by the environment of its parents. Mothers, especially, have major influences on the phenotype of their offspring through maternal effects. Maternal hormones in avian eggs have been thoroughly studied (Schwabl, 1993) and although there are several steroid hormones of maternal origin present in the avian egg, most research has focussed on testosterone (Groothuis et al., 2005; von Engelhardt and Groothuis, 2010). Prenatal testosterone has been shown to affect several behaviours, such as chick begging behaviour, and survival (reviewed in Groothuis et al., 2005). Also, the levels of prenatal testosterone in eggs can differ between the sexes, with higher T concentration in eggs containing male embryos (Badyaev et al., 2006). Thus when considering the heritability of T, besides the environment and genotype of an individual, the maternal effects should also be taken into account.

Future studies should focus on investigating the heritability of T levels in both sexes of different taxa. Heritability of T can, for example, be examined by creating selection lines, where males are selected for high and low T levels, and T levels are subsequently measured in male and female offspring. In addition, the fitness of each selection line can be measured — if the female offspring of males selected for high T levels have a lower fitness compared to female offspring from the low T level selection line, then the evolution of T levels is probably limited by sexually antagonistic selection pressures.

Individual variation in T levels

Another limitation of current studies is the poor knowledge on the adaptive value of the observed natural variation in T levels and the natural variation in duration of the period when T is elevated. For instance, male blue tits display strong variation in T levels between individuals within one population during the same period (Kempenaers et al., 2008). Understanding why there are individual differences in natural T levels is important because the potential to respond to different selection pressures might be regulated by the level of T an individual has (Williams, 2008). For example, consider a population which varies in density each year: in years when the population density is high, it might be beneficial for an individual to have high T levels, as this increases their dominance status and thus the likelihood to obtain resources (e.g., food and territories), ultimately leading to an increase in reproductive success. During these years it is expected that there will be positive selection for high T levels. By contrast, when the population density is low, it might be beneficial to have high T levels because high dominance levels would be unnecessary in this situation, as there is no shortage in resources and high T levels might be costly. During these years it is expected that there will be negative selection for high T levels. A method to study if the variation of natural peak T levels between individuals of the same species has different fitness costs and benefits is to challenge an individual with GnRH. An injection of GnRH, the hormone from the hypothalamus that ultimately stimulates the gonads to produce testosterone, gives a measure of an individual's maximum capacity to produce T. The difference in the strength of the response between individuals to the challenges can be used to measure individual differences in their hormonal phenotype. Subsequently, it can be examined whether certain hormonal phenotypes are correlated to specific behavioural traits. In dark-eyed juncos, for example, it was found that females that show a greater ability to produce T after a GnRH challenge are larger and more aggressive. They also found that more aggressive females have a higher reproductive success (Cain and Ketterson, 2012). Thus, in this species, one would expect positive selection for females able to produce high T levels after being (behaviourally) challenged. More studies should investigate the role of individual variation in peak T levels within individuals of the same sex to understand the adaptive role of T levels in males and females.

How well an individual can respond to these fluctuations in environmental conditions, such as population density, also depends on the phenotypic plasticity of a trait. Phenotypic plasticity is the ability of an organism to change its phenotype in response to changes in the environment (Lawrence, 2011). Hormones play a major role in how an organism responds to changes in the environment as they are one of the links between the environment and the genome (Dufty et al., 2002). An alternation in hormone production due to, for example, a change in day length, can produce different phenotypes from the same genotype (Dufty et al., 2002). In addition, the level of phenotypic plasticity in an organism can influence its fitness, especially in a

variable environment (Nussey et al., 2007). Thus, not only the level of T an individual has, but also its plasticity with regards to T production determines how well it can cope with fluctuating selection pressures.

Studies of T in both sexes

The third limitation is the lack of comparative studies between males and females of the same species. As stated before, the role of T in regulating a phenotypic trait, such as aggression, has been extensively studied in males (Goymann et al., 2007; Wingfield et al., 1990), but less so in females. Until now there are only a few species in which the role of T has been studied in both sexes, and most of these studies did not observe the effects of T on the same behavioural traits in both sexes. I could find only two species in which the role of T on the same behavioural trait in both sexes was studied, and these studies found contradictory results. In the dark-eyed junco male nestling feeding was decreased when their T levels were experimentally increased (Ketterson and Nolan, 1992), whereas elevated T levels did not affect female nestling feeding (O'Neal et al., 2008). In contrast, in the spotless starling, elevated T levels decreased nestling feeding in both sexes (Moreno et al., 1999; Veiga and Polo, 2008). These studies show that not only is there variation in the effect of T on certain behavioural trait between species, but also between sexes of the same species. To really understand the functional role of T levels in females, it is essential to know the fitness costs and benefits of certain T levels on similar phenotypic traits in both sexes. Therefore, if we want to understand the evolution of sexual dimorphism in T levels, future studies should focus on studying the effect of T on similar phenotypic traits in both sexes.

The flutamide enigma

In none of the experiments where I used Flu did I find significant direct effect of this on an individual's condition (**Chapters 2, 3** & **4**), with the exception of a positive effect of Flu on the feather length of nestlings of Flu mothers (**Chapter 2**). I also did not find any difference in T levels in the Flu group compared to the C females (**Chapter 2**). These results lead me to question whether or not Flu an effective androgen receptor blocker?

Flu has been used as an androgen receptor blocker for at least 30 years. During this time, to my knowledge, it has only been used one time before in an adult female bird. Kriner and Schwable (1991) investigated the effects of Flu on territoriality in female European robins, and also found no difference in behaviour and hormone levels between Flu and C females. Flu has been used more often in male birds. Most of these studies did find an effect of flu on behaviour, but flu did not affect T levels in any of these studies except one (see table 6.1).

Why were T levels not affected by the Flu treatment? In mammals Flu can

Table 6.1. Overview of studies that have investigated the effect of the anti-androgen receptor blocker, Flu, on different breeding behaviours in birds. 0 means that there was no effect of the treatment, – indicates that Flu decreased the observed behaviour (or T level), and vice versa for the + symbol. F stands for female and M for male. NA stands for not available. The effects of Flu on T levels or compared to the C group.

	Direction Flu	Direction T	Testosterone levels	Sex	Species	Latin name	Author
Territoriality	0	0	0	F	European Robin	*Erithacus rubecula*	Kriner and Schwable, 1991
Territoriality	0	+	NA	M	Red-Winged Blackbird	*Ageliaus phoeniceus*	Beletsky et al., 1990
Courtship	- / +*	NA	0	M	Golden-collared manakin	*Manacus vitellinus*	Fusani et al., 2007
Feeding rate	+	-	-	M	House sparrows	*Passer domesticus*	Hegner and Wingfield, 1987
Courtship	0	NA	0	M	Vasectomized Texas bobwhite quail	*Colinus virinianus texanus*	Vleck and Dobrot, 1993
Copulation behaviour	0	NA	0	M			
Aggression	-	NA	0	M			
Strutting behaviour	-	+	NA	M	Japanese Quail	*Coturnix coturnix japonica*	Adkins-Regan and Garcia, 1986
Dominance	-	+	0	M	Red-Winged Blackbird	*Ageliaus phoeniceus*	Searcy and Wingfield, 1980
Aggression	-	NA	0	M	Song sparrow	*Melospiza melodia morphna*	Sperry et al., 2010

* First Flu decreased courtship behaviour, but during the third week courtship was increase

increase T levels (in rats: Gomez et al., 2004), but in birds this effect of Flu has not been observed (see table 6.1). In contrast to mammals, Flu administration in birds does not appear to disrupt the negative feedback of T (Sperry et al., 2010). This is probably due to the fact that in birds, oestrogen, rather than androgens, controls androgen levels via negative feedback acting on oestrogen receptors in the hypothalamus and pituitary (Scanes, 2000).

From these previous studies (see table 6.1), we can conclude that reducing the effectiveness of T by blocking the androgen receptors with Flu does affect, at least in males, behaviour in birds. The lack of significant effects of Flu on behaviour in female blue tits might be because these behaviours can potentially be regulated by other steroid hormones, such as oestrogens, although, some behaviours, i.e. aggression, nest building (**Chapter 2**) and mating behaviour (**Chapter 3**), are clearly influenced by T. To test if some of these behaviours are actually (also) oestrogen dependent, one could block aromatase activity so that T cannot be transformed into oestrogen. Another experiment to test if behaviours are T or oestrogen dependent is to block oestrogen receptors in one group, implant a second group of females with oestrogen receptor blockers and T implants (see box 1.1), and the third group will receive an empty implant (control group). If female behaviour is dependent on oestrogen, then the level the behaviour in the group where the oestrogen receptors are blocked would be lower compared to the control group. If the behaviour is T dependent, then the behaviour in the oestrogen blocked/ T elevated group should to be higher.

Concluding remarks

In this thesis I showed that a short-term experimental increase of T within the natural physiological range affected aggressive, nest building and mating behaviour in female blue tits, but did not affect their reproductive success and survival. I also demonstrated that lowering the effectiveness of T did not affect any of the behaviours measured, nor the fitness of these females. These results show that a manipulation of T levels that mimic a more natural situation does not result in a decrease of female fitness, and that lowering the effectiveness of T does not increase female fitness. In contrast, when T was elevated for a longer period than natural in great tit females, T treatments decreased reproductive success dramatically. Combining these results I conclude that present day T levels and the seasonal variation in T levels are most likely not the outcome of sexually antagonistic selection. However, I did not find evidence that currently observed T levels are adaptive for females. Although I did find significant proximate effects (positive effects of T on nest-building activity and aggression), this did not result in ultimate benefits (no increased reproductive success and survival) for T females. Therefore, present day T levels in female blue tits are presumably the result of neutral selection.

References

A

Abitbol, J., Abitbol, P., Abitbol, B., 1999. Sex hormones and the female voice. Journal of Voice 13, 424–446.

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

Adkins-Regan, E., 2008. Do hormonal control systems produce evolutionary inertia? Philosophical Transactions of the Royal Society B-Biological Sciences 363, 1599–1609.

Adkins-Regan, E., Garcia, M., 1986. Effect of flutamide (an antiadrogen) and diethylstilbestrol on the reproductive behaviour of japanes quail. Physiology & Behavior 36, 419–425.

Akcay, E., Roughgarden, J., 2007. Extra-pair paternity in birds: review of the genetic benefits. Evolutionary ecology research 9, 855–868.

Alatalo, R.V., Hoglund, J., Lundberg, A., Rintamaki, P.T., Silverin, B., 1996. Testosterone and Male Mating Success on the Black Grouse Leks. Philosophical Transactions of the Royal Society B-Biological Sciences 263, 1697–1702.

Alonso-Alvarez, C., 2001. Effects of testosterone implants on pair behaviour during incubation in the Yellow-legged Gull *Larus cachinnans*. Journal of Avian Biology 32, 326–332.

Alonso-Alvarez, C., Bertrand, S., Faivre, B., Chastel, O., Sorci, G., 2007. Testosterone and oxidative stress: the oxidation handicap hypothesis. Proceedings of the Royal Society B-Biological Sciences 274, 819–825.

Alvarez, E., Barba, E., 2011. Nest characteristics and reproductive performance in great tits parus major. Ardeola 58, 125–136.

Andersson, M.B., 1994. Sexual Selection. Princeton University Press, Princeton.

Ardia, D.R., Perez, J.H., Clotfelter, E.D., 2010. Experimental cooling during incubation leads to reduced innate immunity and body condition in nestling tree swallows. Philosophical Transactions of the Royal Society B-Biological Sciences 277, 1881–1888.

Arnold, A.P., 2012. The end of gonad-centric sex determination in mammals. Trends in Genetics 28, 55–61.

Arnqvist, G., Kirkpatrick, M., 2005. The Evolution of Infidelity in Socially Monogamous Passerines: The Strength of Direct and Indirect Selection on Extrapair Copulation Behavior in Females. The American Naturalist 165, S26–S37.

Arnqvist, G., Rowe, L., 2005. Sexual conflict. Princeton University Press, Princeton.

B

Badyaev, A.V., Acevedo Seaman, D., Navara, K.J., Hill, G.E., Mendonca, M.T., 2006. Evolution of sex-biased maternal effects in birds: III. Adjustment of ovulation order can enable sex-specific allocation of hormones, carotenoids, and vitamins. Journal of Evolutionary Biology 19, 1044–1057.

Balthazart, J., Arnold, A., Adkins-Regan, E., 2009. Sexual differentiation of brain and behavior in birds, in: Hormones, Brian and Behavior. Academic Press, London, pp. 1746–1787.

