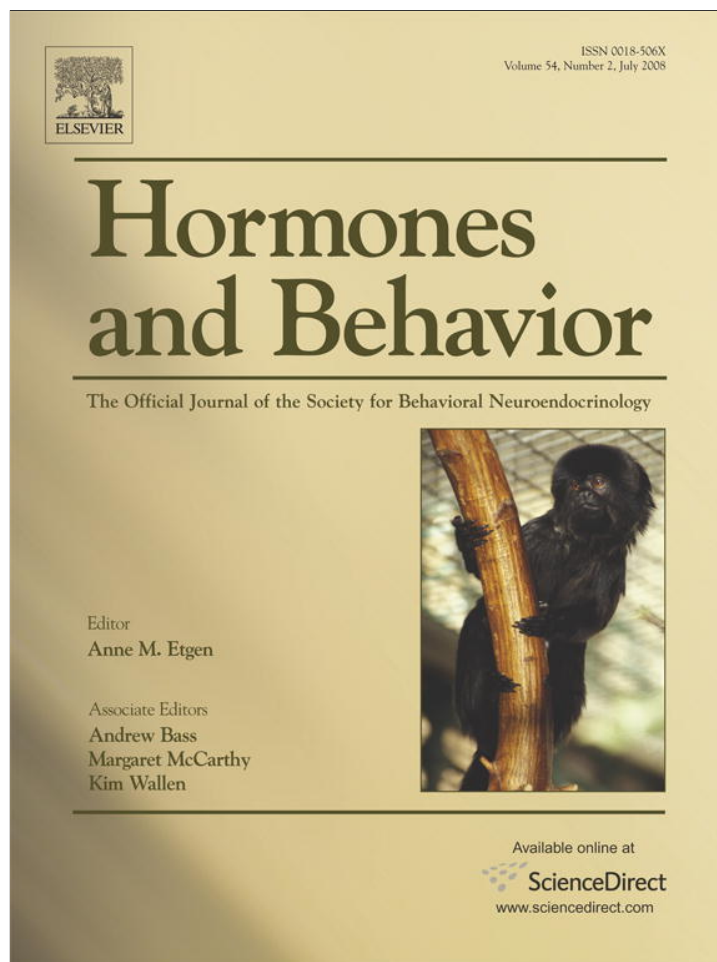


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## Experimental manipulation of testosterone and condition during molt affects activity and vocalizations of male blue tits

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## ABSTRACT

Testosterone (T) is a key hormone regulating behavioral trade-offs in male birds, shifting investment towards sexual and competitive behaviors. However, the role of T in regulating male behavior during the molt has received very little attention, although this is a crucial life-history stage. Since the effect of T on behavior may be condition-dependent, particularly during the costly molt period, we studied the effects of T and condition in a two-way design. We manipulated T under two dietary regimes (standard and improved, resulting in an enhanced condition) in captive blue tits (*Cyanistes caeruleus*) undergoing the first pre-basic molt. T treatment increased song frequency, indicating that song is T-dependent also at this time of year. Males on the improved diet sang less than males in relatively worse condition, providing no support for song as an indicator of male condition. T-treated males exhibited greater locomotor activity than control males, but only when fed the standard diet. Neither T- nor diet-treatment affected plumage maintenance (preening). Although T treatment resulted in a delay in molt progress all birds completed the molt. Taken together our results show that during the molt male birds are sensitive to relatively small fluctuations in T. Similar to its commonly observed effects during the breeding season, T stimulated an increase in song and locomotion. While there might be some benefits associated with such T effects, these must be traded-off against costs associated with conspicuous behavior and increased molt duration.

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## Introduction

Testosterone (T) is a key hormone regulating life-history trade-offs of male vertebrates, driving higher relative investment in sexual traits, such as the pursuit of additional mating opportunities versus care of offspring, or sexual advertising versus immunity (Hau, 2007). Consequently, T generally stimulates behaviors involved in mate attraction or competition (Hau, 2007).

Birds have been extensively used as model systems for studying T-mediated reproductive trade-offs. In agreement with a role for T shifting behavior towards sexual investment, experimentally elevated T during breeding results in an increase in song (Silverin, 1980; Chandler et al., 1994; Enstrom et al., 1997; Hunt et al., 1997; Van Duyse et al., 2000), courtship (Peters, 2002), aggression (Collis and Borgia, 1992; De Ridder et al., 2000), locomotion (Wada, 1982; Lynn et al., 2000), home range (Ketterson and Nolan, 1992; Chandler et al., 1994; Lynn et al., 2000) and territory size (Watson and Parr, 1981; Wingfield, 1984). Conversely, T often reduces behaviors associated with self-maintenance (Lynn et al., 2000), parental care (Ketterson et al., 1992;

Hunt et al., 1999; Peters et al., 2002) and resting behaviors (Lynn et al., 2000) and can decrease survival (Reed et al., 2006).

