The natural plasma testosterone profile of male blue tits during the breeding season and its relation to song output

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In male birds, the gonadal hormone testosterone (T) is known to influence territorial and mating behaviour. Plasma levels of T show seasonal fluctuations which vary in relation to mating system and social instability. First, we determined the natural T profile of male blue tits *Parus caeruleus* during the breeding season. We found that plasma levels of T increased at the onset of nest building. Thus, the increase in circulating T was not associated with territory establishment, nor with the fertile period of the males' mates. In most individuals, T levels dropped to values close to zero during the period of chick feeding. Second, we investigated the relationship between plasma levels of T and male age, size, and singing behaviour. During the mating period, T levels did not differ between 1 yr old and older males and did not correlate with body size or condition. However, song output during the dawn chorus tended to be positively correlated with T levels. Therefore, if high T levels are costly, song output might be an honest indicator of male quality in blue tits. Finally, we show that plasma levels of T are significantly higher during the night than during the day. This pattern has also been observed in captive non-passerine birds, but its functional significance remains unknown.

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Testosterone (T) plays an important role in driving male reproductive behaviour in birds. Temporal patterns in the plasma levels of this sex steroid are triggered by several factors including photoperiod and food availability (Wingfield 1983), as well as social stimuli (Wingfield et al. 1990). Different T profiles during the breeding season have been related to the social systems and the mating strategies of the observed bird species (Wingfield et al. 1990, Beletsky et al. 1995, Vleck and Brown 1999). Outside the breeding season, when competition for nesting sites and copulation partners is low or absent, plasma levels of T are low (nonbreeding baseline, *sensu* Wingfield et al. 1990). When breeding territories are established and when mating takes place, T levels rise up to 20-fold to a breeding baseline (e.g. Ball and Wingfield 1987, Vleck and Brown 1999, Wada et al. 1999). Within the breeding season, large fluctuations in T levels are observed, for instance during social challenges (Wingfield et al.

1990). In all temperate passerines with paternal care, T levels drop rapidly during the incubation phase and are low during the period of nestling feeding (e.g. Ball and Wingfield 1987, Vleck and Brown 1999). T levels may show repeated peaks or remain elevated over the entire breeding season, when multiple clutches, extra-pair copulations, or polygyny are frequent (e.g. Hegner and Wingfield 1986, Beletsky et al. 1995, Wada et al. 1999). As a consequence of the T-mediated trade-off between sexual behaviour and parental care (Hegner and Wingfield 1987, Beletsky et al. 1995, Raouf et al. 1997), high T levels during the late breeding season enable males of these species to acquire additional breeding or copulation partners, at the cost of reduced paternal behaviour.

T is known to affect also the development of morphological, sexually selected traits (e.g. Zuk et al. 1995, Eens et al. 2000, Evans et al. 2000, Peters et al. 2000). Similarly, the role of T in the expression of behavioural

traits involved in sexual selection is well established. Males with elevated T levels defend larger territories (Silverin 1980, Wingfield 1984, Chandler et al. 1994), are more aggressive (Beletsky et al. 1990, but see Romero et al. 1998), and show more elaborate courtship displays (Enstrom et al. 1997, Hill et al. 1999) than males with low T levels. Males with high T levels also sing more (e.g. Silverin 1980, Ketterson et al. 1992, Hunt et al. 1997), although some studies have not found this effect (Beletsky et al. 1995, Saino and Møller 1995). Thus, it is evident that elevated T levels as observed in males before and during the females' fertile period enable behaviours necessary to acquire and defend nest sites and copulation partners. However, males vary greatly in their peak level of circulating T, and this might be related to their reproductive success. Few studies have investigated the effect of this variation on the intensity of sexually selected traits and sexual behaviour (Erickson 1970, Searcy and Wingfield 1980, Borgia and Wingfield 1991).

In this study, we investigated the natural plasma T levels in a population of blue tits *Parus caeruleus* during the breeding season. This species is mostly socially monogamous, but polygyny is not uncommon (Kempenaers 1994). Extra-pair copulations are frequent and females choose extra-pair mates that are older and have longer tarsi than their social partner (Kempenaers et al. 1997). Blue tits are non-migratory and males start singing and defending their territory as early as January/February, long before nest building and egg laying. Only during the fertile period of the mate, do males show a distinct dawn chorus. A previous study on the same population showed that song output was related to female fecundity and is therefore probably under sexual selection (Poesel et al. 2001).