Balthazart, J., Ball, G.F., 2007. Topography in the preoptic region: Differential regulation of appetitive and consummatory male sexual behaviors. Frontiers in Neuroendocrinology 28, 161–178.

Balthazart, J., Charlier, T.D., Barker, J.M., Yamamura, T., Ball, G.F., 2010. Sex steroid-induced neuroplasticity and behavioral activation in birds. European Journal of Neuroscience 32, 2116–2132.

Bedhomme, S., Chippindale, A., Prasad, N., Delcourt, M., Abbott, J., Mallet, M., Rundle, H., 2011. Male-limited evolution suggests no extant intralocus sexual conflict over the sexually dimorphic cuticular hydrocarbons of *Drosophila melanogaster*. Journal of Genetics 90, 443–452.

Bensch, S., Price, T., Kohn, J., 1997. Isolation and characterization of microsatellite loci in a Phylloscopus warbler. Molecular Ecology 6, 91–92.

Birkhead, T.R., Møller, A.P., 1992. Sperm competition in birds: evolutionary causes and consequences. Academic Press, London.

Bjorklund, M., Senar, J.C., 2001. Sex differences in survival selection in the serin, Serinus serinus. J. Evol. Biol. 14, 841–849.

Bjorklund, M., Westman, B., 1986. Mate-Guarding in the Great Tit - Tactics of a Territorial Forest-Living Species. Ornis Scandinavica 17, 99–105.

Bonduriansky, R., Chenoweth, S.F., 200a. Intralocus sexual conflict. Trends in Ecology & Evolution 24, 280–288.

Boonekamp, J.J., Ros, A.H.F., Verhulst, S., 2008. Immune activation suppresses plasma testosterone level: a meta-analysis. Biology Letters 4, 741–744.

Bräuer, G., Chopra, V.P., 1980. Estimating the heritability of hair colour and eye colour. Journal of Human Evolution 9, 625–630.

Bubenik, G., Schams, D., White, R., Rowell, J., Blake, J., Bartos, L., 1997. Seasonal levels of reproductive hormones and their relationship to the antler cycle of male and female reindeer (*Rangifer tarandus*). Comp. Biochem. Physiol. B-Biochem. Mol. Biol. 116, 269–277.

C

Cain, K.E., Ketterson, E.D., 2012. Competitive females are successful females; phenotype, mechanism, and selection in a common songbird. Behavioral Ecology and Sociobiology. 66, 241–252.

Chapman, T., 2006. Evolutionary conflicts of interest between males and females. Current Biolology 16, R744–R754.

Charlesworth, B., 1978. Model for Evolution of Y Chromosomes and Dosage Compensation. Proceedings of the National Academy of Sciences 75, 5618–5622.

Charmantier, A., Blondel, J., Perret, P., Lambrechts, M.M., 2004. Do extra-pair paternities provide genetic benefits for female blue tits *Parus caeruleus*? Journal of Avian Biology 35, 524–532.

Chue, J., Smith, C.A., 2011. Sex determination and sexual differentiation in the avian model: Sex determination in birds. FEBS Journal 278, 1027–1034.

Clotfelter, E.D., O'Neal, D.M., Gaudioso, J.M., Casto, J.M., Parker-Renga, I.M., Snajdr, E.A., Duffy, D.L., Nolan, V., Ketterson, E.D., 2004. Consequences of elevating plasma testosterone in females of a socially monogamous songbird: evidence of constraints on male evolution? Hormones and Behavior 46, 171–178.

Cooper, C.B., Mills, H., 2005. New software for quantifying incubation behavior from time-series recordings. Journal of Field Ornithology 76, 352–356.

Cox, R.M., Calsbeek, R., 2009. Sexually Antagonistic Selection, Sexual Dimorphism, and the Resolution of Intralocus Sexual Conflict. The American Naturalist 173, 176–187.

Cramp, S., Perrins, C.M., 1993a. *Parus caeruleaus* Blue Tit, in: Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palearctic. Oxford University Press, Oxford, pp. 225–248.

Cramp, S., Perrins, C.M., 1993b. *Parus major* Great tit, in: Handbook of the Birds of Europe, the Middle East and North Africa. The Birds of the Western Palearctic. Oxford University Press, Oxford, pp. 255–281.

Croze, F., Etches, R.J., 1980. The physiological significance of androgen-induced ovulation in the hen. Journal of Endocrinology 84, 163–171.

D

Darwin, C., 1859. On the origin of species. Murray, London.

Darwin, C., 1874. The descent of man and selection in relation to sex. Murray, London.

Davies, N.B., Krebs, J.R., West, S.A., 2012. An introduction to behavioural ecology. Wiley-Blackwell, Oxford.

Dawson, D., Hanotte, O., Greig, C., Stewart, I., Burke, T., 2000. Polymorphic microsatellites in the blue tit *Parus caeruleus* and their cross-species utility in 20 songbird families. Mol. Ecol. 9, 1941–1944.

De Heij, M.E., Ubels, R., Visser, G.H., Tinbergen, J.M., 2008. Female great tits Parus major do not increase their daily energy expenditure when incubating enlarged clutches. Journal of Avian Biology 39, 121–126.

De Ridder, E., Pinxten, R., Eens, M., 2000. Experimental evidence of a testosterone-induced shift from paternal to mating behaviour in a facultatively polygynous songbird. Behavioral Ecology and Sociobiology 49, 24–30.

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

Deeming, D.C., Ferguson, M.W.J., 1991. Egg Incubation: Its Effects on Embryonic Development in Birds and Reptiles. Cambridge University Press, Cambridge.

Drevon, T., Slagsvold, T., 2005. When and from whom do female pied flycatchers (*Ficedula hypoleuca*) solicit copulations. Behaviour 142, 1059–1076.

Duffy, D.L., Bentley, G.E., Drazen, D.L., Ball, G.F., 2000. Effects of testosterone on cell-mediated and humoral immunity in non-breeding adult European starlings. Behavioral Ecology 11, 654–662.

Dufty, A., 1989. Testosterone and survival - cost of aggressiveness. Horm. Behav. 23, 185–193.

Dufty Jr, A.M., Clobert, J., Møller, A.P., 2002. Hormones, developmental plasticity and adaptation. Trends in Ecology & Evolution 17, 190–196.

Dunn, E., 1975. Timing of Endothermy in Development of Altrical Birds. Condor 77, 288–293.

E

Edler, R., Goymann, W., Schwabl, I., Friedl, T.W.P., 2011. Experimentally elevated testosterone levels enhance courtship behaviour and territoriality but depress acquired immune response in Red Bishops *Euplectes orix*. Ibis 153, 46–58.

Elekonich, M.M., Wingfield, J.C., 2000. Seasonality and Hormonal Control of Territorial Aggression in Female Song Sparrows (Passeriformes: Emberizidae: *Melospiza melodia*). Ethology 106, 493–510.

Ellegren, H., Parsch, J., 2007. The evolution of sex-biased genes and sex-biased gene expression. Nature Reviews Genetics 8, 689–698.

Enstrom, D.A., Ketterson, E.D., Nolan, Jr, V., 1997. Testosterone and mate choice in the dark-eyed junco. Animal Behaviour 54, 1135–1146.

Evans, R.M., 1990. Effects of low incubation temperatures during the pipped egg stage on hatchability and hatching times in domestic chickens and ring-billed gulls. Canadian Journal of Zoology 68, 836–840.

F

Fairbairn, D.J., Blanckenhorn, W.U., 2007. Sex, size, and gender roles: evolutionary studies of sexual size dimorphism, Oxford biology. Oxford University Press, Oxford.

Foerster, K., Kempenaers, B., 2004. Experimentally elevated plasma levels of testosterone do not increase male reproductive success in blue tits. Behav.Ecol.Sociobiol. 56, 482–490.

Foerster, K., Kempenaers, B., 2005. Effects of testosterone on male-male competition and male-female interactions in blue tits. Behavioral Ecology and Sociobiology 57, 215–223.

Foerster, K., Poesel, A., Kunc, H., Kempenaers, B., 2002. The natural plasma testosterone profile of male blue tits during the breeding season and its relation to song output. Journal of Avian Biology 33, 269–275.

Forstmeier, W., Martin, K., Bolund, E., Schielzeth, H., Kempenaers, B., 2011. From the Cover: Female extrapair mating behavior can evolve via indirect selection on males. Proceedings of the National Academy of Sciences 108, 10608–10613.

G

Garamszegi, L.Z., Eens, M., Hurtrez-Bousses, S., Moller, A.P., 2005. Testosterone, testes size and mating success in birds: A comparative study. Hormones and Behavior 46, 111–112.

Garcia-Vigon, E., Cordero, P.J., Veiga, J.P., 2008a. Exogenous testosterone in female spotless starlings reduces their rate of extrapair offspring. Animal Behavior 76, 345–353.

Garcia-Vigon, E., Cordero, P.J., Veiga, J.P., 2008b. Exogenous testosterone in female spotless starlings reduces their rate of extrapair offspring. Animal Behavior 76, 345–353.

Garnett, M.C., 1981. Body size, its heritability and influence on juvenile survival among great tits, *parus major*. Ibis 123, 31–41.

Gerlach, N.M., Ketterson, E.D., 2013. Experimental elevation of testosterone lowers fitness in female dark-eyed juncos. Hormones and Behavior 63, 782–790.

Gerlach, N.M., McGlothlin, J.W., Parker, P.G., Ketterson, E.D., 2012. Promiscuous mating produces offspring with higher lifetime fitness. Philosophical Transactions of the Royal Society B-Biological Sciences 279, 860–866.

Gomez, F., Manalo, S., Dallman, M., 2004. Androgen-sensitive changes in regulation of restraint-induced adrenocorticotropin secretion between early and late puberty in male rats RID A-7277-2010. Endocrinology 145, 59–70.

Gosler, A., 1993. The great tit. Hamlyn, London.

Goymann, W., Landys, M.M., Wingfield, J.C., 2007. Distinguishing seasonal androgen responses from male-male androgen responsiveness - Revisiting the Challenge Hypothesis. Hormones and Behavior 51, 463–476.

Griffith, S., Stewart, I., Dawson, D., Owens, I., Burke, T., 1999. Contrasting levels of extra-pair paternity in mainland and island populations of the house sparrow (*Passer domesticus*): is there an “island effect”? Biol. J. Linnean Soc. 68, 303–316.

Griffith, S.C., Immler, S., 2009. Female infidelity and genetic compatibility in birds: the role of the genetically loaded raffle in understanding the function of extrapair paternity. Journal of Avian Biology 40, 97–101.

Griffith, S.C., Owens, I.P.F., Thuman, K.A., 2002. Extra pair paternity in birds: a review of interspecific variation and adaptive function. Molecular Ecology 11, 2195–2212.

Groothuis, T.G.G., Muller, W., Von Engelhardt, N., Carere, C., Eising, C., 2005. Maternal hormones as a tool to adjust offspring phenotype in avian species. Neuroscience and Biobehavioral Reviews 29, 329–352.

H

Harris, J.A., Vernon, P.A., Boomsma, D.I., 1998. The Heritability of Testosterone: A Study of Dutch Adolescent Twins and Their Parents. Behaviour Genetics 28, 165–171.

Harrison, X.A., Blount, J.D., Inger, R., Norris, D.R., Bearhop, S., 2011. Carry-over effects as drivers of fitness differences in animals. Journal of Animal Ecology 80, 4–18.

Hau, M., 2007. Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. Bioessays 29, 133–144.

Hedrick, A.V., Temeles, E.J., 1989. The evolution of sexual dimorphism in animals: Hypotheses and tests. Trends in Ecology & Evolution 4, 136–138.

Hegner, R.E., Wingfield, J.C., 1987. Effects of experimental manipulation of testosterone levels on parental investment and breeding success in male house sparrows. AUK 104, 462–469.

Heilman, R., Brugmans, M., Greenslade, F., Davanzo, J., 1976. Resistance of androgen mediated aggressive behavior in mice to flutamide an antiandrogen. Psychopharmacology 47, 75–80.

Hill, W.L., Ballard, S., Coyer, M.J., Rowley, T., 2005. The interaction of testosterone and breeding phase on the reproductive behavior and use of space of male zebra finches. Hormones and Behavior 47, 452–458.

J

Jawor, J.M., Young, R., Ketterson, E.D., 2006. Females competing to reproduce: Dominance matters but testosterone may not. Hormones and Behavior 49, 362–368.

Jennions, M.D., Petrie, M., 2000. Why do females mate multiply? A review of the genetic benefits. Biological Reviews of the Cambridge Philosophical Society 75, 21–64.

