Testosterone-Mediated Effects on Fitness-Related Phenotypic Traits and Fitness

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Submitted February 17, 2008; Accepted October 8, 2008; Electronically published February 23, 2009

abstract: The physiological and behavioral mechanisms underlying life-history trade-offs are a continued source of debate. Testosterone (T) is one physiological factor proposed to mediate the trade-off between reproduction and survival. We use phenotypic engineering and multiple laboratory and field fitness-related phenotypic traits to test the effects of elevated T between two bank vole *Myodes glareolus* groups: dominant and subordinate males. Males with naturally high T levels showed higher social status (laboratory dominance) and mobility (distance between capture sites) than low-T males, and the effect of T on immune response was also T group specific, suggesting that behavioral strategies may exist in male bank voles due to the correlated responses of T. Exogenous T enhanced social status, mate searching (polygon of capture sites), mobility, and reproductive success (relative measure of pups sired). However, exogenous T also resulted in the reduction of immune function, but only in males from the high-T group. This result may be explained either by the immunosuppression costs of T or by differential sensitivity of different behavioral strategies to steroids. Circulating T levels were found to be heritable; therefore, female bank voles would derive indirect genetic benefits via good genes from mating with males signaling dominance.

Keywords: trade-off, immune response, dominance, cost of reproduction, sexual signals, aggression, spacing behavior.

Introduction

Life-history traits commonly trade off with each other, and thus fitness-related phenotypic traits are constrained from evolving independently (Stearns 1992; Roff 2002). According to life-history theory, selection is predicted to favor the evolution of optimal trait values within the constraints imposed by the trade-off (Stearns 1992; Roff 2002; Roff and Fairbairn 2007). Negative covariation between reproduction and survival has stimulated research to understand the physiological and behavioral mechanisms, as well as the constraints, underlying such a trade-off. Physiological factors, such as hormones, often mediate trade-offs, and phenotypic manipulation of hormone status reveals how selection might act on correlated life-history traits while also demonstrating their causal, mechanistic links (reviewed in Ketterson et al. 1996; Ketterson and Nolan 1999).

Reproductive effort in vertebrate males involves the production of sexual signals (ornaments, male-male competition, and aggression) induced by the androgen testosterone (T). Exogenous and endogenous T have been shown to enhance male territoriality (Moore and Marler 1987), aggressive behavior (Salvador et al. 1996; Briganti et al. 1999; McGlothlin et al. 2007, 2008), and male attractiveness to females (Enstrom et al. 1997), while their control on the expression of sexual ornaments shows conflicting results (for [Eens et al. 2000; Evans et al. 2000; Ditchkoff et al. 2001; McGlothlin et al. 2008] or against [Weatherhead et al. 1993; Stoehr and Hill 2001]). The endocrine system is therefore the proximate link between male morphology, behavior, and, ultimately, reproductive success.

Male reproductive effort was proposed to trade-off with genetic resistance to disease organisms, such as parasites, ultimately affecting survival (Hamilton and Zuk 1982). Indeed, elevated T has been found to reduce survival (Marler and Moore 1988; Reed et al. 2006). Therefore, the endocrine system is thought to regulate life-history trade-offs, and its negative feedback with the immune system is defined by the immunocompetence handicap hypothesis (Folstad and Karter 1992). This trade-off may be mediated by both direct and indirect mechanisms: circulating T may bind to androgen receptors on immune cells, directly modulating immune responses (Tanriverdi et al. 2003); alternatively, resources may be redirected away from immune function toward sexual behavior or ornamentation (Wedekind and Folstad 1994), or other hormones, such as glu-

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Am. Nat. 2009. Vol. 173, pp. 475–487.
DOI: 10.1086/597222

cocorticosteroids, elevated by T, may have direct suppressive effects on immune function (Besedovsky and del Rey 1996; Olsen and Kovacs 1996; Råberg et al. 1998; Owen-Asley et al. 2004).

Numerous studies have found evidence for a trade-off between immune function and T in mammals (Barnard et al. 1994), birds (e.g., Saino et al. 1995; Verhulst et al. 1999; Duffy et al. 2000; Casto et al. 2001; Mougeot et al. 2004; Owen-Asley et al. 2004; Greives et al. 2006), and reptiles (e.g., Veiga et al. 1998; Belliure et al. 2004; Mills et al. 2008) or 11-ketotestosterone in fish (Kurtz et al. 2007). However, others found that T had an enhancing effect on (Hasselquist et al. 1999; Evans et al. 2000), showed a positive correlation with (Peters 2000), or had no effect on immune function (Roberts et al. 2007). Some studies have even found opposing effects of T on different immune components in the same individual (Bilbo and Nelson 2001; Brown et al. 2007) and that T-induced immunosuppression is context dependent (Greenman et al. 2005). In conclusion, the life-history literature is abound with discordant results (e.g., Ketterson et al. 1992; Salvador et al. 1996; Saino et al. 1997), and as discussed by Roberts et al. (2004), there is only limited support for both the endocrine-immune trade-off and its role as the mechanism mediating the trade-off between reproduction and survival.

