

Parental responsiveness negatively correlates with fecal testosterone concentration in male mandarin voles (*Microtus mandarinus*)

A. V. Smorkatcheva · T. N. Bychenkova ·
E. L. Zavjalov

Received: 20 July 2008 / Accepted: 18 January 2009
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Abstract The tradeoff between parental effort and mating effort in male animals may be mediated by testosterone (T). The pattern of association between T and paternal care in birds is consistent with this hypothesis, while it is poorly studied and not universal for mammals. We used the correlation approach to test two predictions of T-mediated tradeoff hypothesis for a biparental vole, *Microtus mandarinus*: (1) that T levels in males decrease from before pair formation to after birth of the first litter and (2) that paternal responsiveness of males negatively correlates with their T levels. T concentrations were measured in fecal samples collected before pairing and then immediately before behavioral testing on day 5 after birth of the first litter. Both nonpaternal and low paternal males had high initial T that decreased after birth of pups, though the decrease was only significant in low paternal males. In highly paternal males, the initial T was low and did not change after birth. Our results support the predictions of T-mediated tradeoff hypothesis and reveal individual variation in hormone–behavior relationship.

Keywords *Microtus mandarinus* · Paternal behavior · Testosterone · Life-history tradeoff

Introduction

The tradeoff between parental effort and mating effort is considered to have important influence on life-history evolution (Trivers 1972; Magrath and Komdeur 2003). The first and most obvious cause of this tradeoff is the limited amount of resources, time, and energy that males have to allocate to the alternative behaviors. However, hormones, specifically testosterone (T), can provide mechanistic explanations for the existence of life-history tradeoffs (Wingfield et al. 1990; Ketterson and Nolan Jr 1992). High levels of T usually increase male mating success, while the effect of T on paternal behavior is commonly thought to be negative. Thus, regulation of T secretion in males should balance the benefits of providing parental care with costs of decreased reproduction. This model predicts a negative association between circulating T and amount of paternal behavior (Wingfield et al. 1990; Ketterson and Nolan Jr 1992). At the interspecific level, baseline T concentrations are expected to be lower and the amount of paternal care is expected to be higher in monogamous than in polygynous species. At the interindividual level, T-mediated life-history tradeoff hypothesis predicts a relationship between individual variation in levels of T (or perhaps the ability to increase/decrease T concentration under appropriate conditions) and individual variation in behavior. At the intra-individual level, the tradeoff may permit organisms to adjust their reproductive tactics in response to variations in local conditions (challenge hypothesis, Wingfield et al. 1990). In species with extended paternal care, circulating levels of T should decrease during periods when parental care is required.

A. V. Smorkatcheva (✉) · T. N. Bychenkova
Department of Vertebrate Zoology, St. Petersburg State
University, Universitetskaya nab. 7/9,
199034 St. Petersburg, Russia
e-mail: tonyas1965@mail.ru

T. N. Bychenkova
e-mail: elenin_yachik@mail.ru

E. L. Zavjalov
Institute of Animal Systematics and Ecology,
Frunze Street 11, 630091 Novosibirsk, Russia
e-mail: zavjalov@ngs.ru

These ideas are supported by both comparative and experimental evidence from avian studies. Interspecific variation in patterns of T secretion among birds correlates with interspecific variation in the mating system (Wingfield et al. 1987, 1990). In seasonally breeding species of birds, males tend to have increased baseline T levels during the spring and summer breeding season, which then declines when males provide paternal care (Wingfield et al. 1990; Ketterson and Nolan Jr 1999; Raouf et al. 1997; Hau 2007). Many studies have shown decreases in parental behavior after experimental elevation of testosterone (e.g., Silverin 1980; Hegner and Wingfield 1987; Dittami et al. 1991; Ketterson et al. 1992; Raouf et al. 1997; Peters et al. 2002; Van Roo 2004). In addition, the expected covariation between parental behavior and the magnitude of testosterone elevations that are naturally produced during territorial interactions is demonstrated for male dark-eyed juncos, *Junco hyemalis* (McGlothlin et al. 2007).