Most studies on the role of T in avian life histories have focused on behavioral effects of exogenous T during the breeding season, when mating and reproduction take place, and T levels are at their annual maximum. To our knowledge, no study examined the effects of T on male behavior during the pre-basic molt. T levels generally are at their annual low point during this time (Dawson, 1983; Schleussner et al., 1985; Ketterson and Nolan, 1992). It could be that males then are insensitive to (some of the) effects of additional T, possibly to avoid expression of costly, inappropriate effects of T at that time of the year (Wingfield et al., 2001). On the other hand, a biological role of low T levels during molt may certainly not be off-hand rejected. For example, T plays a central role in regulating male sexual trade-offs (for example see Reed et al., 2006) and the molt period is pertinent to sexual signalling: during the annual replacement of all feathers, males in many species develop their plumage that will determine their sexual attractiveness and competitiveness for the following breeding season. Subtle changes in circulating T during molt may determine male plumage characteristics: for example treatment with slightly elevated T during molt resulted in increased size of the black bib, a male badge of status, in house sparrows *Passer domesticus* (Buchanan et al., 2001). These low T levels also resulted in an increase in metabolic rate (Buchanan et al., 2001), so even low levels of T during molt may be biologically meaningful.

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Therefore it seems plausible that even the small fluctuations in post-breeding T could affect male behavior during the pre-basic molt. Apart from courtship and parental care, many of the behaviors that are T-sensitive during breeding, for example activity levels, are also relevant during molt. T-mediated behavior could be directly or indirectly beneficial during the time of molt. Higher nutrient and energy requirements during molt could be satisfied more easily if a T-induced increase in locomotion or home range allowed more preferred foods to be acquired. Additionally, in species that flock during molt, T can boost dominance thereby facilitating food access (Ekman and Lillien Dahl, 1993) and use of safer feeding places (Lahti et al., 1998; Carrascal and Alonso, 2006). Conversely, high levels of T may be costly. These costs may include exposure to predators (Wingfield et al., 2001), risk of injury (Wingfield et al., 2001), loss of fat reserves (Wingfield, 1984), decreased immune responsiveness (e.g. Evans et al., 2000; Peters, 2000), and heightened energetic demands (Buchanan et al., 2001). Moreover, high (comparable to breeding season) levels of T delay or prevent molt (Runfeldt and Wingfield, 1985; Dawson, 1994).

Since elevated levels of T are often associated with physiological (metabolic, immunological) costs, the effect of T on behavior may be condition-dependent (Folstad and Karter, 1992; Pérez-Rodríguez et al., 2006), particularly during the challenging molt period. Molt is an extremely demanding period during a bird's life-history, with dramatic physiological costs such as a daily energy expenditure of two to three times basal metabolic rate during peak molt (Lindström et al., 1993), 3–4 fold increase in protein-turnover (Murphy and Taruscio, 1995), decreased thermoregulatory ability caused by impaired feather insulation (Klaassen, 1995), reduced ability to produce a physiological stress response (Romero, 2002) and increased predation risk due to impaired flight abilities (Swaddle and Witter, 1997a). Therefore it may be that males in better condition could be more able to express the effects of elevated T.

To examine the effects of T on male behavior during molt and to test the hypothesis that effects of T depend on individual condition, we treated male blue tits (*Cyanistes caeruleus*) in late summer with physiologically relevant levels of T and additionally manipulated condition by offering a standard or improved diet using a two-way design. We then examined behavioral time budget and molt speed in males and females undergoing the post-juvenile molt. We focused our observations on vocalizations, locomotion and resting. In addition, we examined time spent on plumage maintenance (preening). This behavior is often viewed as investment in self-maintenance but it may actually rather be investment in sexual signalling, since preening may improve appearance and attractiveness (Zampiga et al., 2004). We predicted that (1) T would increase song and activity, retard molt progress, and increase preening and (2) the effect of T would depend on condition (diet).

## Methods

### Experimental subjects

Blue tits are socially monogamous, sexually dichromatic small passerines (Hunt et al., 1998). Since T can vary with age as well as experience (Kempnaers et al., 2008), and since timing of adult molt varies individually with breeding success (Cramp and Perrins, 1993), to standardize between experimental subjects, we used only juveniles that had all been raised in similar conditions. Juveniles undergo a body molt in autumn during which they develop their first adult plumage. This is a crucial life-history stage since juvenile mortality is higher than that of adults until November, when juvenile plumage has been completely replaced (Cramp and Perrins, 1993). Although they can show territorial behavior year-round, outside the breeding season blue tits form flocks and compete aggressively for food (Cramp and Perrins, 1993).

We studied a nest-box breeding population of blue tits established in February 2006 on the Bodanrück (47°75'N, 9°07'E), Germany. From the start of the breeding season (1 April), we checked boxes every other day. After chicks hatched, we monitored boxes daily. When nestlings were between 10 and 13 days old we fitted them with a unique metal ring and we took a small blood sample for molecular sex determination. Between 23 and 30 May we brought 14 nest-boxes with nestlings (11–14 days old) and parents into captivity. Parents and chicks were kept in large, naturalistic outdoor

aviaries (300×300×190 cm) where the parents raised their chicks with a diet consisting of several types of adult invertebrates (mealworms, small crickets), invertebrate larvae (of honey bees and ants) and egg food (a mix of mashed boiled hens eggs, crushed rusk and soured milk with added vitamins Mauserpulver (Claus), Vitakalk (MFE) and Korvimin (WDT)). After the fledglings could forage independently, four males and two females of 12 broods were selected randomly and placed separately in similar outdoor aviaries for the remainder of the experiment. No nestlings or parents died and all remaining fledglings were released with their parents in the local area.

Two males and one female of each family were randomly assigned to a standard and an improved diet respectively (see below). One male on the standard diet and one male on the improved diet of each family were randomly assigned to a T treatment and control treatment respectively. Each treatment combination was thus represented once in each family in a 2×2 design.

Removal of blue tits from the wild and animal experimental protocols were approved by the Regierungspräsidium Freiburg (Aktenzeichen 55-8852.15/05 and Registriernr. G-06/05, Aktenzeichen 35-9185.82/3/339 respectively).