Johnson, A.L., 2000. Reproduction in the female, in: Whittow, G.C. (Ed.), Sturkie's Avian Physiology. Academic press, San Diego, pp. 569–594.

K

Kalinowski, S.T., Taper, M.L., Marshall, T.C., 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16, 1099–1106.

Kazama, K., Sakamoto, K.Q., Niizuma, Y., Watanuki, Y., 2011. Testosterone and breeding behavior in male Black-tailed Gulls: an implant experiment. Ornithological. Science 10, 13–19.

Kempenaers, B., 1994. Polygyny in the blue tit: unbalanced sex ratio and female aggression restrict mate choice. Animal behaviour 47, 943–957.

Kempenaers, B., 1995. Polygyny in the Blue Tit - Intra-Sexual and Inter-Sexual Conflicts. Animal Behaviour 49, 1047–1064.

Kempenaers, B., Peters, A., Foerster, K., 2008. Sources of individual variation in plasma testosterone levels. Philosophical Transactions of the Royal Society B-Biological Sciences 363, 1711–1723.

Kempenaers, B., Verheyen, G.R., Vandenbroeck, M., Burke, T., Vanbroeckhoven, C., Dhondt, A.A., 1992. Extra-Pair Paternity Results from Female Preference for High-Quality Males in the Blue Tit. Nature 357, 494–496.

Ketterson, E., Nolan, V., Wolf, L., Ziegenfus, C., 1992. Testosterone and Avian Life Histories - Effects of Experimentally Elevated Testosterone on Behavior and Correlates of Fitness in the Dark-Eyed Junco (*junco-Hyemalis*). The American Naturalist 140, 980–999.

Ketterson, E.D., Nolan, V., 1992. Hormones and Life Histories - an Integrative Approach. The American Naturalist 140, S33–S62.

Ketterson, E.D., Nolan, V., Cawthorn, M.J., Parker, P.G., Ziegenfus, C., 1996. Phenotypic engineering: Using hormones to explore the mechanistic and functional bases of phenotypic variation in nature. Ibis 138, 70–86.

Ketterson, E.D., Nolan, V., Sandell, M., 2005. Testosterone in females: Mediator of adaptive traits, constraint on sexual dimorphism, or both? The American Naturalist 166, S85–S98.

King, R.B., Cline, J.H., Hubbard, C.J., 2004. Heritable variation in testosterone levels in male garter snakes (*Thamnophis sirtalis*). Journal of Zoology 264, 143–147.

Kleiman, D.G., 1977. Monogamy in Mammals. The Quarterly Review of Biology 52, 39.

Kluijver, H.N., 1951. The Population Ecology of the Great Tit, *Parus m. major* L. Ardea 39, 1–137.

Korsten, P., 2006. Avian sex allocation and ornamental coloration: a study on blue tits (Thesis). University of Groningen, Groningen.

Kral, M., Saetre, G., Bicik, V., 1996. Intrasexual aggression of female collared flycatchers (*Ficedula albicollis*): Competition for male parental care? Folia Zoology 45, 153–159.

Kriner, E., Schwabl, H., 1991. Control of winter song and territorial aggression of female robins (*Erithacus rubecula*) by testosterone. Ethology 87, 37–44.

L

Lahaye, S.E.P., Eens, M., Darras, V.M., Pinxten, R., 2012. Testosterone stimulates the expression of male-typical socio-sexual and song behaviors in female budgerigars (*Melopsittacus undulatus*): An experimental study. General and Comparative Endocrinology 178, 82–88.

Lande, R., 1980. Sexual Dimorphism, Sexual Selection, and Adaptation in Polygenic Characters. Evolution 34, 292.

Lande, R., Price, T., 1989. Genetic correlations and maternal effect coefficients obtained from offspring-parent regression. Genetics 122, 915–922.

Langmore, N.E., Cockrem, J.F., Candy, E.J., 2002. Competition for male reproductive investment elevates testosterone levels in female dunnocks, *Prunella modularis*. Proceedings of the Royal Society of London Series B-Biological Sciences 269, 2473–2478.

Lank, D.B., Coupe, M., Wynne-Edwards, K.E., 1999. Testosterone-induced male traits in female ruffs (*Philomachus pugnax*): autosomal inheritance and gender differentiation. Proceedings of the Royal Society of London Series B-Biological Sciences 266, 2323–2330.

Lawrence, E., 2011. Henderson's dictionary of biology. Benjamin Cummings/Pearson, Harlow, Essex; New York.

Littell, R., Henry, P., Ammerman, C., 1998. Statistical analysis of repeated measures data using SAS procedures. Journal of Animal Science 76, 1216–1231.

Logan, C., Wingfield, J., 1995. Hormonal correlates of breeding status, nest construction, and parental care in multiple-brooded northern mockingbirds, *Mimus polyglottos*. Hormones and Behavior 29, 12–30.

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

Lubritz, D., Johnson, B., Robison, O.W., 1991. Genetic parameters for testosterone production in boars. Journal of Animal Science 69, 3220–3224.

Lynn, S.E., 2008. Behavioral insensitivity to testosterone: Why and how does testosterone alter paternal and aggressive behavior in some avian species but not others? General and Comparative Endocrinology 157, 233–240.

Lynn, S.E., Hayward, L.S., Benowitz-Fredericks, Z.M., Wingfield, J.C., 2002. Behavioural insensitivity to supplementary testosterone during the parental phase in the chestnut-collared longspur, - *Calcarius ornatus*. Animal Behaviour 63, 795–803.

M

Magrath, M.J.L., Vedder, O., Van der Velde, M., Komdeur, J., 2009. Maternal Effects Contribute to the Superior Performance of Extra-Pair Offspring. Current Biology 19, 792–797.

Mank, J.E., 2007a. The evolution of sexually selected traits and antagonistic androgen expression in actinopterygiian fishes. The American Naturalist 169, 142–149.

Mank, J.E., 2007b. The evolution of sexually selected traits and antagonistic androgen expression in actinopterygiian fishes RID C-6859-2009. The American Naturalist 169, 142–149.

Mank, J.E., 2009a. The W, X, Y and Z of sex-chromosome dosage compensation. Trends in Genetics 25, 226–233.

Mank, J.E., 2009b. Sex Chromosomes and the Evolution of Sexual Dimorphism: Lessons from the Genome. The American Naturalist 173, 141–150.

Massaro, M., Davis, L.S., Davidson, R.S., 2006. Plasticity of brood patch development and its influence on incubation periods in the yellow-eyed penguin *Megadyptes antipodes*: an experimental approach. Journal of Avian Biology 37, 497–506.

Mazuc, J., Bonneaud, C., Chastel, O., Sorci, G., 2003. Social environment affects female and egg testosterone levels in the house sparrow (*Passer domesticus*). Ecology Letters 6, 1084–1090.

McGlothlin, J.W., Neudorf, D.L.H., Casto, J.M., Nolan, V., Ketterson, E.D., 2004. Elevated testosterone reduces choosiness in female dark-eyed juncos (*Junco hyemalis*): evidence for a hormonal constraint on sexual selection? Proceedings of the Royal Society of London Series B-Biological Sciences 271, 1377–1384.

Meikle, A.W., Stephenson, R.A., Lewis, C.M., Wiebke, G.A., Middleton, R.G., 1997. Age, genetic, and nongenetic factors influencing variation in serum sex steroids and zonal volumes of the prostate and benign prostatic hyperplasia in twins. The Prostate 33, 105–111.

Meitzen, J., Moore, I.T., Lent, K., Brenowitz, E.A., Perkel, D.J., 2007. Steroid Hormones Act Transsynaptically within the Forebrain to Regulate Neuronal Phenotype and Song Stereotypy. Journal of Neuroscience 27, 12045–12057.

Mills, S.C., Grapputo, A., Jokinen, I., Koskela, E., Mappes, T., Oksanen, T.A., Poikonen, T., 2009. Testosterone-Mediated Effects on Fitness Related Phenotypic Traits and Fitness. The American Naturalist 173, 475–487.

Mills, S.C., Koskela, E., Mappes, T., 2012. Intralocus sexual conflict for fitness: sexually antagonistic alleles for testosterone. Proceedings of the Royal Society B-Biological Sciences 279, 1889–1895.

Moller, A., Ninni, P., 1998. Sperm competition and sexual selection: a meta-analysis of paternity studies of birds. Behavioral Ecology and Sociobiology 43, 345–358.

Moller, A.P., Garamszegi, L.Z., Gil, D., Hurtrez-Bousses, S., Eens, M., 2005. Correlated evolution of male and female testosterone profiles in birds and its consequences. Behavioral Ecology and Sociobiology 58, 534–544.

Moore, M.C., 1982. Hormonal response of free-living male white-crowned sparrows to experimental manipulation of female sexual behavior. Hormones and Behavior 16, 323–329.

Moore, M.C., 1984. Changes in Territorial Defense Produced by Changes in Circulating Levels of Testosterone: A Possible Hormonal Basis for Mate-Guarding Behavior in White-Crowned Sparrows. Behaviour 88, 215–226.

Moreno, J., Veiga, J., Cordero, P., Minguez, E., 1999. Effects of paternal care on reproductive success in the polygynous spotless starling *Sturnus unicolor*. Behav. Ecol. Sociobiol. 47, 47–53.

Moss, R., Parr, R., Lambin, X., 1994. Effects of Testosterone on Breeding Density, Breeding Success and Survival of Red Grouse. Proceedings of the Royal Society B-Biological Sciences 258, 175–180.

N

Nakamura, M., 1998. Multiple mating and cooperative breeding in polygynandrous alpine accentors. II. Male mating tactics. Animal Behaviour 55, 277–289.

Nelson, R.J., 2011. An introduction to behavioral endocrinology. Sinauer Associates, Sunderland, MA.

Neri, R.O., Peets, E.A., 1975. Biological aspects of antiandrogens. Journal of Steroid Biochemistry 6, 815–819.

Nespor, A.A., Lukazewicz, M.J., Dooling, R.J., Ball, G.F., 1996. Testosterone induction of male-like vocalizations in female budgerigars (*Melopsittacus undulatus*). Hormones and Behavior 30, 162–169.

Nord, A., Nilsson, J.-A., 2011. Incubation Temperature Affects Growth and Energy Metabolism in Blue Tit Nestlings. The American Naturalist 178, 639–651.

Nur, N., 1984. The Consequences of Brood Size for Breeding Blue Tits II. Nestling Weight, Offspring Survival and Optimal Brood Size. The Journal of Animal Ecology 53, 497.

Nussey, D.H., Wilson, A.J., Brommer, J.E., 2007. The evolutionary ecology of individual phenotypic plasticity in wild populations. Journal of Evolutionary Biology 20, 831–844.

O

O'Neal, D.M., Reichard, D.G., Pavilis, K., Ketterson, E.D., 2008. Experimentally-elevated testosterone, female parental care, and reproductive success in a songbird, the Dark-eyed Junco (*Junco hyemalis*). Hormones and Behavior 54, 571–578.

Oring, L.W., Fivizzani, A.J., Elhalawani, M.E., 1989. Testosterone-Induced Inhibition of Incubation in the Spotted Sandpiper (*Actitis-Mecularia*). Hormones and Behavior 23, 412–423.

P

Panzica, G., Viglietti-Panzica, C., Sanchez, F., Sante, P., Balthazart, J., 1991. Effects of testosterone on a selected neuronal population within the preoptic sexually dimorphic nucleus of the Japanese quail. The Journal of Comparative Neurology 303, 443–456.

Peets, E., Henson, M., Neri, R., 1974. Mechanism of antiandrogenic action of glutamide (alpha-alpha-alpha-trifluoro-2-methyl-4'-nitro-m-propionotoluidide) in rat. Endocrinology 94, 532–540.

Perrins, C.M., 1991. Tits and their caterpillar food supply. Ibis 133, 49–54.

Perrins, C.M., McCleery, R.H., 1989. Laying Dates and Clutch Size in the Great Tit. The Wilson Bulletin 101, 236–253.

Peters, A., 2007. Testosterone treatment of female Superb Fairy-wrens Malurus cyaneus induces a male-like prenuptial moult, but no coloured plumage. Ibis 149, 121–127.

Poesel, A., Kunc, H.P., Foerster, K., Johnsen, A., Kempenaers, B., 2006. Early birds are sexy: male age, dawn song and extrapair paternity in blue tits, *Cyanistes* (formerly *Parus*) *caeruleus*. Animal Behaviour 72, 531–538.

Poissant, J., Wilson, A.J., Coltman, D.W., 2010. Sex-specific genetic variance and the evolution of sexual dimorphism: a systematic review of cross-sex genetic correlations. Evolution 64, 97–107.

