Plasma testosterone levels of male European starlings (Sturnus vulgaris) during the breeding cycle and in relation to song and paternal care

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Summary
In temperate-zone birds, seasonal testosterone (T) profiles often vary among species with different mating systems. The trade-off hypothesis suggests that T levels in male birds represent a trade-off between additional mating effort, stimulated by high T, and paternal effort, inhibited by high T. To study the role of T in mediating the trade-off between paternal and mating effort in the facultatively polygynous European starling (Sturnus vulgaris), we monitored seasonal fluctuations in T levels and song activity, which functions mainly in mate attraction, and we investigated natural covariation between plasma T levels and male parental and song behaviour during the parental phase. T concentrations peaked during nest building, remained high during the fertile period, and then decreased gradually during the period of paternal care. This resembles the profile typically found in polygynous species with biparental care. Seasonal variation in song activity paralleled seasonal changes in T. The proportion of time spent incubating was significantly negatively correlated with T levels in individual males. There was a positive, but non-significant, correlation between song activity at an additional nest box and T levels during incubation. We suggest that this negative correlation between T and incubation behaviour may be partly explained by T increases in males responding to the presence of prospecting females with additional nestbox occupation and mate attraction song, rather than that high T levels stimulate mate attraction song. During the feeding period, characterized by a very low availability of unmated females, variation in T levels was low and there was no covariation between T and feeding. T levels during the parental phase reflect, but do not seem to directly regulate, the trade-off between parental and additional mating effort.

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and other factors such as opportunities for additional matings may influence the resolution of this reproductive trade-off.

Keywords: testosterone, bird song, hormone-behaviour relationships, trade-off.

Introduction

In species where both sexes contribute to one or more aspects of parental care, males must find a balance between investing in paternal care and seeking additional mating opportunities (Ketterson & Nolan, 1994, 1999). Often the opportunity to gain additional matings coincides with periods when males are already providing parental care, implying that males are confronted with the dilemma of how best to allocate time and energy (effort) to these alternative behaviours. This predicted trade-off between parental effort and additional mating effort has attracted much interest in recent years in many taxa including humans (Wingfield et al., 1990; Ketterson et al., 1996; Magrath & Komdeur, 2003; Gray et al., 2006).

Trade-offs in vertebrate life histories are likely to be hormonally mediated since hormones typically influence many behaviours simultaneously (Adkins-Regan, 2005). In birds, the steroid hormone testosterone (T) has been studied intensively because it affects a wide range of behaviours central to male life-history trade-offs (Ketterson et al., 1996). Given that elevated levels of T in male birds have been shown to promote mating behaviours, but also to suppress aspects of parental care, it has often been suggested that T directly mediates the parental effort-mating effort trade-off (Ketterson et al., 1996; Magrath & Komdeur, 2003). In birds, where polygyny and extra-pair fertilizations often are an important component of male reproductive success (Magrath & Komdeur, 2003; Garamszegi et al., 2005), the role of T in this trade-off has been studied intensively in the context of seasonal patterns in androgen levels that seem to vary as a function of mating system, male–male aggression and paternal care (Goymann et al., 2007).

In most temperate-zone birds, males of socially monogamous species with a high degree of paternal care have a single peak of T levels during the first part of the breeding season when territories are established and females are courted, but then show an abrupt decline in T during the parental phase. In contrast, males of polygynous species with little or no paternal care exhibit sustained secretion of T at peak levels for most of the breeding season.
Finally, males in polygynous species with biparental care show a more gradual decline of T during the parental stage (Beletsky et al., 1995; Wada et al., 1999). These observations suggested that fluctuations in T during the breeding season reflect opposite relationships between T levels and several male reproductive behaviours with competing demands for time and energy (Wingfield et al., 1990; Beletsky et al., 1995; Ketterson et al., 1996).