In mammals, the relationship between T and paternal behavior has been examined in only a few species. At the interspecific level, two studies compared hormonal changes in males of Siberian dwarf hamsters, *Phodopus sungorus*, which is considered to be an uniparental species and Djungarian dwarf hamsters, *Phodopus campbelli*, which is biparental (Wynne-Edwards 1995; but see Khrushova and Vasilieva 2008). The results were contradictory (Reburn and Wynne-Edwards 1999; Schum and Wynne-Edwards 2005). The expected temporal dynamics in T levels were demonstrated for men (Berg and Wynne-Edwards 2001), black tufted-ear marmosets, *Callithrix kuhlii* (Nunes et al. 2000), and Mongolian gerbil, *Meriones unguiculatus* (Brown et al. 1995). The manipulative experiments testing the predicted inhibitory effect of T on paternal behavior in rodents give varied results (*Rattus norvegicus*: Quadagno and Rockwell 1972; McCullough et al. 1974; Rosenberg and Herrenkohl 1976; *Meriones unguiculatus*: Clark and Galef 1999; *Mesocricetus auratus*: Marques and Valenstein 1976; *Peromyscus californicus*: Trainor and Marler 2001, 2002; *Microtus ochrogaster*: Wang and De Vries 1993; Roberts et al. 1996; Lonstein and De Vries 1999, 2000, 2002). At the interindividual level, T concentrations are inversely associated with paternal responsiveness in *Meriones unguiculatus* (Clark and Galef 2000; Clark et al. 1997) and *Phodopus campbelli* (Schum and Wynne-Edwards 2005), but tended to be positively correlated with huddling behavior in male *Peromyscus californicus* (Trainor and Marler 2001). The results of two recent studies in men are contradictory (Fleming et al. 2002; Roney et al. 2006). Thus, the pattern of association between T and paternal care is not universal across mammals. Clearly, more experiments with more species are needed to understand whether differences in levels of T may contribute to the existence of the sexual effort–parental effort tradeoff in this vertebrate class.

Due to the ease of husbandry and breeding in the lab as well as the diversity of their mating/rearing systems, arvicoline rodents represent an attractive group to address this problem. The results of manipulative experiments with prairie voles, *Microtus ochrogaster* (Wang and De Vries 1993; Roberts et al. 1996; Lonstein and De Vries 1999, 2000, 2002), are contradictory and do not verify the relevance of the T-mediated tradeoff model. While only prairie voles have been used as a model species in studies of the relationship between T and paternal behavior, several other arvicolines with extensive paternal care (see for review Smorkatcheva 2003) may also be appropriate to test the expected pattern. The mandarin vole, *Microtus mandarinus*, is a specialized fossorial species, distributed in grasslands of Central Asia. Like prairie voles (Getz et al. 1981; Carter and Getz 1993; Getz and McGuire 1997; Solomon and Getz 1997), mandarin voles live in extended communal/cooperative family groups and demonstrate prolonged pair bonds (Smorkatcheva 1999; Tai and Wang 2001). Males are strongly paternal (Smorkatcheva 2003). Therefore, in this study we use the mandarin vole to test, with a correlational approach, the following predictions of T-mediated tradeoff model:

1. T levels in male mandarin voles decrease from before pairing (the period of life when the intensity of male–male competition is probably strongest) to after birth of the first litter (when paternal care is required)
2. Paternal responsiveness of males negatively correlates with their T levels.

Materials and methods

Animals and housing

Mandarin voles used in these experiments were fifth to seventh generation laboratory-reared outbred animals from parental stock captured in western Buryatia, Russia. Breeding pairs were housed in glass aquariums (30 × 60 × 30 cm) half-filled with wood shavings and provided with a wooden box for nesting. Toilet paper served as nest material. Carrots, oats, rabbit chow, and willow twigs were provided ad lib. Additionally, oat shoots were given in small amounts. The voles were maintained on a 16/8-h light/dark cycle (lights on at 0800 h) at an ambient temperature of 18–22°C.