The aim of this study was twofold. First, we describe the temporal changes in natural plasma levels of T in male blue tits during the breeding season. Second, we relate plasma levels of T during the period when T levels are elevated with male characters that are related to their reproductive success, namely age, size, and song characteristics.

Methods

Study area and field methods

We studied a population of blue tits breeding in nest-boxes in a mixed deciduous woodland at Kolbeterberg in Vienna (48°13′N, 16°20′E), Austria. During the breeding season, nestboxes were checked every second day to determine the onset of nest building, laying, hatching, and fledging. In three study seasons (1998–2000), the first nest building activity was ob-

served on 5 March, first eggs were laid on 5 and 6 April, and the last eggs (except for a few replacement clutches) between 29 April and 8 May. The period from 5 March to 8 May includes nest building and egg laying and was therefore defined as the *mating period*. We refer to the periods before and after the mating period as the *territory defence period* and the *chick feeding period*, respectively.

Blue tits were captured at night (20:00–00:00 hours) when they slept inside the nestbox and were placed separately in cotton bags. Blood samples (20–100 μl) from the brachial vein were taken within 30 min of capture. During the mating period some males were caught at day in food-baited potter traps. When the chicks were 8–9 days old, parents were caught inside the nestbox when feeding young. In this case, blood samples were taken within 10 min of capture. After centrifugation, plasma was stored at −70°C until analysis.

Individuals were colour-banded, sexed and aged (1 yr old or older bird, based on plumage characteristics, Svensson 1992). We measured tarsus length to the nearest 0.1 mm using callipers and body mass to the nearest 0.1 g using a digital balance. We calculated body condition as the residuals of a regression of body mass on tarsus length.

Song recording and analysis

The complete dawn song was recorded once for 28 individual males when their females were fertile and analysed using AVISOFT SONAGRAPH 3.0 for Windows. For a detailed description of recording methods, song analysis, and song variables see Poesel et al. (2001). Here we used two measures of song output: mean strophe length (duration of strophes in seconds), and mean percentage performance time (strophe length divided by the sum of the strophe and following pause lengths). As mean percentage performance time declined with recording date (Poesel et al. 2001), we corrected all analyses for recording date. Versatility (number of song type switches during the entire dawn chorus multiplied by the number of different song types) was used as a measure of song complexity. For 10 of the recorded males, the T level was measured during their females' fertile period (song recordings on days 2 to 15, T levels measured on days −3 to 10, 0 = day of the first egg).

Hormone analyses

An enzyme immunoassay (EIA) was used to assess the concentration of T in the plasma samples collected in 1998. In general, we followed the protocol of Palme and Möstl (1993). Five to 20 μl of plasma was

diluted 1:10 with double distilled water, extracted with 5 ml diethyl ether and reconstituted in 50 µl assay buffer. Mean recovery was 84%. The assay sensitivity was 0.06 ng/ml for a plasma sample of 20 µl.

Plasma samples collected in 1999 and 2000 were analysed in a radio immunoassay (RIA). Seven to 50 µl of plasma was diluted to 100 µl with assay buffer. We used a modification (Fusani et al. 2000) of the RIA procedure of Wingfield and Farner (1975). The samples were reconstituted in 160 µl assay buffer. Mean recovery was 90% and the sensitivity of the assay was 0.03 ng/ml for a plasma sample of 20 µl. The intra-assay variation was 2.8%. All samples were measured in a single assay.

All measured plasma levels of T fell within the range of the two standard curves (EIA: 0.3–80 pg/tube, RIA: 0.1–200 pg/tube). When analysing hormone levels corrected for mean recovery, our conclusions did not change, and we report uncorrected values.

In 1999 and 2000, some of the resident males in the study area received T-releasing or control implants during the mating period. We implanted self-dissolving pellets with a release time of two months during the last week of February and the first week of March (K. Foerster and B. Kempenaers unpubl.). T levels during the mating period did not differ between untreated males and males with control implants in these two seasons (n = 23, $\bar{x} = 1.96 \pm 1.48$ ng/ml and n = 6, $\bar{x} = 1.61 \pm 1.73$ ng/ml, resp.; t = 0.497, P > 0.6). Also, T levels of all males (untreated and control-implanted) measured during the mating period did not differ among the three study seasons (each individual used only once, Kruskal-Wallis test, H = 2.165, df = 2, P > 0.3), or between the two T assay methods (EIA: n = 14, $\bar{x} = 1.74 \pm 0.43$ ng/ml and RIA: n = 15, $\bar{x} = 1.95 \pm 0.34$ ng/ml; Mann-Whitney U-test, U = 85.0, P > 0.4). Therefore, we pooled all data obtained during the mating period.