R

Raouf, S.A., Parker, P.G., Ketterson, E.D., Nolan, V., Ziegenfus, C., 1997. Testosterone affects reproductive success by influencing extra-pair fertilizations in male dark-eyed juncos (Aves: *Junco hyemalis*). Proceedings of the Royal Society of London Series B-Biological Sciences 264, 1599–1603.

Reed, W.L., Clark, M.E., Parker, P.G., Raouf, S.A., Arguedas, N., Monk, D.S., Snajdr, E., Nolan, V., Ketterson, E.D., 2006. Physiological effects on demography: A long-term experimental study of testosterone's effects on fitness. The American Naturalist 167, 667–683.

Rhen, T., Ross, J., Crews, D., 1999. Effects of Testosterone on Sexual Behavior and Morphology in Adult Female Leopard Geckos, *Eublepharis macularius*. Hormones and Behavior 36, 119–128.

Rice, W.R., 1996. Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. Nature 381, 232–234.

Rice, W.R., Chippindale, A.K., 2001. Intersexual ontogenetic conflict. Journal of Evolutionary Biology 14, 685–693.

Richardson, D., Jury, F., Blaakmeer, K., Komdeur, J., Burke, T., 2001. Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*). Molecular Ecology 10, 2263–2273.

Ring, H.Z., Lessov, C., Reed, T., Marcus, R., Holloway, L., Swan, G., Carmelli, D., 2005. Heritability of Plasma Sex Hormones and Hormone Binding Globulin in Adult Male Twins. Journal of Clinical Endocrinology & Metabolism 90, 3653–3658.

Roberts, M.L., Buchanan, K.L., Evans, M.R., 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. Animal Behaviour 68, 227–239.

Roberts, M.L., Ras, E., Peters, A., 2009. Testosterone increases UV reflectance of sexually selected crown plumage in male blue tits. Behaioral .Ecology 20, 535–541.

Romanoff, A.L., Smith, L.L., Sullivan, R.A., 1938. Biochemistry and biophysics of the developing hen's egg. III. Influence of temperature. Cornell University Agricultural Experimental Station Memoranda 216, 1–42.

Rost, R., 1990. Hormones and Behavior - a Joint Examination of Studies on Seasonal-Variation in Song Production and Plasma-Levels of Testosterone in the Great Tit *Parus major*. Journal Fur Ornithologie 131, 403–411.

Rosvall, K.A., 2008. Sexual selection on aggressiveness in females: evidence from an experimental test with tree swallows. Animal Behaviour 75, 1603–1610.

Rosvall, K.A., 2013. Life History Trade-Offs and Behavioral Sensitivity to Testosterone: An Experimental Test When Female Aggression and Maternal Care Co-Occur. PLoS ONE 8, e54120.

Rubenstein, D.R., Wikelski, M., 2005. Steroid hormones and aggression in female galapagos marine iguanas. Hormones and Behaior. 48, 329–341.

Rutkowska, J., Cichon, M., Puerta, M., Gil, D., 2005. Negative effects of elevated testosterone on female fecundity in zebra finches. Hormones and Behavior 47, 585–591.

S

Saether, S.A., Saetre, G.-P., Borge, T., Wiley, C., Svedin, N., Andersson, G., Veen, T., Haavie, J., Servedio, M.R., Bures, S., Kral, M., Hjernquist, M.B., Gustafsson, L., Traff, J., Qvarnstrom, A., 2007. Sex Chromosome-Linked Species Recognition and Evolution of Reproductive Isolation in Flycatchers. Science 318, 95–97.

Saladin, V., Bonfils, D., Binz, T., Richner, H., 2003. Isolation and characterization of 16 microsatellite loci in the Great Tit *Parus major*. Molecular Ecology Notes 3, 520–522.

Sandell, M.I., 2007. Exogenous testosterone increases female aggression in the European starling (Sturnus vulgaris). Behavioral Ecology and Sociobiology 62, 255–262.

Scanes, C.G., 2000. Chapter 16 - Introduction to Endocrinology: Pituitary Gland, in: G. Causey Whittow (Ed.), Sturkie's Avian Physiology (Fifth Edition). Academic Press, San Diego, pp. 437–460.

Schifferli, L., 1973. The Effect of Egg Weight on the Subsequent Growth of Nestling Great Tits *Parus major*. Ibis 115, 549–558.

Schlicht, L., Girg, A., Loës, P., Valcu, M., Kempenaers, B., 2012. Male extrapair nestlings fledge first. Animal Behaviour 83, 1335–1343.

Schlinger, B.A., Callard, G.V., 1990. Aromatization mediates aggressive behavior in quail. General and Comparative Endocrinology 79, 39–53.

Schwabl, H., 1993. Yolk is a source of maternal testosterone for developing birds. Proceedings of the National Academy of Sciences 90, 11446–11450.

Searcy, W., Wingfield, J., 1980. The effects of androgen and anti-androgen on dominance and aggressiveness in male red-winged blackbirds. Hormones and Behavior 14, 126–135.

Searcy, W.A., 1988. Do Female Red-Winged Blackbirds Limit Their Own Breeding Densities. Ecology 69, 85–95.

Sheldon, B.C., 1994. Male Phenotype, Fertility, and the Pursuit of Extra-Pair Copulations by Female Birds. Proceedings of the Royal Society B-Biological Sciences 257, 25–30.

Silverin, B., 1980. Effects of long-acting testosterone treatment on freeliving pied flycatchers, *Ficedula hypoleuca*, during the breeding period. Animal Behaviour 28, 906–912.

Silverin, B., Baillien, M., Balthazart, J., 2004. Territorial aggression, circulating levels of testosterone, and brain aromatase activity in free-living pied flycatchers. Horm.Behav. 45, 225–234.

Slagsvold, T., 1993. Female-Female Aggression and Monogamy in Great Tits *Parus major*. Ornis Scandinavica 24, 155–158.

Slatkin, M., 1984. Ecological Causes of Sexual Dimorphism. Evolution 38, 622.

Smith, C.A., Roeszler, K.N., Ohnesorg, T., Cummins, D.M., Farlie, P.G., Doran, T.J., Sinclair, A.H., 2009. The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature 461, 267–271.

Soma, K.K., Scotti, M.-A.L., Newman, A.E.M., Charlier, T.D., Demas, G.E., 2008. Novel mechanisms for neuroendocrine regulation of aggression. Front. Neuroendocrinology 29, 476–489.

Sperry, T.S., Wacker, D.W., Wingfield, J.C., 2010. The role of androgen receptors in regulating territorial aggression in male song sparrows. Hormones and Behavior 57, 86–95.

Staub, N.L., DeBeer, M., 1997. The role of androgens in female vertebrates. General and Comparative Endocrinology 108, 1–24.

Strohbach, S., Curio, E., Bathen, A., Epplen, J.T., Lubjuhn, T., 1998. Extrapair paternity in the great tit (*Parus major*): a test of the "good genes" hypothesis. Behavioral Ecology 9, 388–396.

Stulp, G., Kuijper, B., Buunk, A.P., Pollet, T.V., Verhulst, S., 2012. Intralocus sexual conflict over human height. Biology Letters 8, 976–978.

Suarez, M.E., Wilson, H.R., McPherson, B.N., Mather, F.B., Wilcox, C.J., 1996. Low temperature effects on embryonic development and hatch time. Poultry Science 75, 924–932.

Svensson, L., 1984. Identification Guide to European Passerines. Stockholm.

Svensson, L., 1992. Identification Guide to European passerines. Stockholm.

T

Takahashi, L.K., 1990. Hormonal regulation of sociosexual behavior in female mammals. Neuroscience & Biobehavioral Reviews 14, 403–413.

Taylor, E., DeNardo, D., Jennings, D., 2004. Seasonal steroid hormone levels and their relation to reproduction in the Western Diamond-backed Rattlesnake, *Crotalus atrox* (Serpentes: Viperidae). Gen. Comp. Endocrinology 136, 328–337.

Tell, L.A., 1997. Excretion and metabolic fate of radiolabeled estradiol and testosterone in the cockatiel (*Nymphicus hollandicus*). Zoo Biology 16, 505–518.

Tinbergen, J., Boerlijst, M., 1990. Nestling weight and survival in individual great tits (*Parus major*). Journal of Animal Ecology 59, 1113–1127.

Tokarz, R., 1987. Effects of the antiandrogen cyproterone acetate and flutamide on male reproductive behavior in a lizard (*Anolis Sargrei*). Hormones and Behavior 21, 1–16.

Tokarz, R., Crews, D., 1980. Induction of sexual receptivity in the female lizard, *Anolis carolinensis*: Effects of estrogen and the antiestrogen CI-628. Hormones and Behavior 14, 33–45.

Trivers, R.L., 1972. Parental investment and sexual selection., in: Sexual Selection and the Descent of Man. Aladine, Chicago, pp. 136–179.

V

Van Doorn, G.S., 2009. Intralocus Sexual Conflict. Annals of the New York Academy of Sciences 1168, 52–71.

Van Duyse, E., 2004. Testoserone, male reproductive behaviour and fitness in the great tit (*Parus major*). Universiteit van Antwerpen.

Van Duyse, E., Pinxten, R., Eens, M., 2000. Does testosterone affect the trade-off between investment in sexual/territorial behaviour and parental care in male great tits? Behaviour 137, 1503–1515.

Van Duyse, E., Pinxten, R., Eens, M., 2002. Effects of testosterone on song, aggression, and nestling feeding behavior in male great tits, *Parus major*. Hormones and Behavior 41, 178–186.

Van Duyse, E., Pinxten, R., Eens, M., 2003. Seasonal fluctuations in plasma testosterone levels and diurnal song activity in free-living male great tits. General and Comparative Endocrinology 134, 1–9.

Van Oers, K., Buchanan, K.L., Thomas, T.E., Drent, P.J., 2011. Correlated response to selection of testosterone levels and immunocompetence in lines selected for avian personality. Animal Behaviour 81, 1055–1061.

Van Roo, B.L., 2004. Exogenous testosterone inhibits several forms of male parental behavior and stimulates song in a monogamous songbird: the blue-headed vireo (*Vireo solitarius*). Hormones and Behavior 46, 678–683.

Vedder, O., 2011. All's fair in love and war - Reproduction strategies, intra-specific competition and sexual selection in blue tits. University of Groningen.

Vedder, O., Magrath, M.J.L., Harts, A.M.F., Schut, E., Van der Velde, M., Komdeur, J., 2010a. Reduced extrapair paternity in response to experimental stimulation of earlier incubation onset in blue tits. Behavioral Ecology 21, 9–15.

Vedder, O., Schut, E., Magrath, M.J.L., Komdeur, J., 2010b. Ultraviolet crown colouration affects contest outcomes among male blue tits, but only in the absence of prior encounters. Funct. Ecol. 24, 417–425.

Veiga, J., Vinuela, J., Cordero, P., Aparicio, J., Polo, V., 2004. Experimentally increased testosterone affects social rank and primary sex ratio in the spotless starling. Hormones and Behavior 46, 47–53.

Veiga, J.P., Polo, V., 2008. Fitness consequences of increased testosterone levels in female spotless starlings. American Naturalist 172, 42–53.

Veiga, J.P., Vinuela, J., Cordero, P.J., Aparicio, J.M., Polo, V., 2004b. Experimentally increased testosterone affects social rank and primary sex ratio in the spotless starling. Hormones and Behavior 46, 47–53.

Vleck, C.M., Dobrott, S.J., 1993. Testosterone, Antiandrogen, and Alloparental Behavior in Bobwhite Quail Foster Fathers. Hormones and Behavior 27, 92–107.

Von Engelhardt, N., Groothuis, T.G.G., 2010. Maternal hormones in avian eggs, in: Hormones and Reproduction of Vertebrates. Academic Press, London.

W

Walsh, P., Metzger, D., Higucji, R., 1991. Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10, 506–513.

Walters, M.J., Harding, C.F., 1988. The effects of an aromatization inhibitor on the reproductive behavior of male zebra finches. Hormones and Behavior 22, 207–218.

Warren, R.P., Hinde, R.., 1959. The effect of oestrogen and progesterone on the nest-building of domesticated canaries. Animal Behaviour 7, 209–213.

Webb, D.R., 1987. Thermal Tolerance of Avian Embryos: A Review. The Condor 89, 874.

Whittingham, L.A., Dunn, P.O., Robertson, R.J., 1994. Female Response to Reduced Male Parental Care in Birds: An Experiment in Tree Swallows. Ethology 96, 260–269.