Subject males ($n = 24$) remained with their natal family group until the age of 46–54 days, at which time they were separated from their parents and housed with same-sex littermates. At the beginning of the study, when subjects were between 70 and 120 days of age, each male was housed with an unrelated naive female aged at least 60

days. Immediately before pairing, the first fecal samples were collected (see below). Pairs were then left undisturbed except to inspect females weekly until late pregnancy. After that time, we inspected the nest every other day for birth of pups. We determined the pup's exact age by their external signs (skin pigmentation, presence of the upper and lower incisors, auricle condition).

Behavioral testing

Paternal responsiveness was assessed through behavioral tests similar to those described by Lonstein and De Vries (1999), with some modifications. All tests were conducted on day 5 after birth of the first litter (day 0 = day of birth) between 0900 and 1200 h. This age was chosen because male mandarin voles appear to be less paternal during the first several days after birth than they are later (Smorkatcheva 2003), and the pups become too mobile by the end of their first week. Before the test, the second fecal sample was collected from each subject male. Then we placed the male into the testing arena (glass aquarium). The aquarium was of similar dimensions to the subject's home cage (30 × 60 × 30 cm) and was initially divided with a removable partition into two sections. The section where the male was placed contained some soiled bedding from his home cage, a small amount of food, and small pieces of toilet paper. A clean wooden semi-open box (10 × 15 × 7 cm) was placed into a corner of this section. While voles perceived this box as a safe shelter, the lack of two sides allowed us to observe the male's behavior while inside the box. Each subject was allowed to habituate himself to the new environment for 15 min. While wearing a glove, the experimenter placed one pup from the subject's litter in the farthest corner of the aquarium from where the male was sitting (usually the wooden box). Then the experimenter removed the partition and the male's behavior was recorded for the next 20 min.

We recorded behaviors directed towards pups (sniffing, licking, huddling, manipulating with, and retrieving) and several non-pup-oriented activities (digging, eating, walking, exploratory uprights, and moving food or toilet paper). For pup retrieval, movement of objects, and uprights, the number of occurrences per 20-min period was recorded (all occurrence sampling, Altmann 1974). All other behaviors as well as being in tactile contact with pup (while performing any activities or not) were collected by scan sampling (Altmann 1974) at 5-s intervals, providing 240 data points per testing period. We also recorded latency (number of scans) in approaching the pup. Subjects that did not approach the pup during the 20-min test ($n = 8$) were arbitrarily assigned a latency of 250 scans.

Fecal samples collection

We collected two fecal samples from each experimental male: before pairing and immediately before behavioral testing. To collect each sample, we placed subjects into plastic containers for 90 min. The bottom of the container was covered with blotting paper to soak up urine. Collected fecal samples were dried at 20–22°C and then stored in closed tubes at room temperature before conducting the T assay.

Testosterone assays

We measured the concentration of T in dry feces by radioimmunoassay using Sigma antibodies (rabbit anti-testosterone, T 4276) and Amersham labelled hormones ([1,2,6,7- H^3]-testosterone, Amersham, Boston, MA, USA). Steroids were extracted as follows: Dry feces (50 mg) were homogenized in double-distilled water (3 ml) with a glass grinder. After centrifugation, the supernatants were collected and stored at 20°C until assayed. Steroid hormones were extracted from 0.4 ml of supernatant with 3 ml of ethyl ether, then 2.5 ml extract was removed, transferred to new tubes, vacuum-dried at 45°C, and the residue was resuspended in 100 μ l phosphate buffer, pH 7.4 (Gerlinskaya et al. 1993; Zavjalov et al. 2003). Following the Sigma protocol, we added 100 μ l of diluted antiserum to the tubes containing resuspended samples. After vortexing and a 30-min incubation at room temperature, 100 μ l of [H^3]-testosterone diluted in phosphate buffer was added and samples were incubated again for 1 h at 37°C. After 15 min cooling on ice, free and bound fractions of testosterone were separated by 10-min incubations with 500 μ l of dextran-coated charcoal suspension and centrifugation at 3,000 rpm for 15 min at 4°C. The supernatants from each tube (500 μ l) were added to scintillation cocktails and counted on a liquid scintillation counter (Beckman LS 6500, USA).