### Dietary treatment

Fledglings had *ad libitum* access to egg food (see above), mealworms *Tenebrio molitor* and honey bee *Apis mellifera* larvae before experimental semi-synthetic diets (Table 1) started on 15 July. The standard diet consisted of 20% protein, 0.4% vitamin, 42% carbohydrate and 20% fat and was formulated to have moderately high amounts of carbohydrates and fats and adequate protein compared to natural fruit diets (Pierce et al., 2005; Smith et al., 2007a). The improved diet contained 41% protein, 0.25% lutein, 2% vitamin, 15% carbohydrate and 15% fat. It was formulated to simulate an insect diet (Bairlein, 1998; Smith et al., 2007b) that is more nutritious (higher protein, vitamin and antioxidant (carotenoid) content) and more easily digestible (lower fiber content, more water) than the standard diet.

### Testosterone treatment and assay

On 17 and 18 July, several weeks before the onset of the post-juvenile molt (Cramp and Perrins, 1993), we implanted all birds after confirming that no birds had started to molt. We inserted implants (diameter: 3 mm, height: 1 mm, Innovative Research of America) through a small incision in the skin on the back between the wings. T implants contained 1 mg T and were designed to slowly dissolve and maintain a constant plasma level of T over a period of 90 days. This type of implant, but with a different dose, has been successfully used previously to elevate T in breeding blue tits (Foerster and Kempnaers, 2004, 2005). Control (C) males and females received implants that consisted only of carrier material.

To verify that implants had resulted in elevated T levels, we collected plasma from a sub-sample of birds on 23, 24 and 25 August. After puncture of the wing vein, blood was collected in heparinised capillary tubes. Tubes were placed on ice until centrifugation after which plasma was harvested and stored at -70 °C. Plasma T levels were assayed by direct radio immunoassay (RIA) following Goymann et al. (2006). Briefly, all samples were extracted twice with freshly distilled dichloromethane after overnight equilibration with 1500 dpm tritiated T. After resuspension in assay buffer an aliquot of the redissolved samples was transferred to scintillation vials, mixed with scintillation fluid and counted to an accuracy of 2–3% in a Beckman LS 6000 β-counter to estimate individual extraction recoveries. The remainder was stored at -40 °C until RIA was conducted. Standard curves were set up in duplicates by serial dilution of stock standard solutions in a range of concentrations between 0.39–200 pg. The T antiserum and tritiated T was added to the standard curve, assay controls and duplicate aliquots of each sample. After incubation for 20 h at 4 °C the bound and free fractions were separated by adding 0.5 ml dextran-coated charcoal and centrifugation after 14 min

**Table 1**

Composition (in % wet mass and % dry mass) of the semi-synthetic diets used in this study to manipulate the general condition of molting blue tits

Ingredients	Standard diet		Improved diet	
	% Wet mass	% Dry mass	% Wet mass	% Dry mass
Dextrose	15.14	39.36	3.00	15.01
Casein	7.39	19.20	6.54	32.72
Cellulose	1.92	4.99		
Amino acid mix <sup>a</sup>	1.03	2.69	0.55	2.76
Vitamins <sup>b</sup>	0.15	0.38	0.33	1.67
Salt mix <sup>c</sup>	1.85	4.80	1.17	5.86
Olive Oil	7.39	19.20	3.00	15.01
Agar	1.23	3.20	2.00	10.01
Mealworms	2.34	6.19	2.40	12.01
Water	61.56		80.00	
Lutein powder <sup>d</sup>			1.00	5.00

<sup>a</sup> Amino Acid Mix (Murphy and King, 1982).

<sup>b</sup> AIN Vitamin Mixture 76, MP Biomedicals Inc.

<sup>c</sup> Custom Mineral Mix, Dyets Inc.

<sup>d</sup> FloraGLO, Pfannenschmidt, Lutein content: 5%.



incubation. Supernatants were decanted into scintillation vials, scintillation liquid added, and vials counted. Standard curve and sample concentrations were calculated with ImmunoFit 3.0 (Beckman Inc., Fullerton, CA), using a four parameter logistic curve fit. The lower detection limit of the assay was determined as the first value outside the 95% confidence intervals for the zero standard ( $B_{max}$ ) and was 0.006 ng/ml. The mean recovery rate was 87% and the intra-assay coefficient of variation was 9.6%. Hormone levels were natural log ( $\ln$ ) transformed to achieve a normal distribution; we present back-transformed means  $\pm$  SE.

#### Behavioral observations

To study the effect of T, condition and sex on behavior during molt we performed detailed behavioral observations on 24 T-males, 24 C-males and 24 females between 6 and 11 August, between 0800 and 1200 h, (morning observation period, AM) and between 1500 and 1900 h (afternoon observation period, PM). The observation series was repeated between 4 and 10 September. Between the two observation series, two T-males, one from each diet, died during blood sampling, of unknown causes. In addition one male (C, standard diet) was excluded from all analysis due to injury.

During each AM/PM period, one bird from each treatment combination was randomly chosen and observed for a period of 30 min. The sequence in each observation period was randomized. Observations were conducted from a hide placed next to the aviary 40 min before starting the observation. Every 15 s the observer (R.H.J.M.K.) noted the behavior and classified it as: eating, flying, moving, resting, preening, calling, singing, washing, drinking or out of sight (timed behaviors). In addition the total number of calls and songs during the 30 min observation period was scored (call and song frequency).