Williams, T.D., 2008. Individual variation in endocrine systems: moving beyond the “tyranny of the Golden Mean”. Philosophical Transactions of the Royal Society B-Biological Sciences 363, 1687–1698.

Williams, T.M., Carroll, S.B., 2009. Genetic and molecular insights into the development and evolution of sexual dimorphism. Nature Reviews Genetics 10, 797–804.

Wingfield, J., 1984. Androgens and mating systems - testosterone-induced polygyny in normally monogamous birds. Auk 101, 665–671.

Wingfield, J.C., Hegner, R.E., Dufty, A.M., Ball, G.F., 1990. The Challenge Hypothesis - Theoretical Implications for Patterns of Testosterone Secretion, Mating Systems, and Breeding Strategies. The American Naturalist 136, 829–846.

Wingfield, J.C., Lynn, S.E., Soma, K.K., 2001. Avoiding the “costs” of testosterone: Ecological bases of hormone-behavior interactions. Brain Behavior and Evolution 57, 239–251.

Wingfield, J.C., Visser, M.E., Williams, T.D., 2008. Introduction. Integration of ecology and endocrinology in avian reproduction: a new synthesis. Philosophical Transactions of the Royal Society B-Biological Sciences 363, 1581–1588.

Winkel, W., Winkel, D., 1980. Zum paarzusammenhalt bei Kohl-und Tannenmeise (*Parus major*, *P. caeruleus* und *P. ater*). Vogelwarte 30, 325–333.

Woodley, S., Moore, M., 1999. Ovarian Hormones Influence Territorial Aggression in Free-Living Female Mountain Spiny Lizards. Hormones and Behavior 35, 205–214.

Zysling, D.A., GreiveS, T.J., Breuner, C.W., Casto, J.M., Dernas, G.E., Ketterson, E.D., 2006. Behavioral and physiological responses to experimentally elevated testosterone in female dark-eyed juncos (*Junco hyemalis carolinensis*). Hormones and Behavior 50, 200–207.

Samenvatting

Inleiding

Bij veel diersoorten verschillen mannelijke en vrouwelijke individuen substantieel van elkaar in morfologie, fysiologie en gedrag. De biologische term hiervoor is seksuele dimorfie. Denk hierbij aan het verenkleed bij pauwen, de spiermassa bij mensen en het baltsgedrag van zangvogels. Verschillen in morfologie, fysiologie en gedrag tussen de geslachten zijn het gevolg van genetische en fysiologische mechanismen, zoals de aanwezigheid van geslachtschromosomen en -hormonen. Hormonen zijn signaalstoffen die afgegeven worden aan het bloed. Zij spelen een belangrijke rol bij het reguleren van morfologie, fysiologie en gedragingen van een individu.

Steroïde hormonen zijn van grote betekenis voor de regulatie van seksuele dimorfie. Het steroïde hormoon testosteron (T) speelt een belangrijke rol bij verschillende reproductieve gedragingen van mannetjes. In veel seizoenaal broedende zangvogels nemen de plasma T-waarden aan het begin van het broedseizoen toe. Deze toename valt samen met de periode waarin er veel territoriale agressie en paringen plaatsvinden. Na een experimentele verhoging van testosteron laten mannetjes een toename zien in territoriale agressie en paringsgedrag. Dit kan positive gevolgen hebben voor territorium grootte en broedsucces. Maar een verhoging van T kan ook negatieve gevolgen hebben, zoals de afname van ouderzorg en overleving. Na de piek in T-waarden aan het begin van het broedseizoen nemen de T-waarden dan ook langzaam af zodra de periode met ouderzorg begint. De T-waarden blijven daarna laag tot aan het begin van het volgende broedseizoen. Vrouwtjes van verschillende diersoorten laten dezelfde seizoenale variatie zien in T-waarden als mannetjes. Deze T-waarden zijn echter lager dan bij mannetjes,. In tegenstelling tot de mannelijke situatie, is er tot dusver weinig bekend over de functionele rol van T bij vrouwtjes.

Hypothese 1. T in vrouwtjes is bijproduct door selectie bij mannetjes (T bij vrouwtjes non-adaptief)

Interseksuele genetische correlatie zou één van de verklaringen zijn voor de huidige T-waarden en seizoenale variatie in T-waarden in vrouwelijke individuen. Het genoom van mannetjes en vrouwtjes is vrijwel gelijk. Deze interseksuele genetische correlatie zorgt ervoor dat natuurlijke of seksuele selectie voor een bepaald kenmerk in het ene geslacht, vaak een gecorreleerde respons oplevert in dezelfde richting in het andere geslacht. Dus wanneer selectie in het ene geslacht een kenmerk bevoordeelt dat nadelig is voor het andere geslacht, dan kan dit de evolutie van dit kenmerk afremmen in beide geslachten. Deze tegengestelde selectiedruk wordt antagonistische seksuele selectie genoemd. Uiteindelijk kan seksuele genetische correlatie ertoe leiden dat mannen en vrouwen méér gelijkaardig in fenotype worden dan ‘optimaal’ zou zijn voor elk geslacht afzonderlijk. Deze antagonistische seksuele selectie kan er ook toe leiden dat T-waarden in vrouwen mogelijk niet adaptief - of zelfs schadelijk –

zijn, wat de verdere toename van T in mannen en een afname van T in vrouwen kan beperken.

Hypothese 2. T in vrouwtjes is selectief neutraal (er vindt geen selectie plaats op T)

De evolutie van hetzelfde kenmerk in beide geslachten hoeft niet altijd beperkt te worden door het voorkomen van dezelfde genen bij mannen en vrouwen. Wanneer het niveau van een kenmerk geen invloed heeft op de fitness van het individu dan wordt dit kenmerk selectief neutraal genoemd. Een kenmerk kan in het ene geslacht evolueren naar een optimum, als bij het andere geslacht geen sprake is van selectie op ditzelfde kenmerk. Indien T-waarden in vrouwen een selectief neutraal kenmerk zijn, zal de hoogte van de waarden niet van invloed zijn op de fitness van vrouwten. De verdere evolutie van T in mannen is daarmee onafhankelijk van de expressie bij vrouwen.

Hypothese 3. T in vrouwtjes is een *adaptief* kenmerk. Er vindt effectieve selectie op plaats

Tot slot zouden de huidige T-waarden bij vrouwen verklaard kunnen worden door aan te nemen dat zij zijn aangepast aan de ecologie van de vrouw. Ergo, er zijn voordelen verbonden aan de productie van T als vrouw. Bijvoorbeeld, een toename van T aan het begin van het broedseizoen zou nodig zijn voor vrouwtjes om hun territoriale agressie te reguleren, net als bij mannetjes. Echter, om allerlei redenen zou het kunnen dat de optimale hoogte van T waardes verschilt tussen geslachten. Bij grote verschillen in T-optima kan antagonistische seksuele selectie een nivellerende werking hebben in de expressie. Dat kan in het volgende scenario. Als een kenmerk constant onder antagonistische seksuele selectiedruk staat, dan zal uiteindelijk natuurlijke en/of seksuele selectie die fenotypen bevoordelen die een minder sterke genetische correlatie tussen de sexen voor dat kenmerk vertonen. Dit komt omdat deze fenotypen niet beperkt worden in het tot uiting brengen van de optimale waarde voor een kenmerk door het andere geslacht. Hierdoor hebben deze fenotypen een hogere kans om te overleven en hun genen (via hun nakomelingen) door te geven aan de volgende generatie. Kortom, T zal onafhankelijk in mannen en vrouwen naar een optimale waarde kunnen evolueren wanneer de evolutie van T in beide geslachten niet beperkt wordt door genetische correlatie.

De functionele rol van een hormoon kan getest worden met behulp van het manipuleren van hormoonspiegels. Men kan onderzoeken of huidige T-waarden in evolutionaire zin optimaal zijn voor vrouwtjes. Hiertoe kunnen T-waarden experimenteel verhoogd worden met behulp van met T gevulde implantaten onderhuids aan te brengen. Dit doet men in één groep vrouwtjes, om vervolgens de fitness van deze

groep te vergelijken met de fitness van een niet gemanipuleerde controlegroep kan. Fitness kan gemekten worden als bijvoorbeeld: het aantal nakomelingen dat een vrouwtje produceerd en haar overleving. Tot nu toe heeft een aantal studies al aangetoond dat experimenteel verhoogde T bij vrouwtjes effect heeft op verschillende reproductieve parameters, zoals toename van agressie en afname van legselgroottes en aantal uitgevlogen jongen. Uit dergelijke studies wordt vaak geconcludeerd dat de huidige T-waarden in vrouwtjes het gevolg zijn van antagonistische seksuele selectie. Er zijn echter enkele methodologische bezwaren bij dergelijke studies. Ten eerste wordt het testosteron vaak over een langere periode verhoogd dan in de natuurlijke situatie. De implantaten worden namelijk niet verwijderd wanneer gestart wordt met broedzorg. Als er dan een effect van T op bijvoorbeeld het gedrag van vrouwtjes wordt gevonden later in het broedseizoen, zegt dit alleen iets over de tijdsperiode waarin vrouwtjes gevoelig zijn voor T. De verkregen resultaten verschaffen derhalve geen inzicht in de functionele rol van T bij het begin van het broedseizoen. Verder liggen de experimenteel verhoogde T-waarden vaak buiten het fysiologische bereik van de soort. Dit laat weliswaar toe te concluderen dat T-waarden tot het niveau van mannetjes niet adaptief zijn voor vrouwtjes, maar dergelijke studies geven geen uitsluitsel over de vraag of de huidige (d.w.z. veel lagere) T-waarden in vrouwtjes adaptief zijn. Daarnaast werd nog geen studie verricht die zowel verhoogde als verlaagde T-concentraties bij vrouwtjes in een natuurlijke populatie beschouwde. Dit is nodig om te testen of huidige T-waarde het gevolg zijn van antagonistische seksuele selectie. Namelijk, uitgaande van deze hypothese is de verwachting dat vrouwtjes met verhoogde T-waarden een lagere fitness moeten hebben dan de controlevrouwtjes. Maar daarnaast zou je ook verwachten dat vrouwtjes met verlaagde T-waarden een hogere fitness zouden moeten hebben dan controlevrouwtjes. Dit laatste verwacht je omdat deze vrouwtjes niet het optimale niveau voor hun T-waardes kunnen bereiken door antagonisctische seksuele selectie die veel hogere waardes in de mannetjes selecteert.

Doel en methoden

Om na te gaan wat de functionele rol is van T op het gedrag van vrouwtjes pimpelmezen (*Cyanistes caeruleus*), verhoogde ik daarom T-waarden binnen het fysiologische bereik van individuen in een natuurlijke populatie. Hierbij werd gebruik gemaakt van implantaten gevuld met T. In een andere groep verlaagde ik de effectiviteit van T door middel van implantaten gevuld met een androgeen receptor blokker (flutamide, Flu). Vervolgens vergeleek ik verschillende gedragingen, reproductieve succes en overleving van deze twee groepen met elkaar en met een controlegroep (C). Deze laatste had alleen lege implantaten had gekregen. Het reproductieve succes werd gemeten als het aantal uitgekomen eieren en uitgevlogen kuikens. De manipulatie vond plaats aan het begin van het broedseizoen. In deze periode neemt de

natuurlijke productie van T in vrouwtjes toe. Ze beginnen met nestbouw en verdedigen hun territoria. Direct na de start van de eileg, wanneer natuurlijk T-waarden dalen naar basale waarden, werden de implantaten verwijderd. Mijn predictie was dat Flu-vrouwtjes een hogere fitness hebben dan T- of C-vrouwtjes indien de huidige T-waarden het gevolg zijn van antagonistische seksuele selectie. Als C-vrouwtjes een hogere fitness hebben dan T-vrouwtjes en Flu-vrouwtjes, dan zijn de huidige T-waarden adaptief voor vrouwen . En als er geen verschil in fitness is tussen de experiementele groepen, dan is de selectie voor T-waarden neutraal in vrouwen.

Naast onderzoek naar de functionele rol van de piek in T-waarden onderzocht ik tevens in welke mate het seizoenale patroon van T-waarden adaptief is in vrouwtjes. Hiervoor heb ik de T-waarden experimenteel verhoogd door middel van implantaten in vrouwelijke koolmezen (*Parus major*) gedurende een langere periode dan dat van nature het geval is. Door deze aanpak kon ik onderzoeken of vrouwtjes nog steeds gevoelig waren voor verhoogde T-waarden na verlaging van hun natuurlijke T-waarden en wat de kosten en baten zijn van het al dan niet gevoelig blijven voor T. Ik voorspelde daarbij dat als vrouwtjes gevoelig bleven voor T en dit kosten met zich meebracht, dat dan het huidige seizoenale patroon van T adaptief is voor vrouwen.