The assays were validated for use with vole fecal steroid extracts by determining accuracy and parallelism (Chard 1978). Sensitivity of the assay was determined from the 95% confidence interval of zero standards with mean values of 5 pg/tube. The inter- and intra-assay variations were 10.3 and 6.7, respectively. To determine parallelism, we prepared a five-point, twofold dilution series of fecal samples in phosphate buffer and compared it with the standard curve for testosterone. There were no significant differences between the slope of the standard curve (−2.66) and the slopes of lines generated from fecal samples (−2.43) of assayed voles ($t = 0.8$, $df = 8$, $P > 0.5$).

Data analyses

We used Pearson correlation coefficients to test if there were correlations between T concentrations in naive males before pairing (T_1) and in fathers immediately before behavioral testing (T_2) to make sure that the intrasample differences in T reflected individual variation rather than transient fluctuations in hormone levels.

A principal components analysis was used to organize 12 behavioral measures (10 activities plus being in contact with pup plus latency to approach pup) into a small number of underlying factors. Four behavior-related principal components (eigenvalues above 1) were extracted from the data matrix (Table 1). The first principal component (PC1) accounted for 33.2% of the total variance and comprised contact with pup, latency to approach pup, and all pup-oriented activities but retrieval. In the following statistical analyses, we used scores from PC1 as our measure of parental responsiveness.

We carried out two types of analyses. First, we tested the correlations between each of the three T measures (T_1 : concentration before pairing, T_2 : concentration before behavioral testing, the magnitude of the decrease in T) and parental responsiveness scores using Pearson correlation coefficients.

Second, we categorized all males as nonpaternal (NP), low paternal (LP), or highly paternal (HP) based on their parental responsiveness scores (see “Results” section for details). Then we used a mixed ANOVA to analyze the effect of sample period (within-subjects variable with two levels: T_1 , T_2) and category of males (between-subjects variable with three levels: NP, LP, HP) on T concentration. We also tested the effect of male category (NP, LP, and HP) on the magnitude of T decrease with a one-way ANOVA. As pairwise comparisons were of especial

Table 1 Loadings of the four principal components of behaviors measured during 20-min trials

Behavior	PC1	PC2	PC3	PC4
Latency to approach pup	-0.89	-0.23	0.08	0.03
Huddle pup	0.62	-0.34	-0.47	-0.21
Lick pup	0.79	-0.18	0.22	0.11
Manipulate with pup	0.74	-0.09	0.16	0.24
Sniff pup	0.62	0.13	0.62	-0.09
Retrieve pup	0.48	-0.13	-0.18	-0.61
In contact with pup	0.88	-0.26	-0.13	-0.06
Walk	0.40	0.70	-0.15	0.38
Upright	0.22	0.75	-0.04	0.13
Eat	-0.13	0.74	-0.03	-0.47
Dig	0.01	0.64	0.41	-0.33
Bring objects	0.11	0.74	-0.45	0.08

interest, post-hoc analyses (Tukey’s HSD tests) were conducted independently to determine whether F was significant (Zwick 1993; Hsu 1996). Given small sample sizes, we calculated effect sizes (d) with confidence intervals (Hedges and Olkin 1985) for all nonsignificant pairwise contrasts, and where effect sizes were at least 0.50 (medium to large), we presented the results of retrospective power analyses. Both effect-size calculations and power analyses were conducted for respective t -tests for independent (NP vs. LP vs. HP) or dependent (T_1 vs. T_2) variables. When t -test power is low, it means that the Tukey test provides even less power to detect the differences (Conagin et al. 2008).

Because T values were not normally distributed, we analyzed the log-transformed data. The magnitude of the decrease in T (dT) was calculated as $\log T_1 - \log T_2$.

All tests were two-tailed. Statistical significance in all cases was $P < 0.05$. All statistical analyses were done using Statistica 7.1.

Results

We found a strong positive correlation between T_1 and T_2 ($r = 0.64$; $P < 0.001$). Paternal responsiveness was inversely correlated with both T_1 ($r = -0.55$, $P = 0.006$; Fig. 1a) and T_2 ($r = -0.47$, $P = 0.021$; Fig. 1b), but, although the slope was negative, it was not significantly correlated with a decrease in T ($r = -0.23$; $P = 0.275$; Fig. 1c).