Data analysis

Statistical analyses follow Sokal and Rohlf (1995) and were performed using SPSS 10.0 and StatXact 3 for Windows. Plasma levels of T during the mating period were normally distributed after log transformation. We applied this transformation when testing for correlations of T levels with sampling date and time, and with male characteristics. Untransformed data are presented as means ± SE, transformed data as back-transformed means with upper and lower 95% confidence limits. We applied two-tailed tests and used Bonferroni corrections in the case of multiple comparisons.

Some males were caught repeatedly over the three years of this study and all data are presented in Fig. 1. For each statistical test we used only one data point per individual, selected at random.

Results

Temporal changes in plasma levels of T

The natural profile of circulating T in blue tit males during the breeding period changed from low non-breeding levels in late winter to high levels at the time of nest building (Fig. 1a). High T levels were observed from 17 March onwards, two weeks before the first females in the study area were fertile. Individual males showed elevated T levels as early as 27 days before their social mate became fertile (Fig. 1b). T levels remained elevated during egg laying, but dropped when males
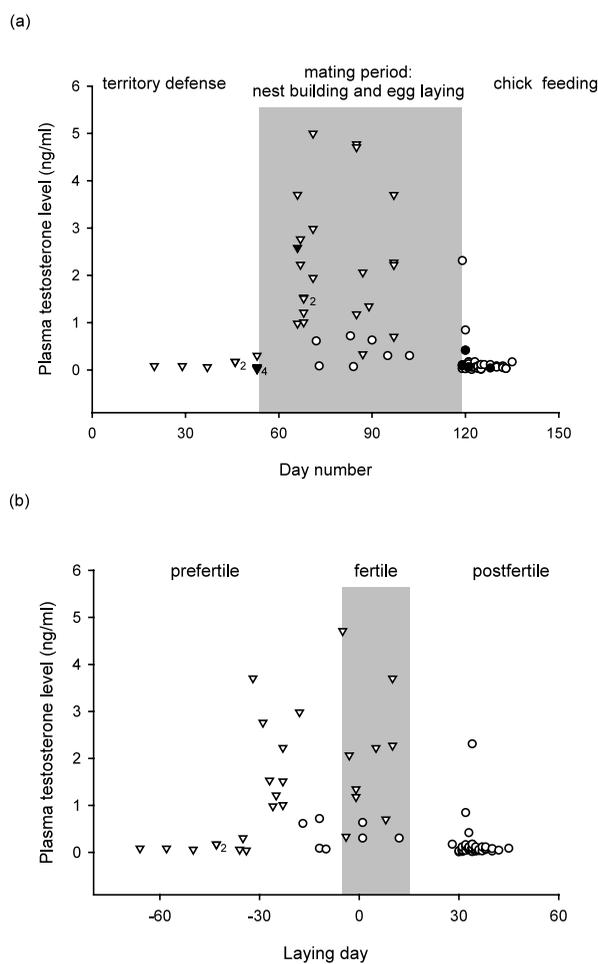

Fig. 1. Natural profile of plasma testosterone levels in resident male blue tits during the breeding season. Data are from 68 individuals in three seasons. Circles: T levels measured during day, triangles: T levels measured during night. Multiple data points are labelled with numbers. (a) T levels in relation to sampling date (1 = 1 Jan). Filled circles: socially polygynous males, filled triangles: birds not found breeding. The shaded area indicates the mating period. (b) T levels in relation to egg laying of the social mate (0 = day when the first egg was laid). The shaded area indicates the maximum fertile period of blue tit females, starting at day −5 and ending at day 15 (maximum clutch size: 16).

were feeding nestlings (Fig. 1a). Four polygynous males that were sampled during the nestling phase had T levels comparable to those of monogamous males (Fig. 1a).