Belangrijkste bevindingen

In **hoofdstuk 2** heb ik de effecten van verhoogde T en Flu onderzocht op onder andere nestbouwactiviteit, start van de eileg, territoriaal gedrag en reproductief succes van pimpelmeesvrouwtjes. De experimenteel verhoogde T-waarden hadden een sterk effect op de nestbouw. Nadat het nestmateriaal was verwijderd, direct na implantatie, begonnen de T-vrouwtjes gemiddeld twee dagen eerder met het herbouwen van hun nest vergeleken met C-vrouwtjes. Daarnaast waren de T-vrouwtjes eerder klaar met het bouwen van hun nest vergeleken met de andere twee groepen. Flu had geen significant effect op de nestbouwactiviteit van de vrouwtjes. De start van de eileg verschilde niet significant tussen de experimentele groepen. Naast de effecten op nestbouw hadden de experimenteel verhoogde T-waarden invloed op het territoriale gedrag. T-vrouwtjes waren agressiever tegenover een opgezet pimpelmeesvrouwtje dan de vrouwtjes van de andere experimentele groepen. Na het verwijderen van de implantaten werden geen verschillen gevonden tussen de experimentele groepen voor legselgrootte, fractie uitgekomen eieren, nestmortaliteit en aantal uitgevlogen kuikens. Er werd bijgevolg geen effect van Flu of T op het reproductieve succes van de vrouwtjes gemeten. Aan de hand van mijn resultaten uit dit experiment concludeer ik dat het onwaarschijnlijk is dat de huidige T-waardes bij vrouwtjes het gevolg zijn van antagonistische seksuele selectie. Omdat ik ook geen negatieve effecten vond in de verhoogde T-groep en de gedragseffecten van de verhoogde T-waardes als positief geïnterpreteerd kunnen worden, lijkt het mij waarschijnlijker dat T-waarden in vrouwtjes eerder adaptief zijn. De resultaten van

mijn studie staan in schril contrast met deze uit eerdere studies. Mogelijk oorzaak hiervan zijn de hierin toegepaste onnatuurlijk hogere waardes van T, bovendien over een onnatuurlijke tijdsinterval.

In **hoofdstuk 3** onderzocht ik de rol van T op het paringsgedrag van vrouwelijke pimpelmezen. Door van alle kuikens en hun ouders bloed af te nemen en daar het DNA van te analyseren kon ik precies achterhalen welk kuiken afkomstig was van een buitenechtelijke copulatie (d.w.z. met een andere man dan de sociale partner). Hierdoor kon ik het aantal buitenechtelijke kuikens over meerdere jaren meten, namelijk in het jaar dat de hormoonwaardes van de moeders waren gemanipuleerd en het jaar daarop. In de T-groep werden minder nesten gevonden met ten minste één buitenechtelijk jong dan in de Flu- en C-groepen, die bovendien onderling niet met elkaar verschilden. In het jaar volgend op de manipulatie was er geen verschil meer tussen de drie experimentele groepen. Deze resultaten wijzen erop dat experimenteel verhoogde T-waarden leiden tot een verlaging van het aantal buitenechtelijke jongen bij vrouwen. Hoe T het paringsgedrag van vrouwtjes pimpelmezen beïnvloedt, is niet geheel duidelijk. Een verklaring zou kunnen zijn dat de T-vrouwtjes minder aantrekkelijk worden gevonden. Deze verklaring heb ik onderzocht in een pilote experiment, waarbij mannetjes pimpelmezen konden kiezen tussen T- en Flu-vrouwtjes. Ik vond geen verschil in voorkeur van de mannetjes voor vrouwtjes tussen de experimentele groepen. Deze resultaten wijzen er op dat het lager aantal nesten met buitenechtelijke kuikens in de T-groep mogelijk niet verklaard kan worden door een verschil in aantrekkelijkheid tussen de experimentele groepen. Een alternatieve verklaringen zou kunnen zijn dat de vrouwtjes met experimenteel verhoogde T-waarden meer tijd investeerden met het weghouden van andere vrouwtjes in hun territorium en minder met het zoeken van buitenechtelijke paringen. Deze alternatieve verklaring lijkt plausiebel aangezien ik in de voorgaande studie (**hoofdstuk 2**) heb aangetoond dat T agressie verhoogt bij pimpelmees vrouwtjes.

In **hoofdstuk 4** heb ik onderzocht of een verhoging of een verlaging van T effecten had op het reproductieve succes en overleving van pimpelmeesvrouwen gedurende twee broedseizoenen. Ten eerst bleek dat zowel een verhoging van T-waarden als het blokkeren van T aan het begin van het broedseizoen geen effect had op het reproductieve succes van de vrouwtjes in beide jaren. Ten tweede had geen van de manipulaties een effect op de overleving van de vrouwtjes. Uit dit onderzoek blijkt dat het experimenteel verhogen van T-waarden aan het begin van het broedseizoen niet leidt tot de verlaging van fitness, maar dat het verlagen van de effectiviteit van T ook niet leidt tot een verhoging van de fitness van vrouwtjes, consistent met de resultaten uit hoofdstuk 2.

In **hoofdstuk 5** werd onderzocht of het seizoenale patroon van T-waarden adaptief is in vrouwtjes, met name of de afname van T tijdens de broedzorg functioneel is. Om dit te testen heb ik de T-waarden van vrouwtjes koolmezen voor een langere periode dan natuurlijk voorkomt gemanipuleerd: van vlak voor de start van eileg tot

het einde van het broedseizoen. Vervolgens heb ik gekeken naar de effecten van de hormoonmanipulatie op broedgedrag en reproductief succes. T had sterke negatieve effecten op de incubatietemperatuur van de vrouwtjes. De gemiddelde dag- en nachttemperatuur in de nesten van de T-vrouwtjes was drie graden lager dan in nesten van C-vrouwtjes. Deze lagere temperaturen hadden ook effect op het uitkomstsucces van de eieren van de T-vrouwtjes. Dit succes lag lager bij de eieren van T-vrouwtjes. Ook het aantal jongen dat uitvloog was lager in deze groep. Deze resultaten wijzen er op dat koolmeesvrouwtjes gevoelig blijven voor verhoogde T-waarden later in het broedseizoen, wanneer natuurlijke T-waarden al weer gedaald zijn naar hun basale waarde. De negatieve effecten van de blijvende gevoeligheid voor T laten zien dat het mogelijk adaptief is voor vrouwtjes om alleen maar een piek in T-waarden te hebben aan het begin van hun broedseizoen. Deze resultaten tonen een tegengesteld patroon dan dat van de mannetjes koolmezen. Deze bleken ongevoelig voor T later in het broedseizoen. Verschillen tussen geslachten in gevoeligheid voor T later in het broedseizoen kunnen erop wijzen dat dit kenmerk geëvolueerd is via geslachtsspecifieke processen.

Slotbeschouwing

Tijdens mijn onderzoek werd aangetoond dat een kortdurende verhoging van T binnen de fysiologische range effect heeft op agressie, nestbouw en paringsgedrag in vrouwtjes pimpelmezen. Er werd echter geen effect van T gevonden op reproductief succes of overleving. Daarnaast bleek dat een experimentele verlaging van de effectiviteit van T door middel van Flu, geen meetbare gevolgen had op de bestudeerde kenmerken, gedragingen, en fitnesscomponenten. Wanneer T op een natuurlijkere manier wordt gemanipuleerd, blijkt dit bijgevolg niet tot een meetbare verlaging in fitness te leiden. Daar tegenover staat dat het verlagen van de effectiviteit van T ook niet leidt tot een verhoging in fitness. In tegenstelling tot deze resultaten, heb ik in een andere experiment gevonden dat wanneer T voor een langere periode werd verhoogd buiten het fysiologische bereik van koolmeesvrouwtjes, T reproductief succes drastisch verlaagde. Op basis van deze gecombineerde resultaten concludeer ik dat de huidige T-waarden en seizoenale variatie in T hoogstwaarschijnlijk niet het gevolg zijn van seksuele antagonistische selectie. Aan de andere kant heb ik ook geen bewijs gevonden dat T-waarden in vrouwtjes op dit moment adaptief zijn. Al vond ik wel directe positieve effecten van T (positieve effecten op nestbouwactiviteit en agressie), dit heeft niet geleid tot blijvende voordelen (geen verhoging van het reproductieve succes en overleving) voor vrouwtjes. Dat laatste resultaat is echter mogelijk niet helemaal betrouwbaar aangezien voor het verkrijgen van significante effecten op fitness veelal veel grotere steekproeven nodig zijn. Daarom zou ik voorzichtig willen concluderen dat de huidige T-waarden in vrouwtjes pimpelmezen tijdens het paringsseizoen adaptief zijn of selectief neutraal.

Gearfetting

-Oerset troch Alle Radema-

Ynlieding

By in bulte diersoarten forskille manlike en froulike yndividuen substansjeel fan inoar yn morfology, fysiology en gedrach, sa as it fearrendek by pauwen, de spiermassa by minsken en it baltsgedrach by sjongfûgels. Forskillen yn morfology, fysiology en gedrach tusken de geslachten binne it gefolch fan genetyske en fysiologyske meganismen, sa as de oanwêsichhyt fan geslachtshormonen. Hormonen binne sinjaalstoffen dy't ôfjoun wurde oan it bloed. Trochdat de produksje derfan mei ûnder ynfloed stiet fan ûmjouwingsfaktoren en se it hiele lichem birikke, kinne se de morfology, fysiology en it hâlden en dragen fan in yndividu op in yntegrearre manear ôfstimme op syn/har ûmjowing.

Steroïde hormonen binne fan grutte bitsjutting foar de regulaesje fan seksuele dymorfy. It steroïde hormoon testosteron(T) spilet in wichtige rol by foskaat reproduktyf hâlden en dragen fan mantsjes. Yn in bulte seizoenael briedende sjongfûgels nimme de plasma T-wearden oan it bigjin fan it briedseizoen ta. Dizze tanimming falt togearre mei it tiidrek hweryn't der folle territoariale agresje en pearingen pleatsfine. Nei in eksperimintele forheging fan testosteron litte mantsjes in tanimming sjen yn agresje en pearingsgedrach. Mar in forheging fan T kin ek negative gefolgen hawwe, sa as de ôfnimming fan âldersoarch en oerlibjen. Nei de pyk yn T wearden oan it bigjin fan it briedseizoen nimme de T-wearden dan ek stadichoan ôf sa gau as de perioade mei âldersoarch bigjint. De T-wearden bliuwe dêrnei leech ta oan it bigjin fan it folgjende briedseizoen. Wyfkes fan forskate diersoarten litte deselde seizoenale faryaesje sjen yn T-wearden as mantsjes. Dizze T-wearden binne lykwols leger as by mantsjes, sels op pyknivo. Yn tsjinstelling ta by mantsjes, is der oant safier net folle bikend oer de funksjonele rol fan T by wyfkes.

Hypotheze 1. T yn wyfkes is ien byprodukt troch seleksje by mantsjes (T by wyfkes is net adaptyf)

Ien fan de forklearingen foar de hjoeddeiske T-wearden en seizoenale faryaesje yn T-wearden yn froulike yndividuen soe ynterseksuele genetyske korrelaesje wêze kinne. It genoom fan mantsjes en wyfkes is suver itselde. Dizze ynterseksuele genetyske korrelaesje soarget derfoar dat natuerlike of seksuele seleksje foar in bipaeld skaaimerk yn it iene geslacht, faeks in korrelearre respons opsmyt yn deselde rjochting yn it oare geslacht. Dus as seleksje yn it iene geslacht in skaaimerk bifoardielt dat neidielich is foar it oare geslacht, dan kin dit de evoluesje fan dit skaaimerk ôfremje yn beide geslachten. Dizze tsjinstelde seleksjedruk wurdt antagonistyske seksuele seleksje neamd. Uteinlik kin seksuele genetyske korrelaesje der ta liede dat mantsjes en wyfkes mear gelykaerdich wurde as dat optimael wêze soe foar elts geslacht ôfsûnderlik. Dizze antagonistyske seksuele seleksje kin der ek ta liede dat T wearden yn wyfkes mooglik net adaptyf of sels skealik binne, hwat de fierdere tanimming fan T

yn mantsjes en wyfkes en in ôfnimming fan T yn wyfkes biheine kin.