Based on the scores from the principal component analysis, male subjects were divided into three categories (Fig. 2). The first category (NP) included eight males that did not make any contact with pups and thus had very low PC1 scores (between -1.25 and -1.27). The second category (LP, $n = 9$) consisted of males with intermediate PC1 scores (between -0.48 and 0.21), and the third category (HP, $n = 7$) consisted of males with very high PC1 scores (0.93 – 2.12).

The comparison of T_1 and T_2 in NP, LP, and HP males is presented in Fig. 3. The main effects of both within-subject ($F_{1,21} = 5.81$, $P = 0.025$) and between-subjects ($F_{2,21} = 6.00$, $P = 0.009$) variables on T levels were statistically significant. The effect of sample period \times category interaction was of borderline statistical significance ($F_{2,21} = 3.30$, $P = 0.057$). The general effect of paternal responsiveness on T level was negative (Fig. 3). However, only HP males significantly differed in their T_1 levels from both NP and LP males (respectively, $P = 0.014$ and 0.016), while there was no difference in T_1 between NP and LP males ($d = 0.060 \pm 49$). Tukey test revealed no statistically significant differences in T_2 between NP, LP, and HP individuals although the effect sizes for these comparisons

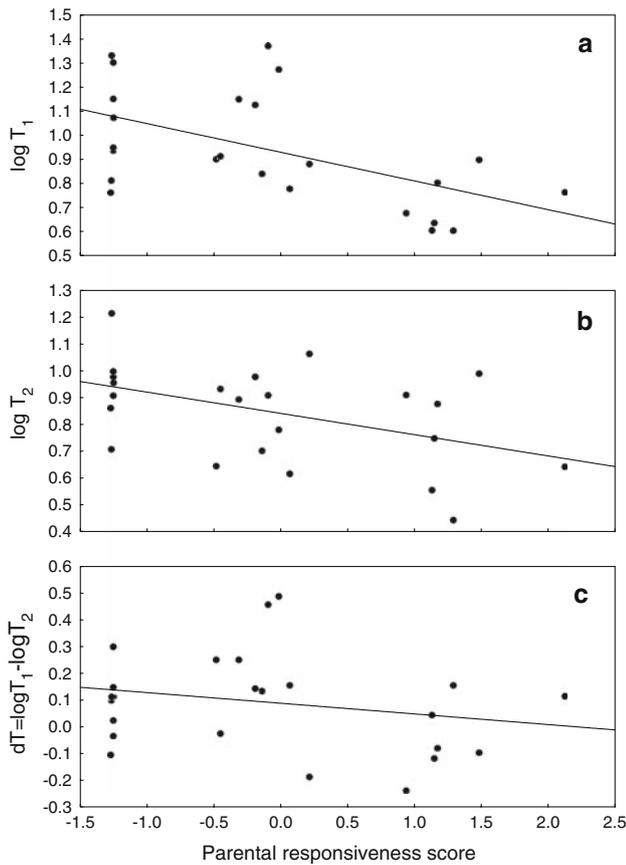


Fig. 1 Relationships between parental responsiveness and **a** initial testosterone levels (T_1 , ng/ml, log transformed), **b** testosterone levels after birth (T_2 , ng/ml, log transformed), and **c** the magnitude of T decrease ($\log T_1 - \log T_2$)

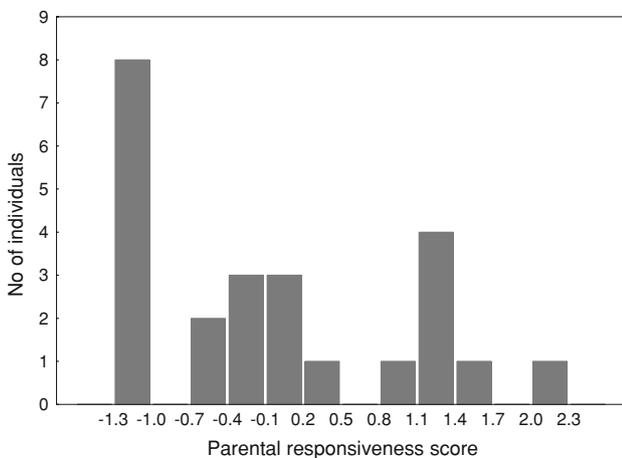


Fig. 2 The distribution of scores by PC1 (paternal responsiveness) in 24 subjects

were medium to large (NP vs. LP: $d = 0.79 \pm 0.98$; NP vs. HP: $d = 1.24 \pm 0.56$; LP vs. HP: $d = 0.54 \pm 1.00$). The low power for these comparisons (0.32, 0.55, and 0.18, respectively) suggests that the lack of statistical