The aviaries contained small bushes to provide shelter for the birds. As a result, birds were sometimes on the other side of a bush and thereby out of the line of sight of the observer (mean  $\pm$  SD =  $7.6 \pm 3.4$  min out of sight). Since certain behaviors were always visible (eating, drinking and washing could take place only at the available food and water sources) or audible (singing and calling) to the observer, we adjusted those behaviors that could occur while out of sight (flying, moving, resting and preening) proportional to their likelihood when in sight, so that the percentage of time spent on all behaviors totaled 100.

#### Molt and condition measurements

On 31 July and 11 September birds were caught for scoring of molt. Since juvenile blue tits do not molt primaries, molt stage was scored by visual assessment of the progress of molt of body feathers that change coloration between the juvenile to the adult plumage. Molt was scored on a scale ranging from 1 to 3: (1) less than 1/3 of the body feathers molted, (2) between 1/3 and 2/3 of the body feathers molted and (3) more than 2/3 of the body feathers molted (Bairlein, 1995). Additionally we measured tarsus length to the nearest 0.1 mm using calipers (first capture only), body mass to the nearest 0.1 g using a digital balance and we ranked fat by visual assessment of the furcular and abdominal fat deposits on a scale from 0 (no fat) to 8 (bulging fat deposit) (Kaiser, 1993).

#### Statistical analyses

In all analyses we used mixed models with bird number nested in family (for repeated measures analysis since we included both observation periods in the same model) as a random term to avoid the problem of pseudo-replication at the individual and family level. To determine the effects of T and diet we constructed models comparing C- and T-males. To determine the effects of sex and diet we constructed separate models comparing C-males and females.

To analyze the effect of diet on body condition we used the restricted maximum likelihood (ReML) method with response variables body mass and fat score, which were analyzed separately. The fixed models consisted of diet, hormone treatment group and diet-hormone interaction as well as tarsus length to account for skeletal size in the body mass model. To test for the effect of T and condition on molt progress we used GLMMs (generalized linear mixed models). Molt score was the response variable in a Poisson model. As fixed terms we included diet, hormone treatment group and diet-hormone interaction and as random factor we included observation period.

To analyze the effect of T and body condition on timed behaviors eating, flying, moving, resting and preening (expressed as percentage of total observation time) we used binomial GLMMs with logit-link function and total observation time as binomial total. Of the timed behaviors, singing ( $0.2 \pm 0.1\%$ ), calling ( $1.7 \pm 0.2\%$ ), washing ( $0.3 \pm 0.1\%$ ), and drinking ( $0.6 \pm 0.1\%$ ) were too infrequent for statistical analyses. To analyze the effect of T and body condition on vocalizations we therefore used the frequency of vocalizations. Since call and song frequency followed a Poisson distribution they were log transformed to normalize residuals of ReML models. As fixed effects, we fitted diet, hormone treatment group and diet-hormone interaction. In all analyses of behavior we controlled for time of day (AM/PM) since birds called and flew more and rested and preened less during the morning (calls: Wald ( $w$ ) = 37.86,  $P < 0.001$ , flying:  $w = 30.60$ ,  $P < 0.001$ , resting:  $w = 14.42$ ,  $P < 0.001$ , preening:  $w = 22.96$ ,  $P < 0.001$ ; eating:  $w = 0.00$ ,  $P = 0.90$ , moving:  $w = 0.00$ ,  $P = 0.98$ , songs:  $w = 0.91$ ,  $P = 0.34$ ). Additionally we controlled for period since birds sang and flew more and called and preened less in the first period (songs:  $w = 19.23$ ,  $P < 0.001$ , flying:  $w = 5.28$ ,  $P = 0.022$ , calls:  $w = 6.07$ ,  $P = 0.014$ , preening:  $w = 12.01$ ,  $P < 0.001$ ; eating:  $w = 1.86$ ,  $P = 0.17$ , moving:  $w = 1.45$ ,  $P = 0.23$ , resting:

$w = 2.96$ ,  $P = 0.09$ ). We included both observation periods in the same analysis, controlling for differences between periods by including period as a fixed factor, since we were interested in consistent treatment effects rather than between-period variance.

We derived minimal models from maximal models by excluding the least significant terms from the models by stepwise deletion. The final models included only significant ( $P < 0.05$ ) terms. In case of a significant diet-hormone interaction we analyzed the effects of T within each diet group separately. We present Wald ( $w$ ) statistics (that follow a chi-square distribution with d.f. = 1), and  $P$ -values as predicted by the final model. Values are presented as raw means  $\pm$  SE. For all statistical analyses we used GenStat 8.1 (GenStat, 2005).