The ‘trade-off hypothesis’ has been further supported by many hormone manipulation studies demonstrating that males with experimentally elevated T levels during the parental phase indeed showed reduced paternal care (Beletsky et al., 1995; de Ridder et al., 2000; Peters et al., 2002; Schoech et al., 1998; van Roo, 2004; but see van Duyse et al., 2000; Lynn et al., 2005 for exceptions) and, at the same time, increased song and other sexual and aggressive behaviours (Stoehr & Hill, 2000; van Duyse et al., 2000, 2002). Until now, very few studies have examined correlations between natural plasma T levels and both mate-attraction and parental effort in unmanipulated males to elucidate further the role of T in mediating male reproductive trade-offs (Adkins-Regan, 2005).

The European starling (*Sturnus vulgaris*) is a facultatively polygynous songbird (Pinxten & Eens, 1990). Monogamous males provide extensive parental care during both incubation and nestling rearing (Pinxten et al., 1993; Sandell et al., 1996). Polygynous male starlings mainly attract additional females during the laying, incubation or early nestling period of their first clutch. They predominantly assist their primary females in incubation and feeding nestlings, but nevertheless primary females usually receive less male assistance than monogamous females (Pinxten & Eens, 1994). Parental investment strongly varies among males, making the starling an appropriate species for studying the possible role of T in regulating the balance between mating and paternal effort during the parental phase.

In the present study, we investigated the relationship between plasma T levels and the expression of male behaviour in free-living starlings. Specifically, we examined seasonal fluctuations in plasma T concentrations and song activity (as a measure of mate attraction behaviour). We also investigate the relationship between T levels and male incubation and feeding behaviour. Studies investigating the association between T and song production are scarce in free-living male songbirds (Rost, 1990; Weatherhead et al., 1993; Saino & Möller, 1995; Johnsen, 1998; see Gil & Gahr, 2002) and, as far as we know, no previous studies have investigated individual correlations between plasma T levels and incubation or feeding behaviour.
Materials and methods

Study areas and field methods

This study was carried out in different nest box colonies around Antwerp, Belgium, during 1998-2000. For a description of these colonies, see Pinxten et al. (1989) and de Ridder et al. (2000). The studied population is usually single-brooded and first clutches are always laid highly synchronously (Pinxten et al., 1990). Each year, starlings were caught as soon as possible after their arrival in the colonies to mark them with individually numbered metal and colour rings, allowing easy identification in the field. We captured the starlings while they were roosting in the nest boxes at night (only during the pre-nesting stage; see below), with traps while they were visiting the nest boxes, or with a long stick on which a metal plate was fixed to close the opening of the nest box (only during the incubation stage; see below). All birds were sexed by using bill and eye colour, and we also determined age (first-year versus older; Svensson, 1984). Blood samples were collected from males throughout the breeding period which was divided into five reproductive stages: (1) pre-nesting (beginning of February until mid-March): males had arrived in the colony but were not yet defending a nest box against other males; (2) nest building (mid-March until the beginning of April): males had occupied nest boxes and attracted a female, and nests were being built; (3) fertile: from five days before the first egg is laid until the day the penultimate egg is laid (Pinxten & Eens, 1997); (4) incubation (eggs are incubated for about 11-12 days) and (5) nestling feeding (starling nestlings fledge at about 21 days of age). Information on the breeding stage of each male was obtained from daily inspections of the nest boxes and from behavioural observations. Male ownership of the nest boxes, mate attraction behaviour (male starlings trying to attract a female spend a lot of their time singing close to or into their nest box), attempts at polygyny (when males were singing at a nest box other than the nest box in which their mate was building a nest or had initiated a clutch) and paternal care activities were evaluated frequently (see also below). Blood was sampled by puncturing the alar vein. We collected approximately 400 µl blood into heparinised haematocrit capillary tubes immediately after capture. The blood samples were then stored temporarily in a cool box filled with freezer packs. Within 6 h, they were centrifuged at 7000 rpm for 13 min and the plasma was stored at −70°C until hormonal assay was performed. Since some starlings are ‘trap shy’, it
was impossible to capture all birds during all reproductive stages (see also Ball & Wingfield, 1987). We sampled as many birds as possible during each breeding stage. Totally, we captured 84 individuals. Most males \( N = 57 \) were captured only once, while 19 males were captured twice, seven three times and one four times. Average time span between repeated capture of the same male was 17 days (range: 4-77 days). All research was carried out in accordance with the laws and ethical guidelines of Flanders and Belgium. Results from earlier studies indicate that behaviour and survival are not affected by the blood sampling technique used in this study (Ball & Wingfield, 1987; de Ridder et al., 2000).