Samples that were collected at night had higher T concentrations than samples collected at day (mating period only, night: $n = 22$, $\bar{x} = 2.31 \pm 0.29$ and day: $n = 7$, $\bar{x} = 0.39 \pm 0.10$; $U = 4.0$, $P < 0.001$). Also, T levels at night were more variable than T levels measured during day (mating period only, Conover test for equality of variances with unequal means, $T = 506.0$, $P = 0.01$). During the mating period, T levels did not vary with date or sampling hour (ANCOVA: day/night sample: $F_{1,24} = 27.58$, $P < 0.001$, sampling date: $F_{1,24} = 0.54$, $P > 0.4$, sampling hour: $F_{1,24} = 2.54$, $P > 0.1$). To test for differences in T levels between the three periods of the breeding season, we compared only samples obtained at either night or day. At the time of territory defence in January and February, all samples were collected at night, and their T levels were significantly lower than those of samples obtained at night during the mating period (territory defence: $n = 9$, $\bar{x} = 0.11 \pm 0.03$ and mating period: $n = 19$, $\bar{x} = 2.32 \pm 0.32$; $U = 0.0$, $P < 0.001$). During the mating period, most birds were captured at night, while all birds were captured at day when feeding chicks. However, considering only daytime samples, T levels still dropped significantly from the mating period to the chick feeding period (mating period: $n = 7$, $\bar{x} = 0.39 \pm 0.10$ and chick feeding: $n = 42$, $\bar{x} = 0.16 \pm 0.06$; $U = 57.5$, $P = 0.008$).

Testosterone and male characteristics

To correlate male characteristics and T levels, we considered only samples taken at night during the mating period. Samples taken during the day were too few for meaningful analyses. Male T levels did not differ between 1 yr old males and older individuals (1 yr old: $n = 12$, $\bar{x} = 1.92$ [CI 1.19, 3.11] and older males: $n = 9$, $\bar{x} = 1.76$ [CI 1.12, 2.76]; $t = 0.29$, $P > 0.7$). T levels were not correlated with male size (tarsus: $n = 22$, $r_p = 0.17$, $P > 0.4$) or male condition ($n = 17$, $r_p = -0.05$, $P > 0.8$). Song output during the dawn chorus measured as mean percentage performance time tended to increase with T level (partial correlation corrected for recording date: $n = 7$, $r = 0.86$, $P = 0.03$; significance level after Bonferroni: $P = 0.017$). Mean strophe length and song versatility were not significantly related to T level ($n = 7$, $r_p = 0.62$, $P > 0.1$ and $n = 5$, $r_p = 0.42$, $P > 0.4$, respectively). Mean percentage performance time was positively related to male body mass and condition in the prebreeding period (partial correlation: $n = 28$, $r = 0.51$, $P = 0.007$ and $r = 0.51$, $P = 0.007$, respectively; significance level after Bonferroni: $P = 0.0125$), and also tended to correlate with male body mass later during

chick feeding (partial correlation: $n = 25$, $r = 0.44$, $P = P = 0.032$).

Discussion

This study shows that the profile of plasma levels of T in male blue tits during the breeding season follows the typical pattern of socially monogamous temperate passerines. The increase in T levels was not associated with territory establishment, or with the fertile period of the males' mates. T levels during the mating period seem to correlate with song output during the dawn chorus, which is a sexually selected trait (Poesel et al. 2001).

The natural profile of plasma levels of T in male blue tits showed a single, distinct peak at the time when competition for breeding sites and copulation partners is high. T levels increased at the beginning of nest building, long before females were fertile. This is similar to results from other studies showing that T levels increase at the onset of nest building (e.g. Silverin et al. 1986, Schoech et al. 1991) or, in migratory species, when females arrive at the breeding sites (e.g. Silverin and Wingfield 1982, Hunt et al. 1997). Blue tit males in our study population occupied their territories during winter (own obs.). Therefore, most males that later bred on the study plot had already established a territory long before the onset of nest building (either by occupying the same area as during the preceding spring or by establishing a territory sometime during winter). However, some 1 yr old males arrived in the study area as late as March and tried to occupy territories. Aggressive male-male interactions were frequent at the time when T levels started to rise, but also occurred earlier (own obs.). Mate guarding was less intensive during early nest building compared to when females were fertile (K. Foerster and B. Kempenaers unpubl.) Copulations were observed almost exclusively during the female's fertile period (own obs.). Thus, high T levels in blue tit males were less correlated with mate guarding than with territorial behaviour, as predicted by the "challenge hypothesis" (Wingfield et al. 1990). However, territories are also defended during the period of low T levels in winter.