## Results

### Condition

There was no significant diet-T interaction on mass and fat scores (mass:  $w = 0.12$ ,  $P = 0.73$ , fat:  $w = 1.65$ ,  $P = 0.20$ ). There was no effect of T on mass ( $w = 0.09$ ,  $P = 0.76$ ). T-males tended to have a higher ( $2.3 \pm 0.1$ ) fat score than C-males ( $2.1 \pm 0.1$ ) ( $w = 2.96$ ,  $P = 0.09$ ). Males fed the improved diet had higher body mass ( $11.9 \pm 0.1$  g) and higher fat score ( $2.3 \pm 0.1$ ) than those receiving the standard diet ( $11.7 \pm 0.1$  g,  $2.0 \pm 0.1$ ; mass:  $w = 6.00$ ,  $P = 0.014$ , fat:  $w = 5.10$ ,  $P = 0.024$ ). Likewise, C-males and females fed the improved diet had higher body mass ( $w = 9.06$ ,  $P < 0.001$ , controlling for sex) and fat scores ( $w = 7.99$ ,  $P = 0.005$ ) than birds on the standard diet. Generally, C-males had higher body mass ( $11.9 \pm 0.1$  g) than females ( $11.3 \pm 0.1$  g) ( $w = 11.71$ ,  $P < 0.001$ ) but there was no significant sex difference in fat score (C-males:  $2.1 \pm 0.1$ , females:  $1.9 \pm 0.1$ ;  $w = 2.43$ ,  $P = 0.15$ ). Although male birds on the improved diet were heavier and fatter compared to males on the standard diet, their condition fell in the natural range of molting male juvenile blue tits captured in the local area at that time of year (mass:  $12.1 \pm 0.2$  g, range: 11.5–12.7, fat:  $1.6 \pm 0.2$ , range: 1–2;  $n = 8$ ) (A. Peters unpublished data).

### Testosterone levels

T implants resulted in significantly ( $w = 17.37$ ,  $P < 0.001$ ) elevated T levels, on average  $1.9 \pm 0.7$  ng/ml (range 0.25–12.0 ng/ml,  $n = 11$ ), which is around the maximum level observed in C-males (range 0.26–2.1 ng/ml, mean  $0.55 \pm 0.2$  ng/ml,  $n = 13$ ). T levels in females ( $0.74$  ng/ml, range 0.34–1.24 ng/ml,  $n = 15$ ) were similar to those in C-males ( $w = 2.07$ ,  $P = 0.15$ ). Lack of a sex difference is similar to free-living molting blue tits: average natural T levels during post-juvenile molt (late August–early September 2004) in blue tits from the local area did not differ between males and females ( $t = 1.33$ ,  $P = 0.19$ ,  $n = 33$  males, 13 females, A. Peters unpublished data) although absolute levels were much lower (mean 0.10 ng/ml, range: 0.06–0.23 ng/ml). Diet had no effect on T levels ( $w = 2.07$ ,  $P = 0.15$ ; and no significant interaction:  $w = 0.23$ ,  $P = 0.63$ ) in the males, or in the C-males and females with sex in the model ( $w = 1.70$ ,  $P = 0.19$ ; and no significant interaction:  $w = 0.88$ ,  $P = 0.35$ ).

### Behavior

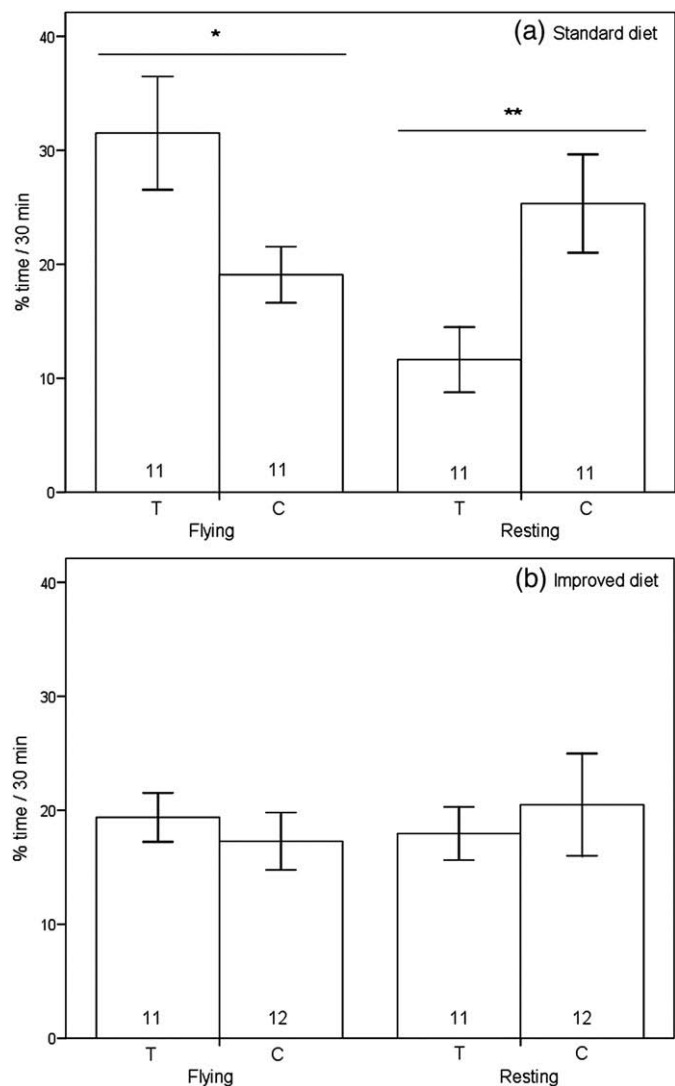
On average male birds spent  $3.6 \pm 0.3\%$  of their time eating,  $21.7 \pm 1.9\%$  flying,  $47.5 \pm 2.3\%$  moving,  $18.7 \pm 2.2\%$  resting and  $4.9 \pm 0.8\%$  preening. There was a significant interaction between diet and T on time spent flying and resting (Table 2). On the standard diet T-males spent more time flying and less time resting than C-males (flying:  $w = 5.25$ ,  $P = 0.02$ ; resting:  $w = 7.61$ ,  $P = 0.006$ , Fig. 1a). This pattern was absent in males fed the improved diet (flying:  $w = 0.76$ ,  $P = 0.39$ ; resting:  $w = 0.01$ ,  $P = 0.91$ , Fig. 1b), which showed levels of resting and flying similar to C-males on the standard diet. There were no significant diet-T interactions or significant differences between T-males and C-males in time spent eating, moving and preening (Table 2). Birds on the improved diet spent more time eating ( $4.4 \pm 0.3\%$ ) than birds on the standard diet ( $2.9 \pm 0.2\%$ ) (Table 2). Males called on average  $46.2 \pm 4.3$  times and sang  $2.2 \pm 0.7$  times per half hour