Hypotheze 2. T yn wyfkes is selektyf neutraal (der fyn gjin seleksje plak op T)

De evoluesje fan itselde skaaimerk yn beide geslachten hoecht net altiten biheint to wurden troch it foarkommen fan deselde genen by mantsjes en wyfkes. As it nivo fan in skaaimerk by, bygelyks, wyfkes gjin ynfloed hat op harren fithyt (bygelyks mjitten as it oantal neikommelingen dat se produsearje en harren oerlibjen), dan wurdt dit skaaimerk selektyf neutraal neamd. Dit hâldt yn dat as de mate fan ekspresje fan in skaaimerk gjin foar – of neidielen bisoarget yn it iene geslacht, dit skaaimerk frij is om to evolueren yn de rjochting dy 't optimael is foar it oare geslacht. Dus as hjoeddeiske T wearden yn wyfkes selektyf neutrael binne dan wurde de wearden yn wyfkes noch wol bipaeld troch deselde genen as yn mantsjes, mar de hichte fan de wearden is net fan ynfloed op de fithyt fan wyfkes. De fierdere evoluesje fan T yn mantsjes wurdt dus net biheind as T-wearden by wyfkes selektyf neutral binne.

Hypotheze 3. T yn wyfkes is ien adaptyf skaaimerk. T wurdt effektyf selektearre

By einbislút soene de hjoeddeiske T-wearden by wyfkes forklearre wurde kinne troch oan to nimmen dat se oanpast binne oan de ekology fan it wyfke. Bygelyks, in tanimming fan T oan it bigjin fan it briedseizoen soe nedich wêze foar wyfkes om harren territoariale agresje to regulearjen, krekt as by mantsjes. Lykwols, om allerhande reden soe it kinne dat de optimale hichte fa T-wearden forskilt tusken geslachten. As de optimale wearde fan in skaaimerk sterk foskilt tusken de geslachten, dan kin dat der ta liede dat antagonistyske seksuele seleksjedruk minder sterk wurdt. Dat kin yn it folgjende senario. As in skaaimerk konstant ûnder antagonistyske seksuele seleksjedruk stiet, dan sil úteinlik natuerlike- en/of seksuele seleksje dy mutanten bifoardiele, dy 't in minder sterke genetyske korrelaesje tusken de seksen foar dat skaaimerk fortoane. Dit komt om 't dizze mutanten net biheind wurde yn it ta uting bringe fan de optimale wearde foar in skaaimerk troch it oare geslacht. Hjirtroch hawwe de mutanten in hegere kâns om to oerlibjen en harren genen(troch harren neikommelingen) troch to jaen oan de folgjende generaesje. Koartsein, as de evoluesje fan T yn beide geslachten net biheind wurdt troch genetyske korrelaesje dan sil T ûnôfhinklik yn mantjes en wyfkes nei in optimale wearde evoluearje kinne.

De funksjonele rol fan in hormoon kin teste wurde mei help fan it manipulearjen fan hormoonspegels. Troch T eksperiminteel to forheegjen yn ien groep wyfkes en dêrnei de fithyt fan dizze groep to forgelykjen mei de fithyt fan in kontrôle, net manipulearre groep, kin men ûndersykje oft hjoeddeiske T- wearden optimael binne foar wyfkes. Oant nou ta hat in oantal studys al oantoand dat eksperiminteel forhege T by

wyfkes effekt hat op forskate reproduktive parameters, sa as tanimming fan agresje en âfnimming fan lechselgruttes en oantal útfleine jongen. Ut soksoarte studys wurdt faeks konkludearre dat de hjoeddeiske T-wearden yn wyfkes it gefolch binne fan antagonistyske seksuele seleksje. Der binne lykwols inkele biswieren by soksoarte fan studys. Earst wurdt it testosteron faeks oer in langere perioade forhege as de natuerlike perioade, om 't de ymplantaten net fuorthelle wurde as der bigoun wurdt mei briedsoarch. As der dan in effekt fan T op bygelyks it gedrach fan wyfkes foun wurdt letter yn it briedseizoen, seit dit allinnich hwat oer de tiidsperioade hweryn 't wyfkes gefoelich binne foar T. De bikommen resultaten forskaffe m.o.w. gjin ynsicht yn de funksjonele rol fan T bij it bigjin fan it briedseizoen. Fierder lizze de eksperiminteel forhege T-wearden faeks bûten it fysiologysk birik fan de soart. Dit lit wolriswier ta to konkludearjen dat T-wearden oant it nivo fan mantsjes net adaptyf binne foar wyfkes, mar soksoarte studys jouwe gjin útslútsel oer de fraech oft de hjoeddeiske (d.w.s. folle legere) T-wearden yn wyfkes adaptyf binne.

Dêrneist wurdt noch study dien, dy 't sawol forhege as forlege T- konsintraesjes by wyfkes yn in natuerlike populaesje biskôgde. Dit is nedich om to testen oft hjoeddeiske T-wearden it gefolch binne fan antagonistyske seksuele seleksje. Nammentlik, ûtgeande fan dizze hypothese, soene je forwachtsje dat wyfkes mei forhege T-wearden in legere fithyt hawwe moatte as de kontrôle wyfkes. Mar dêrneist soene je ek forwachtsje dat wyfkes mei forlege T-wearden in hegere fithyt hawwe moatte soene as de kontrôle wyfkes. Dit lêste forwachtsje je, om 't dizze wyfkes net it optimale nivo foar harren T-wearden birikke kinne,troch antagonistyske seksuele seleksje dy 't folle hegere wearden yn de mantsjes selekteard.

Doel en metoaden

Om nei to gean hwat de funksjonele rol is fan T op it gedrach fan wyfkesblaumiezen(*Cyanistes caeruleus*), forhege ik T-wearden binnen it fysiologyske birik fan soksoarte yndividuen troch middel fan ymplantaten fold mei T. Yn in oare groep forlege ik de effektiviteit fan T troch middel fan ymplantaten fold mei in androgeen reseptor blokker(flutamide, Flu). Forfolgens forgelike ik forskaat hâlden en dragen, reproduktyf sukses en oerlibjen fan dizze twa groepen mei inoar, en mei in kontrôle(K) groep dy 't allinnich ymplantaten krigen hie. It reproduktive sukses waerd mjitten oan 'e hân fan it oantal útbrette aeijen en útfleine piken. De manipulaesje foun pleats oan it bigjin fan it briedseizoen, as de natuerlike T produksje yn wyfkes tanimt en as se oan it nêstbouwen binne en harren territoria fordigenje. Fuortendaliks nei it bigjin fan de aeiliz, as natuerlike T-wearden dalen nei basale wearden, waerden de ymplantaten fuorthelle. Myn prediksje wie dat Flu-wyfkes in hegere fithyt hawwe as T- of C-wyfkes hwannear 't de hjoeddeiske T-wearden it gefolch binne fan antagonistyske seksuele seleksje. As C-wyfkes in hegere fithyt hawwe as T-wyfkes en Flu-wyfkes, dan binne de hjoeddeiske T- wearden adaptyf foar wyfkes. En as der gjin

forskil yn fithyt wie tusken de eksperimintele groepen dan is de seleksje foar T-wearden neutrael yn wyfkes.

Neist ûndersyk nei de funksjonele rol fan de pyk yn T-wearden ûndersocht ik ek yn hokker mate it seizoenale patroan fan T-wearden adaptyf is yn wyfkes. Hjirfoar haw ik de T-wearden eksperiminteel forhege troch middel fan ymplantaten yn froulike blausyskes(*Parus major*), yn de tiid fan in langere perioade as fan nature foarkomt. Troch dizze oanpak koe ik ûndersykje oft wyfkes noch hieltiten gefoelich wiene foar forhege T-wearden nei forleging fan harren natuerlike T-wearden en hwat de kosten en baten binne fan it al as net gefoelich bliuwen foar T. Ik foarsei dêrby dat as wyfkes gefoelich bleaunen foar T en dit kosten meibringe soe, dat dan it hjoeddeiske patroan fan T adaptyf is foar wyfkes.

Wichtichste útkomsten

Yn **haedstik 2** haw ik de effekten fan forhege T en Flu ûndersocht op ûnder oaren nêstbou aktiviteit, bigjin fan de aeiliz, territoariael gedrach en reproduktyf sukses fan blaumieskwyfkes yn forliking mei inoar en de kontrôlewyfkes. De eksperiminteel forhege T-wearden hawwe in sterk effekt op de nêstbou fan blaumieskwyfkes. Nei 't it nêstmateriael fuorthelle wie, fuortendaliks nei de ymplantaesje, bigounen de T-wyfkes troch inoar hinne nommen twa dagen earder mei herbouwen fan harren nêst, forlike mei C-wyfkes. Dêrneist wiene de T-wyfkes earder klear mei it bouwen fan harren nêst forlike mei de oare twa groepen. Flu hjat gjin signifikant effekt op de nêstbouaktiviteit fan de wyfkes. It bigjin fan de aeiliz forskilde net signifikant tusken de eksperimintele groepen. Neist de effekten op nêstbou hiene de eksperiminteel forhege T-wearden ynfloed op it territoriale gedrach fan blaumieswyfkes. T-wyfkes wiene agressiver tsjinoer in opset blaumieswyfke as de wyfkes fan de oare eksperimintele groepen. Nei it fuortheljen fan de ymplantaten waerden gjin forskillen foun tusken de eksperimintele groepen oangeande lechselgrutte, gedieltelik útkommen aeijen, nêstmortaliteit en oantal útfleine piken. Der waerd by gefolch gjin effekt fan Flu of T op it reproduktive sukses fan de wyfkes mjitten. Oan 'e hân fan myn resultaten út dit eksperimint konkludearje ik dat it ûnwierskynlik is dat de hjoeddeiske T-wearden by wyfkes it gefolch binne fan antagonistyske seksuele seleksje. Om 't ik ek gjin negative effekten foun yn de forhege T-groep en de gedrachseffekten fan de forhege T-wearden as posityf ynterpretearre wurde kinne, liket it my wierskynliker dat T-wearden yn wyfkes earder adaptyf binne. De resultaten fan myn study steane yn skril kontrast mei dizze út eardere study 's, dy 't únnatuerlike hegere wearden fan T oer in únnatuerlik tiidsynterfal hantearren.

Yn **haedstik 3** úndersocht ik de rol fan T op it pearingsgedrach fan froulike blaumiezen. Troch fan alle piken en harren âlden bloed ôf to nimmen en dêr it DNA fan to analysearjen koe ik sekuer achterhelje hokker pyk âfkomstich wie fan bûtenechtlike kopulaesje(d.w.s. mei in oar mantsje as de sosiale partner). Hjirtroch koe ik it oantal

bûtenechtlike piken oer mear jierren mjitte, nammentlik yn it jier dat de hormoonwearden fan de memmen manipulearre wienne en it jier dêrop folgjend. Yn de T-groep waerden minder nêsten foun mei minstens ien bûtenechtlik jong as yn de Flu- en en C-groepen, dy 't boppedat ûnderling net forskilden fan inoar. Yn it jier folgjend op de manipulaesje wie der gjin foskil mear tusken de trije eksperimintele groepen. Dizze resultaten wiize der op dat eksperiminteel forhege T-wearden liede ta in forleging fan it oantal bûtenechtlike jongen by wyfkes. Hoe 't T it pearingsgedrach fan wyfkesblaumiezen biynfloedet, is net hielendal dúdlik. In forklearing soe wêze kinne dat de T wyfkes minder oantreklik foun wurde. Dizze forklearing haw ik ûndersocht yn in "piloateksperimint" , hwerby 't mantsjesblaumiezen kieze koene tusken T- en Fluwyfkes. Ik foun gjin forskil yn foarkar fan de mantsjes foar wyfkes tusken de eksperimintele groepen. Dizze resultaten wiize der op dat it leger oantal nêsten mei bûtenechtlike piken yn de T-groep mooglik net forklearre wurde kin troch in forskil yn oantreklikens tusken de eksperimintele groepen. In alternative forklearring soe wêze kinne dat de wyfkes mei eksperiminteel forhege T-wearden, mear tiid ynvestearden yn it fuortreagjen fan oare wyfkes yn harren terrytoarium en minder mei it sykjen fan bûtenechtlike pearingen. Dizze alternative forklearring liket plausibel om 't ik yn de foargeande study (**haedstik 2**) oantoand haw dat T agresje forheget by wyfkesblaumiezen.