**Behavioural observations**

In 1998 and 2000, we observed male song activity and paternal care activities to study the relationship between these behaviours and plasma T levels in individual birds. Observations were made with a telescope from permanent or transportable hides. Males’ behaviour was recorded on cassette tape and later transcribed. Singing behaviour was observed during the nest building, fertile, incubation and feeding stage. During the nest building and fertile stage, we watched each male for 15-30 min. Males were observed for 2 h during incubation, and for 1 h during the feeding period. Every minute, we recorded at the signal of an electronic timer whether the males were singing or not (Eens et al., 1990, 1994). Therefore, we could determine the proportion of time spent singing for each male. In all cases, we also noted where a male was singing. Incubation behaviour was recorded on days 5-6 (1 h) and on days 8-9 (1 h) in 1998 (average values were used for statistical analysis; differences between both days were not significant; Wilcoxon matched pairs signed ranks test: \( z = -0.53, p = 0.59 \)), while in 2000 observations were made on days 5-6 (2 h). An observation period started when the male or the female entered the nest box. We measured the total proportion of time spent in the nest box by the male. In 1998, we collected blood on days 4-6 of the incubation period. With the exception of two individuals, blood samples were taken after the first observation period. In 2000, blood samples were always taken immediately after observation (days 5-6). Incubation behaviour was observed at 15 nests. Two males succeeded in attracting an additional female. One of them attracted an additional female during the incubation period of his first mate while the other one attracted a second female during the
fertile period of his first mate. Since we could observe nest boxes of both the primary and secondary female together, we could determine that this male did not assist his secondary female in incubating the eggs. For both males we used only the observations made at the nest of the primary female. During the feeding period, we recorded male feeding rates for 1 h on days 12-14 in 1998 and on days 12-13 in 2000. Blood samples were collected on days nine-ten in 1998 and on days 13-14 in 2000 (the latter always after behavioural observation). Feeding efforts of 11 males were monitored. Two of the males of which we recorded feeding rates were observed to feed the nestlings without assistance of their females. We excluded these males from the statistical analysis, because they probably compensated in terms of feeding trips for the lack of female help in feeding. All observations took place between 0700 and 1500 h in all weather, except steady rain.

Hormone assay

Plasma levels of T were quantified using a commercial double antibody system purchased from ICN (Costa Mesa, CA, USA). Briefly, in order to avoid interference from plasma proteins, 50 µl of plasma from each sample was extracted and added to 400 µl of a 50:50 mixture of cyclohexane-ethylacetate of which 300 µl was then evaporated by vacuum centrifugation. The dried samples were then diluted by PBS containing 0.1% BSA and further treated as recommended by the manual provided by the manufacturer (see Eens et al., 2000). The specification sheets provided by the company indicate that 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%). T standards ranged from 0.02 to 10 ng/ml but the effective detection interval ranged from 0.013 to 6.67 ng/ml, owing to the concentration effect of the extraction procedure. The intra-assay coefficient of variation was 6.6% while the inter-assay coefficient of variation was 10.1%.