**Table 2**  
Statistical results of mixed model analysis of two-way T and diet treatments on behavior of molting juvenile male blue tits (T-males:  $n=22$ , C-males:  $n=23$ )

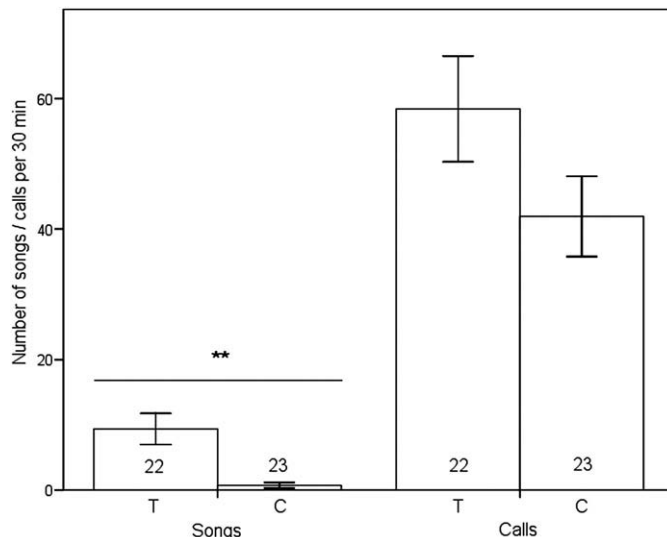
	Diet-T interaction		Diet		T	
	Wald	P	Wald	P	Wald	P
Eating (%)	1.23	0.27	15.61	<0.001	0.37	0.54
Flying (%)	5.33	0.021				
Moving (%)	0.03	0.87	1.48	0.22	0.00	0.96
Resting (%)	4.31	0.038				
Preening (%)	0.57	0.45	0.01	0.91	0.65	0.42
Songs (no.)	0.25	0.61	4.64	0.031	9.30	0.002
Calls (no.)	3.03	0.08	0.70	0.40	1.81	0.18

Wald statistics and *P*-values were derived by excluding a term from the full final model containing only significant terms.

observation period. There was no significant diet-T interaction on the frequency of songs or calls (Table 2). T-males sang more often than C-males (Fig. 2, Table 2) but there was no effect of T on calls. Additionally, there was a significant effect of diet on singing: males on the improved diet ( $1.2 \pm 0.6$ ) sang less than males on the standard diet ( $5.3 \pm 1.8$ ) (Table 2).



**Fig. 1.** T treatment increases the time spent flying and decreases the time spent resting in molting male blue tits when fed a standard diet (a) but not when fed an improved diet (b) that enhanced body condition. T=T, C=control. Numbers indicate sample sizes. Values are raw means  $\pm$  SE, \* $P < 0.05$ , \*\* $P < 0.01$ .



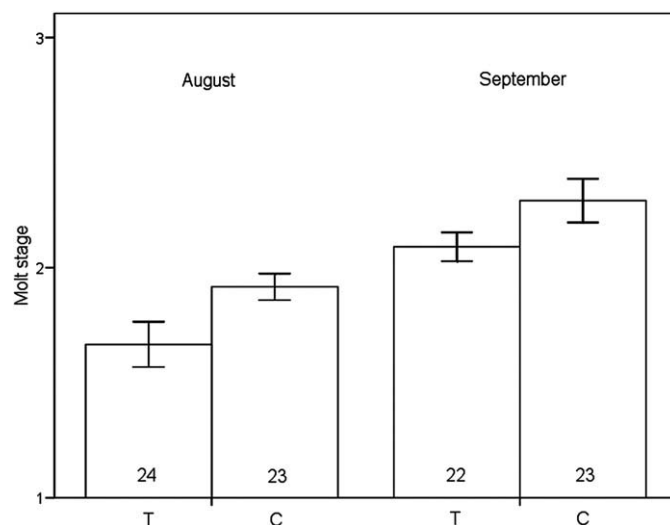
**Fig. 2.** T treatment increases song activity, but not other vocalizations, of molting male blue tits. Depicted are number of calls and songs per half hour observation period in T- and C-implanted males. Numbers indicate sample sizes. Values are raw means  $\pm$  SE, \*\* $P < 0.01$ .

*Molt progress*

In early August 37 of 47 (79%) males were in mid-molt (stage 2) and ten males in stage 1. In early September 36 of 45 (80%) males were in mid-molt and nine males in stage 3. There was no significant diet-T interaction on the progress of molt ( $w=0.56$ ,  $P=0.46$ ) and no effect of diet ( $w=0.61$ ,  $P=0.46$ ) but T-males molted significantly slower than C-males (Fig. 3). Nonetheless, all birds completed the molt: by mid October no males were molting and all had achieved a complete adult plumage.