Yn **haedstik 4** haw ik ûndersocht oft in forheging of in forleging fan T effekten hat op it reproduktive sukses en oerlibjen fan wyfkesblaumiezen twa briedseizoenen lâns. As earste die bliken dat sawol in forheging fan T-wearden as it blokkeren fan T oan it bigjin fan it briedseizoen gjin effekt hat op it reproduktive sukses fan de wyfkes yn beide jierren. Twaddens hat gjin fan de manipulaesjes in effekt op it oerlibjen fan de wyfkes. Út dit ûndersyk docht bliken dat it eksperiminteel forheegjen fan T-wearden oan it bigjin fan it briedseizoen net liedt ta de forleging fan fithyt, mar dat it forleegjen fan effektiviteit fan T ek net liedt ta in forheging fan de fithyt fan wyfkes, konsistent mei de resultaten út haedstik 2.

Yn **haedstik 5** waerd ûndersocht oft it seizoenale patroan fan T-wearden adaptyf is yn wyfkes, mei nammen oft de ôfnimming fan T tiidens de briedsoarch funksjoneel is. Om dit to testen, haw ik de T-wearden fan wyfkesblaumiezen foar in langere peryoade as natuerlik foarkomt, manypulearre, fan krekt foar it bigjin fan de aeiliz oant it ein fan it briedseizoen. Dêrnei haw ik sjoen nei de effekten fan de hormoonmanypulaesje op briedgedrach en reproduktyf sukses. T hat sterke effekten op de ynkubaesjetemperatuer fan de wyfkes. De gemiddelde dei- en nachttemperatuer yn de nêsten fan de T-wyfkes wie trije graden leger as yn de nêsten fan C-wyfkes. Dizze legere temperaturen hawwe ek effekt op it útkomsukses fan de aeijen fan de T-wyfkes. Dit sukses lei leger by de aeijen fan T-wyfkes. Ek it oantal jongen dat útfleech wie leger yn dizze groep. Dizze resultaten wiize der op dat blaumieswyfkes gefoelich binne foar forhege T-wearden letter yn it briedseizoen, hwannear 't natuerlike T-wearden al wer sakke binnen nei harren basale wearde. De negative effekten fan de bliuwende gefoe-

lichhyt foar T litte sjen dat it mooglik adaptyf is foar wyfkes om allinne mar in pyk yn T-wearden to hawwen oan it bigjin fan it briedseizoen. Dizze resultaten toane in tsjinsteld patroan as dit biskrean by mantsjesblaumiezen. Dizzen, die bliken, wiene ûngefoelich foar T letter yn it briedseizoen. Forskillen tusken geslachten yn gefoelichhyt foar T letter yn it briedseizoen kinne der op wiize dat dit skaaimerk evoluearre is troch gelachtsspesifike prosessen.

Einbiskôging

Tiidens myn ûndersyk waerd oantoand dat in koartduorjende forheging fan T binnen de fysiologyske oarder effekt hat op agresje, nêstbou en pearingsgedrach yn wyfkesblaumiezen. Der wurdt lykwols gjin effekt foun op reproduktyf sukses of oerlibjen. Dêrneist die bliken dat in eksperimintele forleging fan de effektiviteit fan T troch middel fan Flu, gjin mjitbere gefolgen hat op de bistudearre skaaimerken, it hâlden en dragen en fithytskomponenten. As T op in natuerlikere wei manypulearre wurdt, blykt dit by gefolch net ta in mjitbere forleging fan fithyt to lieden. Dêr tsjinoer stiet dat it forleegjen fan de effektiviteit fan T ek net liedt ta in forheging fan fithyt. Yn tsjinstelling ta dizze resultaten, haw ik yn in oar eksperymint foun dat as T foar in langere peryoade forhege waerd bûten it birik fan wyfkesblaumiezen,T reproduktyf sukses drastysk forlege. Op groun fan dizze kombynearde resultaten konkludearje ik dat de hjoeddeiske T-wearden en seizoenale faryaesje yn T nei alle wierskyn net it gefolch binne fan seksuele antagonistyske seleksje. Oan de oare kant haw ik ek gjin biwiis foun dat T-wearden yn wyfkes op dit stuit adaptyf binne. Al foun ik wol direkte posytive effekten fan T(posytive effekten op nêstbouaktyviteit en agresje), dit liede net ta bliuwende foardielen(gjin forheging fan it reproduktive sukses en oerlibjen) foar wyfkes. Dat lêste resultaet is lykwols mooglik net hielendal bitrouber om ’t foar it krijen fan signyfikante fithytseffekten almeast folle gruttere stekproeven nedich binne. Dêrom soe ik foarsichtig konkludearje wolle dat de hjoeddeiske T-wearden yn wyfkesblaumiezen tiidens it pearingsseizoen adaptyf binne of selektyf neutrael.



LANDGOED VOSBERGEN
KGS
KRAUS-GROENEVELD STICHTING
• VRIJE WANDELING OP WEGEN EN PADEN VAN ZONSOPGANG TOT ZONSONDERGANG
• HONDEN AAN DE LIJN
OVERIGENS VERBODEN TOEGANG Art. 461 W.v.S.











SICCO
LEO
REIND
PETER
MAKE
OSCAR

Dankwoord
Acknowledgement

Het dankwoord. De plek om iedereen te bedanken die op welke manier dan ook heeft bijgedragen aan het voltooien van dit proefschrift. Eerst een lijstje maken, wie wel (of wie niet) te bedanken, glaasje wijn, pen in de hand en schrijven maar...

Een aantal mensen wil ik persoonlijk bedanken. Als eerste wil ik Luc bedanken. Zonder jou was dit proefschrift er nooit geweest. Jouw vertrouwen in mij en durf om mij aan te nemen zal ik nooit vergeten. Ook al was je meer een begeleider op afstand, ik waardeer je inbreng in mijn project zeer. Van de discussies waarbij je de advocaat van de duivel speelde heb altijd genoten. Bovendien hebben die zeker de kwaliteit van mijn proefschrift verhoogd.

Als tweede wil ik Jan bedanken. Ook jouw geloof in mij heeft er voor gezorgd dat ik mijn project tot een goed einde heb kunnen brengen. Je enthousiaste en menselijke manier van begeleidding heeft er voor gezorgd dat ik weer vertrouwen heb gekregen in mijn kunnen. Ik zal je “prima’s” bij track-changes missen.

Als derde wil ik Ton bedanken. Jij raakte als laatste bij mijn project betrokken en bleek onmisbaar te zijn. Zonder jouw kennis van mijn onderzoeksveld had mijn project zeker minder succesvol uitgepakt. Ik waardeer de tijd die je altijd probeerde te maken om te overleggen, ook al betekende dit dat je tot laat door moest werken. Ook heb ik veel plezier beleefd aan onze wetenschappelijke discussies over mijn resultaten en alles daarom heen.

Voordat ik verder ga met het bedanken van de mensen die mij de afgelopen drie jaar hebben geholpen, wil ik eerst een stapje teruggaan in de tijd. Zoals sommige van jullie weten, ben ik met mijn project begonnen in Antwerpen. Ook al heeft het grootste gedeelte van mijn onderzoek in Groningen/Gent plaatsgevonden, dit proefschrift was er nooit gekomen zonder de bijdrage van een aantal mensen in Antwerpen. Ann, heel erg bedankt voor je hulp in het veld en je vriendschap. Alex, ook heel erg bedankt voor je vriendschap en gastvrijheid. Verder wil ik Stefi, Evi, Alain, Hector en Jonas bedanken voor alle steun in moeilijke tijden, maar ook voor de plezante avonden en sportieve momenten die we samen hebben gehad.
Het doen van veldonderzoek is een zware job. Zonder alle assistenten, mede-onderzoekers en studenten had ik die klus nooit geklaard. Daarom bedankt voor alle hulp: Peter W., Oscar, Peter K., Elske, Wouter, Amarins, Maarten, Katrien, Toke, Christiaan, Vincent, Sicco, Leo, Reinder, Eric en Thijs.
Bonnie en Ilse, bedankt voor jullie hulp in het lab. Roelie, Sjoerd, Saskia en alle andere dierverzorgers, bedankt voor het verzorgen van de pimpelmezen, ook toen alle andere dieren al verhuisd waren.

Als geen ander weet ik dat de werkplek ook een belangrijke rol speelt in het slagen van een project. Mijn collega’s in Groningen hebben mij vanaf het begin thuis doen voelen, mede dankzij mijn kamergenoten: Martijn, Janne, Janske, Reinder, Elske,

Oscar, Lucie, Frank, Sjouke, Minke, Isabella, Seyed Mehdi en Jildou. Natuurlijk wil ik ook de rest van Dierecologie, BESO en diergedrag bedanken voor de fijne tijd die ik Groningen heb gehad. In het bijzonder wil Martina bedanken. Martina, thank you for being my partner in crime. Nathan and Bram thank you very much for your comments and suggestions on my thesis. Leave Alle, tige betanke datsto yn sa ien koarte tiid myn gearfeting hast oerset nei it Frysk. Ik hie it sels nea kind.

Ook wil ik de Terec vakgroep in Gent bedanken. Ondanks dat ik maar voor korte periodes aanwezig was, voelde ik mij wel altijd onderdeel van de groep. Hartelijk dank daarvoor.

Verder wil ik mijn paranimfen, Aafke en Janne, bedanken. Aafke, vanaf ons eerste jaar biologie konden we het al goed vinden. Ook al spreken we elkaar minder door de afstand, ik waardeer onze vriendschap zeer. Heel erg bedankt dat je mijn paranimf wilt zijn. Ik ben benieuwd naar onze toekomst samen bij het jeugdnatuurbureau. Janne, toen ik je vroeg als paranimf was je dat nog nooit geweest. Maar nu ben je dat al voor de tweede keer en na mij zeker nog één keer (heb je al een spaarkaart?). Eén ding weet ik zeker, dat mijn promotie in goede handen is bij iemand die al zo ervaren is. Daarnaast wil ik je ook bedanken voor alle steun op de momenten dat ik er doorheen zat. En voor alle leuke en gezellige eigen huis- en tuinactviteiten.

Natuurlijk wil ik ook de mensen bedanken die niet direct hebben bijgedragen aan mijn proefschrift. Leave Doede en Kicky, bedankt foar jim steun, fertrouwe en leafde. Sûnder sokke âlder wie ik noait sa fier kommen. Leave Amarins en Jesse tige betanke foar jimme help yn it fjild en dêr bûten. Ik hoopje dat ik no wer mear tiid ha om by jimme, Marcel, Pepijn, Merel en Abel lânse te komen. Lieve Hans, Hilde, Matthijs en Hanne, bedankt voor de gastvrijheid, en voor de gezellige avonden en uitstapjes. Ik hoop dat er nog vele zullen volgen. Lieve Bonobo's, jullie zijn de leukste, vreemdste en gezelligste huisgenoten die iemand maar kan wensen. Bedankt voor jullie begrip dat ik niet altijd kon mee kolonisten, omdat ik moest overwerken ;-).

Tot slot wil ik graag Wouter bedanken (ja ik heb je toch aan het einde gedaan :-P). Bedankt voor al je steun in moeilijke tijden, je geduld, hulp en zetten in de rug. Ik ben benieuwd naar onze toekomst samen. En ook al is het verleden daar geen garantie voor, zie ik hem met veel vertrouwen te gemoed.

Ik zie u graag, dikke tút!

Haren, 10 oktober 2013

List of authors

Prof. dr. Veerle M. Darras
Laboratory of Comparative Endocrinology, K.U. Leuven, Leuven, Belgium

Prof. dr. Marcel Eens
Research Ethology Group, University of Antwerp, Antwerpen, Belgium

Prof. dr. Ton Groothuis
Behavioural Biology Group, Centre for Behaviour and Neurosciences, University of Groningen, the Netherlands

Prof. dr. ir. Jan Komdeur
Behavioural Ecology and Self Organization, Centre for Ecological and Evolutionary Studies, University of Groningen, the Netherlands

Prof. dr. Luc Lens
Terrestrial Ecology Unit, Department Biology, Ghent University, Belgium

Dr. Oscar H. Vedder
Institute of Avian Research 'Vogelwarte Helgoland', An der Vogelwarte 21, D-26386, Wilhelmshaven, Germany

Dr. Marco van der Velde
Behavioural Ecology and Self Organization, Centre for Ecological and Evolutionary Studies, University of Groningen, the Netherlands

M. Amini Nasab
Behavioural Ecology and Self Organization, Centre for Ecological and Evolutionary Studies, University of Groningen, the Netherlands

Dr. Kees van Oers
Animal Ecology, Netherlands Institute of Ecology, Wageningen, the Netherlands

Prof. dr. Rianne Pinxten
Research Ethology Group, University of Antwerp, Antwerpen, Belgium