Statistical analyses

Analyses were conducted using the statistical software programs SAS/STAT (SAS, 1988) and SPSS/PC (SPSS, 1986). All statistical analyses followed procedures outlined in Siegel (1956) and Sokal & Rohlf (1981). To investigate seasonal changes in plasma T concentrations and song activity, we
performed three-way repeated measures ANOVAs (mixed model – REML procedure) with reproductive stage, year and age as fixed factors. Because repeated observations were made on single individuals, residual values may be correlated. We therefore explicitly modelled these potential correlations and compared five types of correlation structure (i.e., no correlation, compound symmetry, serial autocorrelation, toeplitz and unstructured) by likelihood ratio test. After selecting the ‘most likely’ correlation structure, the number of degrees of freedom of the $F$-tests of the fixed effects were adjusted using sattertwaite formulas (Littell et al., 1996). Comparisons between reproductive stages were made using Tukey post-hoc tests. Due to a low number of second-year birds in some reproductive stages, three-way interactions could not be tested. ANCOVAs with male incubation or feeding behaviour as response variable, year as factor and T as covariate were used to investigate the relationship between T levels and paternal care activities. In 1998, the incubation behaviour of two males was observed one day after the birds were captured. As this capture may have affected a male’s incubation behaviour afterwards, we also re-analysed the relationship between incubation behaviour and T concentrations, excluding observations made after capture. The relationship between T and song activity during the incubation phase was analysed by logistic regression analysis with song activity as response variable (whether or not a male was seen to sing). In all analyses, average values were calculated for each reproductive stage for each individual to avoid problems with pseudoreplication. To meet assumptions required for parametric statistics, proportions were subjected to arcsine square-root transformation while T levels were log-transformed. When our data did not meet the criteria for the use of parametric statistics, we used non-parametric tests. Differences were considered significant when $p < 0.05$. All statistical tests are two-tailed, and values are presented as means $\pm$ SE.

**Results**

*Seasonal changes in T concentrations and song expression*

A three-way ANOVA revealed a highly significant effect of reproductive stage on plasma T concentrations of male starlings ($F_{4,36} = 7.87, p = 0.0001$; Figure 1). Tukey post-hoc tests showed that T concentrations during feeding were significantly lower than those during the pre-nesting, nest
building and fertile period. Incubation T levels were significantly lower than nest building levels. All other differences between reproductive stages were non-significant. Age and all two-way interactions had no significant effect ($p > 0.15$ in all cases). Although there was a significant year effect ($F_{2,35} = 7, p = 0.004$), the profile of the T concentrations did not differ significantly between years ($p > 0.2$ for all interactions of year with the other variables). Therefore, data of separate years could be pooled.

Figure 2 shows changes in male song activity during the breeding season. Differences in song expression between reproductive stages were highly significant ($F_{3,27} = 9.13, p = 0.0003$). Tukey post-hoc tests showed that the proportion of time spent singing during incubation and feeding was significantly lower than the proportion of time spent singing during the nest building and fertile stages. Year, age and two-way interaction effects were not significant ($p > 0.2$ in all cases).

**Paternal care and song in relation to T**

**Incubation period**

Figure 3 presents the relationship between the proportion of time spent incubating and plasma T levels. Analysis of covariance revealed that the correlation between the proportion of time spent incubating and plasma T concentrations was negative and significant ($F_{1,13} = 7.36, p = 0.017$). The interaction between year and T was not significant ($F_{1,11} = 1.09, p = 0.32$),
Figure 2. Male song activity (proportion of time spent singing) during the nest building (NB), fertile, incubation and feeding period. Data shown are mean ± SE. Figures denote sample sizes.

Figure 3. Male incubation behaviour (proportion of time spent incubating) in relation to plasma T concentrations. Open symbols represent males of 1998, while full symbols represent males of 2000. Circles represent males that were not observed singing during the incubation period, and triangles represent males that showed song activity at an additional nest box.

which means that statistical analysis could be done for both years together. When considering only observations made before any capture, our data also showed a significant negative relationship between the proportion of time spent incubating and T levels ($F_{1,11} = 4.75$, $p = 0.05$). The interaction effect was also not significant ($F_{1,9} = 0.06$, $p = 0.82$), which allowed us to pool the data of separate years. Four out of the 15 (27%) males were observed singing at an additional nest box during the incubation period (Fig-
Figure 4. Absolute feeding rates (number of male feeding trips/hour) in relation to plasma T concentrations. Open symbols represent males of 1998, while full symbols represent males of 2000. Circles represent males that were not observed singing during the feeding period, and triangles represent males that showed song activity at an additional nest box.