*Sex differences*

Differences between the sexes were examined by comparing C-males and females. Males tended to preen ( $5.5 \pm 1.2\%$ ) and rest ( $21.6 \pm 2.5\%$ ) more than females ( $3.3 \pm 0.9\%$ ,  $15.9 \pm 1.3\%$ ) (Table 3). Males and females did not significantly differ either in time spent eating, flying



**Fig. 3.** Molt stage (stage  $1 < 1/3$ , stage  $2 = 1/3 - 2/3$  and stage  $3 > 2/3$  body molt) of T-males and C-males in August and September. Numbers indicate sample sizes. Values are raw means  $\pm$  SE. T treatment resulted in significantly slower molt progress ( $w=7.96$ ,  $P=0.005$ ), but all birds eventually molted a complete fresh plumage.

**Table 3**

Sex differences in, and the effect of dietary manipulation of condition on, behavior of molting blue tits (C-males:  $n=23$ , females:  $n=24$ )

	Diet–sex interaction		Diet		Sex	
	Wald	<i>P</i>	Wald	<i>P</i>	Wald	<i>P</i>
Eating (%)	2.06	0.15	19.14	<0.001	0.20	0.66
Flying (%)	2.27	0.13	1.59	0.21	1.57	0.21
Moving (%)	0.07	0.80	3.32	0.07	0.92	0.34
Resting (%)	1.23	0.27	1.89	0.17	3.53	0.06
Preening (%)	0.05	0.83	0.45	0.50	3.27	0.07
Songs (no.)	0.65	0.42	3.14	0.08	0.93	0.34
Calls (no.)	1.86	0.17	0.06	0.81	0.06	0.80

Wald statistics and *P*-values were derived by excluding a term from the full final mixed model containing only significant terms.

or moving or in frequency of songs and calls (both were extremely low) (Table 3). There were no significant diet–sex interactions on any behavior (Table 3) and no sex difference in molt progress ( $w=0.57$ ,  $P=0.45$ ).

## Discussion

We showed that an exogenous dose of T affected behavior of male blue tits during their first molt, and this effect partly depended on condition. In agreement with the well-documented effects of T during breeding, T treatment had a positive effect on song frequency. Additionally, T also increased locomotion (flying) and decreased resting, but on the standard diet only. As expected, T-treated males molted slower than C-males. To our knowledge this is the first study to indicate that T can mediate behavior of male birds during molt. Additionally, similar circulating T in males and females and T-independent sex differences in plumage maintenance behavior indicate that differences between the sexes are not related to T.

T in implanted males was on average increased to the maximum levels observed in control-implanted males kept in the same conditions and sampled at the same time. Overall, T levels were several times higher in our captive birds than in free-living molting blue tits captured during molt (2 years earlier). While we cannot explain why this should be the case, the relative magnitude of the T treatment compared to control males is within the optimal range for a physiologically relevant manipulation. Likewise, diet treatments successfully manipulated body condition: males as well as females on the improved diet were heavier and fatter. They were not unnaturally obese, since body mass and fat scores were at the extreme end of the natural variation in molting juveniles in our study area at that time of the year. Birds that were offered the improved diet spent a slightly greater proportion of their time eating (Table 2) which allowed them to increase their food intake per day (M.L. Roberts, R.H.J.M. Kurvers and A. Peters, unpublished data) and hence produce more fat stores.

### Song and locomotion

T treatment resulted in a moderate increase of song activity during molt: irrespective of diet, T-males sang more than C-males (Table 2, Fig. 2). Increase in song frequency is a classic response of male birds to exogenous T during the breeding season (Silverin, 1980; Chandler et al., 1994; Enstrom et al., 1997; but see Peters, 2002), including species where other behavior (aggression/nestling feeding) is insensitive to additional T (Hunt et al., 1997; Van Duyse et al., 2000). Our results extend this stimulation of song activity by exogenous T to the molt period. Although unmanipulated blue tits during molt sing infrequently, song could be important in flocks, when birds compete aggressively for food (Cramp and Perrins, 1993). In passerines, dominance in such non-breeding flocks is closely linked to song, with dominant individuals singing more (Wiley et al., 1993; Krams, 2000). An increase in song during molt can thus be beneficial by

improving access to food and use of safer feeding places. An additional potential benefit could be an advantage in obtaining a mate, since song in passerines also serves for mate attraction (Andersson, 1994). Although blue tits remain loosely paired outside the breeding season, the extent to which juveniles invest in mate attraction at this time of year is unknown (Cramp and Perrins, 1993).

There was no evidence for a condition-dependent effect of T on singing, or a stimulatory effect of improved condition on song activity. Song output in blue tits may signal male quality during the (pre-) breeding season, but significant relationships between song production and condition in breeding blue tits have not been replicated among studies (see Parker et al., 2006 for an overview). Although blue tits sing at other times of the year, albeit more infrequently, even less is known about the role of song outside the breeding season. Our results do not support the view of song as a signal of male condition. Rather they suggest that song could act as a signal of need or motivation. Birds on the standard diet, in relatively worse condition, sang more than birds on the improved diet: presumably their need or motivation to improve their food access was greater than that of the birds in improved condition.

Food access can also be improved by increased home range size, achieved through increased locomotion. As expected, T treatment increased the time spent flying, and decreased the time spent resting. However, this effect only occurred in males fed the standard diet, not in males on the improved diet. This indicates that the effect of T may depend on condition, and thereby on motivation or need, a hypothesis that is not often considered when explaining variation in response to (exogenous) T (Kempnaers et al., 2008). A T-induced increase in locomotion (and home range) may be only useful when food resources are limited in a bird's territory.