Elevated T levels were observed over a period of 4.5 weeks, which is comparable to the pattern found in other temperate monogamous species (Wingfield et al. 1990, Vleck and Brown 1999). Birds with prolonged competition over access to extra-pair mates, with multiple clutches, or polygyny maintain high T levels over a long period (Hegner and Wingfield 1986, Vleck and Brown 1999, Wada et al. 1999). In our population extra-pair paternity occurs frequently (59.5% of all broods in 1998 contained at least one extra-pair young, own unpubl. data). However, females produced only one clutch (with the exception of a few replacement

clutches) and laying was highly synchronous (breeding synchrony index for 1998–2000: 69–72% see Kempenaers 1993). Therefore, the availability of possible extra-pair mates for males was limited to a short time interval that overlapped greatly with the fertile phase of the social partner. Thus, in this population few females were fertile during the chick-feeding period and males did not face the usual trade-off between mating and parental effort. In other species, T has been shown to play an important role in the regulation of the behaviours involved in this trade-off (Beletsky et al. 1995, Raouf et al. 1997). Males with high T levels during the nestling stage feed nestlings less, or not at all (e.g. Silverin 1980, Ketterson et al. 1992, Schoech et al. 1998, Hunt et al. 1999). In blue tits, both monogamous and polygynous males had low levels of circulating T during the nestling stage, comparable to levels before the mating season (with two exceptions, see Fig. 1) and provided about half of the food to the nestlings (own unpubl. data).

We found that plasma levels of T were higher and more variable during the night than during the day. Higher T levels during night or a peak at the end of the dark phase have been reported in domestic ducks (Balthazart 1976), domestic fowl (Bachman et al. 1987, Schanbacher et al. 1974), and ring doves (Balthazart et al. 1981). These studies describe circadian patterns of T levels in captive birds, but the functional significance of the observed differences is unclear (Balthazart et al. 1981). To our knowledge, our study provides the first data on free-living individuals and on a passerine. Our results may have been influenced by the sampling methods; handling time during day was generally shorter than during night, when birds were transported to a shed before bleeding. However, several studies have shown that T levels were not influenced significantly by long handling times (Wingfield et al. 1982, Silverin 1998, Kitaysky et al. 1999).

Some studies indicate that the relative concentration of circulating T during the period when T levels are elevated correlates with the intensity of aggressive and sexual behaviour (Searcy and Wingfield 1980, Borgia and Wingfield 1991, Enstrom et al. 1997). However, it is still unclear whether the individual variation in T levels during the mating period affects male attractiveness to females (Hill et al. 1999). Few studies have investigated correlations between T levels and male characteristics in free-living birds. Single measurements of T levels may depend strongly on time of day (this study) and on the state of the animal prior to sampling. Social interactions quickly raise plasma levels of T in some bird species (Wingfield and Wada 1989, Wingfield et al. 1990, Wikelski et al. 1999). Thus, if only one sample is taken per individual, large fluctuations over time might mask relations of T levels with male characteristics. We were not able to take multiple measurements during the mating period. Thus, our

non-significant results should be interpreted cautiously, as they might be caused by high individual variation in T levels.

Although blue tit females prefer older and larger males as extra-pair partners (Kempenaers et al. 1997), we did not find any evidence that these characteristics were related to T levels during the mating period. However, we found that males with higher T levels during this period tended to have a higher song output at dawn. Furthermore, males with higher song output were in better condition before and during the breeding season. Song is a dynamic behavioural trait that plays an important role in mate choice in passerines (Andersson 1994). In the blue tit, song output might be under sexual selection, since males with a higher song output were paired to females that laid earlier in the season (Poesel et al. 2001). Several studies have shown that individual variation in the avian song control system and syringe control is influenced by T (e.g. Luine et al. 1980, Smith et al. 1997). Also, T affects the frequency of spontaneous song during the day (e.g. Ketterson et al. 1992, Hunt et al. 1997, but see Saino and Møller 1995). In this study we show that within a period of continuous song (dawn chorus), males with higher T levels tend to have a higher song output. To our knowledge only one other study has so far related a song characteristic (other than song rate) to T levels during the breeding season: Galeotti et al. (1997) found that male barn swallows *Hirundo rustica* with high T levels produced longer rattles than males with low T levels. Interestingly, that study also showed that a characteristic of the rattle (peak amplitude frequency) was related to body condition. Similarly, we found a relation between song output and condition in blue tit males.

It is probably costly for a male to maintain high T levels over a long period, as T has been shown to be immunosuppressive in birds (Verhulst et al. 1999, Peters 2000, but see Hasselquist et al. 1999). If high T levels are costly and the intensity of a sexually selected trait depends on T, then this trait might signal male quality (Folstad and Karter 1992). Thus, our data indicate that song output during the dawn chorus might provide an honest signal of male quality.

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