The relationship between song activity and T during incubation was positive, but not statistically significant (logistic regression analysis; change in deviance $= 2.5$, df $= 1$, $p = 0.11$). Since year did not prove to be significant (logistic regression analysis; change in deviance $= 1.07$, df $= 1$, $p = 0.3$), data from both years were pooled.

Discussion

Seasonal pattern of T and song

In our starling population, T increased in the beginning of the breeding season and peaked during the nest building and fertile period when males were defending nest boxes, attracting females and guarding their mates.
This early season peak in T levels is consistent with the pattern reported in males of most north-temperate species studied to date (Wingfield & Farner, 1978a,b; Morton et al., 1990; Johnsen, 1998). T gradually decreased during the parental stage in our population: T levels during the incubation period did not differ from those during the fertile period. This seasonal T pattern differs from that observed in strictly monogamous species showing a sharp(er) decline at the onset of paternal behaviours (Wingfield et al., 1990). The decrease in T in our population was also much less pronounced than in starling populations in England (Dawson, 1983) and the United States (Ball & Wingfield, 1987), where polygyny attempts are absent or very rare (Wright & Cuthill, 1990; Pinxten et al., 1989). These findings are consistent with the ‘challenge hypothesis’ (as recently revisited by Goymann et al., 2007), stating that not only male-male interactions over territories and mates (as originally proposed by Wingfield et al., 1990, 2000), but also androgen responses to receptive females (Peters et al., 2001) and to non-social environmental cues (such as ownership of nest boxes) tend to drive T secretion up, whereas expression of parental care by males requires that T declines. Male starlings have indeed been experimentally shown to respond to the presence of females and to nest box ownership with an increase in T levels (Gwinner et al., 1987, 2002; Pinxten et al., 2003). The presence of prospecting females during the incubation and early nestling period (Pinxten et al., 1989; Pinxten & Eens, 1994), and male-male aggression over access to these females and particularly ownership of additional nest boxes to attract them (Gwinner et al., 1987, 2002) may thus be responsible for the more gradual decline in T during the parental phase in our population (see below). Our finding that seasonal changes in the T profile paralleled changes in song activity, which mainly functions in mate attraction (Eens et al., 1991, 1993; Eens, 1997), supports this view.

Role of T in mediating the trade-off between mating and paternal behaviour

In (polygynous) birds with biparental care, males may face a trade off between parental effort and additional mating effort (through extra social mates or extra-pair copulations) during the parental phase. The trade-off hypothesis (derived from the challenge hypothesis explaining variation in seasonal T patterns) suggests that male plasma T directly regulates (at the proximate level) the allocation of reproductive effort between mating behaviour (requiring high T levels) and paternal behaviour (requiring low T levels) during
the parental phase (Ketterson et al., 1996). A previous experimental study in our population suggested that T mediates, at least partly, the trade-off between mating and parental effort in male starlings. Elevated T levels during the parental phase decreased both incubation and feeding behaviour, and induced a shift towards mate attraction behaviour during the incubation period but not during the nestling period. The latter may be explained by the strongly reduced opportunities to attract additional females during this period (de Ridder et al., 2000). Therefore T is probably not the only factor underlying the allocation of reproductive effort between mating and parental behaviour in starlings and social factors such as the availability of unmated females may also be important.