While there are potential beneficial effects of T-induced increase in song and locomotion, these behaviors during the molt may also be costly. Molting birds are known to decrease conspicuous behavioral patterns (Newton, 1966; Wingfield and Soma, 2002) to avoid predation. An increase in song as well as locomotion makes birds more conspicuous (Krams, 2001; Post and Götmark, 2006). Moreover, since molt is energetically costly (Payne, 1972; Lindström et al., 1993), the costs of molt will be higher for birds that spend more time in flight (Møller et al., 1995). This indicates that males need to strictly balance favorable with potentially harmful behavioral effects of increasing their T levels, a balancing act that is further complicated by direct effects of T on molt progress.

### Molt progress

There is ample experimental evidence that exogenous T can delay (Runfeldt and Wingfield, 1985; Schleussner et al., 1985; Dawson, 1994; Stoehr and Hill, 2001; Clotfelter et al., 2004) or even prevent molt (Nolan et al., 1992; Dawson, 1994; but see Peters et al., 2000 and Peters, 2007 for T-stimulated molt). The comparatively modest experimental increase of T in molting juvenile male blue tits in our study also resulted in delayed molt progress (Fig. 3), but the effect was rather mild compared to the sometimes dramatic effects (delay in molt completion of several months or no feather replacement) observed in some of the other studies (that also employed higher T levels, similar to breeding levels). Although all T-males in our experiment completed the molt by mid October, a slower molt rate may be costly if birds do not complete their molt before dispersal or migration starts and/or lower temperatures arrive. Additionally, being in a state of molt for a longer period may be disadvantageous, in view of the physiological costs involved in the process (see Introduction).

However, despite the plethora of physiological costs that are involved in the process of molt, improved condition did not enhance molt progress or override the T-induced molt delay in our experiment. Possibly, conditions under the standard diet were sufficiently benign for the process of molt to proceed optimally. Indeed, it appears that a



normally proceeding molt program is of such importance for birds, that molt is fairly resistant to forms of malnutrition that free-living birds may encounter (Murphy and King, 1991), and that it can proceed even under circumstances of severe food deprivation (Murphy et al., 1988; Swaddle and Witter, 1997b) although feather growth and quality may be affected (Swaddle and Witter, 1994; McWilliams and Leafloor, 2005).

#### Plumage maintenance

Contrary to our prediction, there was no significant effect of diet/condition or exogenous T on investment in plumage maintenance (preening). Maintenance of plumage in birds may be important for mate choice (Griggio and Hoi, 2006) and in blue tits, UV/blue color of the male crown is an important sexual signal (Hunt et al., 1998; Andersson et al., 1998; Delhey et al., 2003, 2007a, 2007b; Johnsen et al., 2005), related to T levels during breeding (Peters et al., 2006). Crown color declines through the year (Delhey et al., 2006), underscoring the importance of plumage maintenance. Indeed, preening may improve the appearance of plumage and male attractiveness (Dowling et al., 2001; Zampiga et al., 2004). Therefore we had expected this behavior to be condition- and/or T-dependent. Possibly a self-maintenance function of plumage care is more or equally important to investment in sexual signalling. Nonetheless, males preened 1.6 times more than females, although this difference was not quite significant (Table 3). Similarly, in barn swallows *Hirundo rustica* and Seychelles warblers *Acrocephalus sechellensis* males preened more than females during the breeding season (Møller, 1991; Dowling et al., 2001). Therefore it seems that males might indeed invest more in preening behavior as part of their sexual investment strategy, but this investment is not dependent on their condition or T, at least not during molt. Possibly, T affects investment in plumage maintenance at other times of the year, closer to the breeding season, when importance of a well-maintained plumage becomes more imperative (see also Delhey et al., 2006).

#### Sex differences

Differences in behavior between the male T treatments were not correspondingly observed between the sexes; males spent a similar amount of time flying, rested marginally more than females and there was no sex difference in molt speed. This lack of a sex difference in behavior agrees with our observation that in captive as well as in free-living birds, during the autumn molt, males have similar T levels as females. Therefore it seems that T does not control behavioral and morphological differences between the sexes during molt, although our experiment demonstrated that individual variation in T can affect male investment strategies.

#### Conclusion

Exogenous administration of T affected male blue tits during the molt period. Similar to its effects during the breeding season, T shifted investment away from self-maintenance (resting, molt progress) towards behaviors relevant for aggressive competition (singing, flying), although T did not stimulate investment in a putative sexual behavior (preening). These results thereby do not provide support for the T insensitivity hypothesis, which states that birds may become insensitive to the behavioral effects of T during certain periods of the year (Wingfield et al., 2001). Insensitivity to T has been demonstrated for the parental phase in arctic breeding birds (Hunt et al., 1997; Lynn et al., 2002) but also in the closely related great tit, *Parus major* (Van Duyse et al., 2000). Our study shows that also during molt male blue tits are sensitive to effects of exogenous T in the expected manner. However, this experiment cannot exclude the possibility of a partial decrease in sensitivity compared to other times of the year. Since T can have costly effects – it delays molt and can increase energy

expenditure (through effects on metabolic rate (Buchanan et al., 2001) or behavior (this study)) – a quick decline to low levels in T after breeding, as is usually observed (e.g. Dawson, 1983; Schluessner et al., 1985; Ketterson and Nolan, 1992), appears to be an adaptive strategy for male birds. Our results indicate that nonetheless, subtle fluctuations in low molt levels of T may be instrumental in governing male trade-offs during this important, but neglected, life-history stage.

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