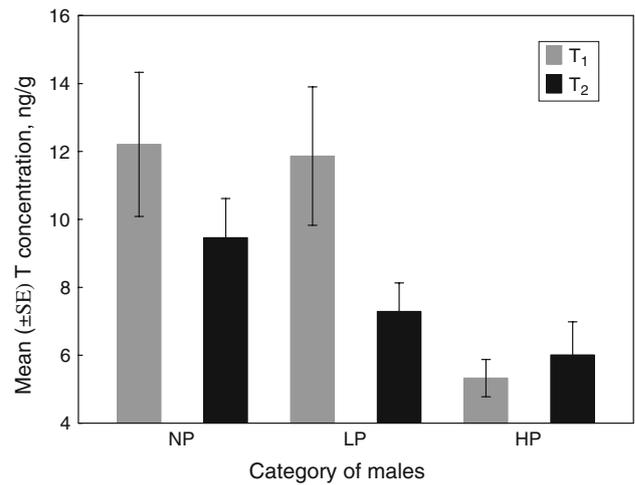


Fig. 3 The comparison of T_1 and T_2 in nonpaternal (NP), low paternal (LP), and highly paternal (HP) males

significance may be due to small sample size rather than to a lack of biological differences.

Overall, T_1 was higher than T_2 , but the difference was significant only for LP males ($P = 0.027$). In fact, for HP males there was no decrease in T levels at all ($d = -0.21 \pm 0.58$; Fig. 3). NP males showed decreased T levels, but the decrease was not statistically significant. Again, the lack of statistical significance was apparently due to small sample size ($d = 0.77 \pm 1.01$; power = 0.46).

As was expected from the results of previous analyses, the one-way ANOVA that examined the effect of male category on dT also showed borderline statistical significance ($F_{2,21} = 3.30$, $P = 0.057$). LP males showed greater decreases in T levels compared to HP males ($P = 0.046$). Other differences in dT were nonsignificant. Again, sample sizes appeared to provide insufficient power to detect the differences despite medium or even large effect sizes (NP vs. LP: $d = 0.57 \pm 0.98$, power = 0.20; NP vs. HP: $d = 0.87 \pm 1.06$; power = 0.34).

Discussion

In general, our correlational study with biparental *Microtus mandarinus* supported both predictions: T concentrations were higher in naive males than in new fathers, and there was a negative correlation between T concentrations and levels of paternal behavior. These results are consistent with published correlative data for men (Berg and Wynne-Edwards 2001; Storey et al. 2000; Fleming et al. 2002; Gray et al. 2006) and males of two nonhuman species with extensive paternal care—black tufted-ear marmosets (Nunes et al. 2000) and Mongolian gerbils (Brown et al.

1995). At the same time, the more detailed analysis of individual variation in paternal responsiveness and temporal dynamics of T revealed that the pattern of association between these variables is not so simple. In accordance with the expected pattern, HP male mandarin voles had lower initial and post-birth T levels than other categories of males. However, contrary to the prediction, their T levels did not decrease after birth of pups. Those males that showed paternal behavior at low levels (LP) had initial concentrations of T that were as high as seen in NP males. T levels decreased after birth in both LP (in which the decrease was statistically significant) and NP (in which small sample sizes appear to prevent the detection of significance) individuals. Among these two categories, LP males demonstrated lower mean T_2 concentrations and a greater decrease in T than did NP males. Although none of these measures differed significantly between categories (probably due to small sample sizes), the greater paternal responsiveness of LP in comparison with NP category might be attributable to one or both of these differences. Our results show that the pattern of association between T and paternal responsiveness at the intra-individual level may not be uniform even within one species. The similar variation in T dynamics relative to birth of offspring has been reported for men (Berg and Wynne-Edwards 2001) and may occur in other nonhuman mammals. This variation may contribute to the inconsistency in the results from two studies that examined changes in new dwarf hamster fathers (Reburn and Wynne-Edwards 1999; Schum and Wynne-Edwards 2005).