In this study we used a correlative approach to investigate the role of T in mediating the reproductive trade-off. We found that natural T-levels were significantly negatively correlated with male incubation effort, while there was no relation with feeding effort. As far as we know, our study is the first to report a negative association between natural T and the proportion of time spent incubating in a male songbird. Our correlational data thus confirm previous experimental data that high T suppresses incubation behaviour in starlings and other birds (Oring et al., 1989; de Ridder et al., 2000; McDonald et al., 2001; van Roo, 2004). The mechanism by which (experimentally elevated) early season peak T-levels during the parental phase might interfere with care behaviour in birds remains unclear at present (Schoech et al., 1998), but the preponderance of information suggests that full expression of paternal care occurs only if T decreases and prolactin, which mediates parental behaviours, becomes elevated in the correct temporal sequence (Buntin, 1996). The negative relationship between natural (and thus moderate) T-levels and incubation found in the present study might be explained by incubation behaviour inhibiting T (e.g., through prolactin). An alternative explanation may be that mate attraction behaviour, stimulated by high T levels, constrains incubation which correlated negatively with T levels. We found a positive, but statistically non-significant, relationship between song activity at an additional nest box (as a measure of mate attraction) and T levels during incubation. The lack of a significant positive relationship could be explained by our small sample size (only 4 out of 15 males were observed singing at an additional nest box). However, the few other correlative studies investigating how variation in plasma T relates to individual variation in song also found no evidence for a dose-dependent relationship, which could
be explained by individual differences in the responsiveness of the brain song system to T (Gil & Gahr, 2002). However, there is increasing evidence that a minimum threshold level of T (the breeding baseline T) is sufficient for full expression of singing behaviour (Wingfield et al., 1990), but that the presence of species-typical stimulus factors (presence of conspecific male or female as well as a nest site) largely determines whether males will actually start singing (Ball et al., 2002), thus implying that T does not stimulate mate attraction song in a dose-dependent way. This is supported by several observations in our population where some males during the parental phase show mate attraction behaviour at an additional nest box in the morning, when prospecting females are mainly present in the colony, and then switch to incubation and feeding behaviour in the afternoon, or even rapidly switch from paternal care to mate attraction in response to a prospecting female in the morning. This confirms that during incubation and feeding, T levels are low enough for not suppressing paternal care, but high enough to elicit song behaviour in response to the appropriate stimulus. In agreement with this, Smith (1995) demonstrated that when monogamous male starlings’ opportunities to attract additional females were experimentally increased by providing them with additional nestboxes, they decreased their paternal effort and instead invested in mate attraction during the incubation period. This clearly illustrates the plasticity of the trade-off within individual males (Magrath & Komdeur, 2003) and also suggests that T is not the key factor mediating this trade-off.

Although these findings suggest that, as contrasted to the predictions of the trade-off hypothesis, high T levels are not necessary to stimulate mate attraction song, we did find that all four males that showed song activity at an additional nest box had high T levels (and low incubation rates), compared to most other males (Figure 3). Given that (1) male starlings respond to the presence of a female by temporarily shifting their activity from paternal behaviour to song at an additional nest box, and (2) ownership of an additional nest box and, probably to a lesser extent, presence of unmated females, have been shown to elicit short term elevations in T (Gwinner et al., 2002; Pinxten et al., 2003), the observed relationship between high T and mate attraction may not result from high T stimulating mate attraction but from mate-attraction behaviour increasing T levels, as predicted by the challenge hypothesis. This may also partly contribute to the negative correlation between T and incubation behaviour we found. The observation that a
few other males had high T levels and low incubation rates, without showing mate attraction behaviour (at least not in the colony; Figure 3), may suggest that other factors may increase T levels during the parental phase. Likewise, the physiological capacity to produce and secrete androgens in response to stimuli has been shown to vary among males (Jawor et al., 2006).

In contrast to during the incubation period, T levels during the feeding period tended to be lower, showed less variation (Figure 4) and were not related to paternal effort. Although these results should be interpreted cautiously due to the rather small sample size and limited observation durations, they may also be explained by the strongly reduced presence of stimuli (unmated females) to elicit short-term T increases at the time when blood samples were taken (days 9-14). In our population, males can attract additional females only during the early part (first 10 days) of the feeding period (Pinxten et al., 1989; Pinxten & Eens, 1994), due to the unavailability of unmated females later in the season. Likewise, in the present study, only one male was observed to show mate attraction behaviour during the feeding period. That this male had relatively low T levels may be due to a lower androgen response to females later in the breeding season, and confirms that low T levels are sufficient to activate mate attraction behaviour.

To conclude, our correlative results suggest that natural plasma T levels reflect, but do not directly mediate, the trade-off between parental effort and additional mating effort in male starlings. For T dependent traits such as mate attraction, there seems to be more to the regulation of their expression than simply the concentration of T in the plasma. Several other factors such as the availability of unmated females may influence the resolution of the reproductive trade-off.

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