The results of our work are difficult to compare with those of published studies on another arvicoline species, *Microtus ochrogaster*, because different approaches and methods were used. The association between T and parental responsiveness in the prairie vole has been examined by manipulative methods only (Wang and De Vries 1993; Roberts et al. 1996; Lonstein and De Vries 1999, 2000, 2002). The authors of these experiments used castration or treatments resulting in supraphysiological levels of T, while we correlated variation in paternal behavior with natural individual variation in T. In addition, prairie voles used in these experiments were not fathers of pups but were virgin males. Most of these studies provide no evidence for the interference of T with parental care. Although postnatal injections of T reduced alloparental behavior in juvenile males (Roberts et al. 1996), the effect of similar treatment on the behavior of adult animals was not statistically significant (Lonstein and De Vries 2000). Neonatal castration significantly reduced the percentage of males that displayed parental behavior (Lonstein and De Vries 2002). The influence of castration during adulthood is inconsistent, with one study reporting a decrease in pup-oriented behavior (Wang and De Vries 1993) and a second

study reporting no effect (Lonstein and De Vries 1999). Taken together, the results of experiments with *Microtus ochrogaster* and their disagreement with our findings in *Microtus mandarinus* appear to support the indirect and/or dose-dependent effect of T on paternal behavior in *Microtus*. Further research into the modulation of paternal care by androgens in male voles is clearly warranted.

Independent of the causal mechanisms underlying the established association between T and paternal responsiveness, this study demonstrates that male mandarin voles with chronically low levels of T were strongly paternal, while ones with chronically high T levels did not show paternal behavior under the same conditions. In *Meriones unguiculatus*, males with greater life-time circulating levels of T are not only less parental but also more sexually active and have greater copulatory success than are those with relatively low T titres (Clark and Galef 2000; Clark et al. 1997). The positive correlation between T and individual traits that determine male mating effort and mating success has been shown for several species of voles (*Arvicola terrestris*: Moshkin et al. 1984; *Microtus pennsylvanicus*: Turner et al. 1980, 1983; Ferkin et al. 1994; Perrot-Sinal et al. 1998; *Clethrionomys gapperi*: Schulte-Hostedde et al. 2003; *Microtus brandti*: Zhang and Shi 2005).

Brandt's vole, *Microtus brandti*, is not only closely related to *Microtus mandarinus* (Musser and Carleton 2005) but also shares many of the mandarin vole's social traits: high degree of sociality, prolonged pair-bonds, communal and cooperative breeding, paternal and alloparental care (Zhang and Zhong 1981; Fang and Sun 1991; Yin and Fang 1998; Zöphel 1999; Gromov 2003, 2005). In this latter species, dominant males are more aggressive and have higher serum T levels than subordinate males (Zhang and Shi 2005). If this is the case for *Microtus mandarinus*, the variation in T levels may at least partially underlie individual variation in the sexual effort–parental effort tradeoff in this species. Importantly, the T-mediated conflict between mating effort and parental effort should persist even if acquiring mates and caring for offspring do not overlap in time. So, the tradeoff is relevant independently of whether or not male mandarin voles are engaged in extra-pair copulations.

To summarize, the results of our study supported the negative association between T concentration and parental responsiveness of male voles at both the intra-individual and inter-individual levels. Individual variation in the temporal dynamics of T relative to the birth of pups should be taken into account in subsequent investigations.

Acknowledgments Two anonymous referees helped substantially to improve a previous version of this article. We are grateful to Nancy Solomon for her critical comments and for editing the English. The

authors also thank Natalia Rostova for her help in statistics. The research was supported by the program “Biological Diversity and Dynamics of Gene Pools” of the Presidium of the Russian Academy of Sciences (grant 6.1.2) and by Russian Foundation of Basic Research (project 07-04-00142a).

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