In this article, we investigate T-mediated trade-offs in a small mammalian species, the bank vole *Myodes* (*Clethrionomys*) *glareolus*, which overcomes many problems that may have potentially led to the current ambiguous literature. First, one difficulty involves both acquiring long-term measures of fitness and relating fitness to mechanisms behind the trade-off (Reed et al. 2006). The bank vole has a short life span in the wild (0.5–2 years), with most individuals surviving no more than one breeding season (Ostfeld 1985; Macdonald 2001). As the bank vole's life span is within the duration of this study, we are able to interpret field reproductive success as fitness. In addition, male fitness, measured as male dominance and number of pups sired, has been correlated to plasma T levels in laboratory and field experiments, respectively (Mills et al. 2007*a*, 2007*b*). Second, an effective method for measuring the immune system is problematic due to the large number of interrelated immune components, as unexpected negative or positive correlations might arise when only a single immune measure is taken (Norris and Evans 2000; Zuk and Stoehr 2002). Circulating immunoglobulin G (IgG), one measure of the innate immune system—the first line of defense—representing a state of immunological readiness (Greives et al. 2006), is one immune measure that has been developed in the bank vole (Oksanen et al. 2003). In addition, by experimentally challenging the immune system with novel antigens and measuring concomitant host-specific antibody titers against these antigens, one gets a good estimate of host resistance to a variety of pathogens (Svensson and Skarstein 1997; Svensson et al. 1998; Hasselquist et al. 1999), and a measure of humoral adaptive immunity has been successfully established in the bank vole (Oksanen et al. 2003). Third, previously ambiguous results may also have arisen from the choice of study species. Males, whose sexual signals consist of status badges alone, may be able to temporally uncouple the feedback between T and immune response, yet if the signal is accompanied by social interactions, social costs would ensure that signals honestly reflect a male's current T level and immune response (Tibbetts and Dale 2004). The bank vole uses dominance to advertise quality through competition with other males for access to females (Hoffmeyer 1982). Direct male-male competition for sexually receptive females is common in microtine rodents (e.g., Madison 1980; Kawata 1985; Ostfeld 1985) and is therefore the likely social cost enforcing dominance in males. We report the results of experiments conducted in the lab and in field enclosures on the heritability of T and the relationship between T, a set of potentially fitness-related phenotypic traits, and fitness.

Methods

Species and Laboratory Experiment

The bank vole *Myodes* (*Clethrionomys*) *glareolus* is a common microtine rodent in the Palaearctic region (Stenseth 1985) and has a polygynandrous breeding system (Mills et al. 2007*a*) in which males provide no material resources to the female or offspring. Breeding female bank voles defend an exclusive territory against other females, but their home ranges (feeding areas) greatly overlap (Koskela et al. 1997). In comparison, males have territories overlapping with several female territories (Mazurkiewicz 1971). Females breed in postpartum estrus, showing preferences for dominant males based on cues in their urine (Kruczek 1997), and the preputial gland, the main source of male sexual attractants, is T dependent (Radwan et al. 2006). In our study area, females give birth to up to four litters over the breeding season, the litter size ranging from two to 10 (on average, five; Koivula et al. 2003). Male T levels are higher at low breeding density, and breeding by both males and females declines with increasing density (Oksanen et al. 2007); furthermore, synchronous breeding acts as a counterstrategy against infanticide in the bank vole (Poikonen et al. 2008).

Experimental animals were first-generation laboratory-born bank voles originally captured in Konnevesi, central Finland (62°37′N, 26°20′E), and were housed in standard mouse cages measuring 43 × 27 × 15 cm, maintained on

a 16L : 8D photoperiod. Water and food were continuously available. Paternal parameters, such as T level, were known for the experimental animals. After pretreatment plasma T had been measured in our experimental animals (methods described below), males received either a T-filled implant or an empty implant. Male mating success was measured in the laboratory 1 week after implantation, and a novel antigen was injected into all males to measure immune response (methods described below). Experimental animals were then released to outdoor enclosures for the field experiment (fig. 1).

Field Experiment

A 2 × 2 full factorial field experiment was performed, which consisted of two treatments: plasma T group (high- and low-T reflecting male dominance) and implant (empty and T filled). Four males, one from each treatment (low T + empty implant, low T + T-filled implant, high T + empty implant, high T + T-filled implant), were randomly assigned to 25 enclosure groups, which were released to 25 × 0.2-ha outdoor enclosures in June 2002 (for details of enclosures and vegetation, see Oksanen et al. 2003). Measurements of male fitness were required; therefore, six gravid females (ensuring that they would enter postpartum estrus in enclosures) were also released to each enclosure, and male fitness was measured from a female's second litter. Females had previously been paired with random males not used in this experiment. In order to monitor births and survival and measure endocrine and immune parameters, 20 Ugglan special multiple-capture live traps, baited with oats and potatoes, were distributed in each enclosure in a 5 × 4 grid with 10 m between traps. The field experiment ran until November 2002. A detailed time schedule of the experiment is provided in figure 1.

T Measurements

Retro-orbital blood samples were collected from each individual male (see methods in Oksanen et al. 2003) to measure plasma T using a radioimmunoassay kit (TESTO-CTK, DiaSorin, Byk-Sangtec Diagonstica GmbH, Dietzenbach, Germany), and the methods, test for parallelism, and repeatability are fully described elsewhere (Mills et al. 2007*a*). Fifty μL of blood samples competed for 3 h at 37°C with 500 μL of ^{125}I-labeled T for antibody-binding sites in tubes coated with a T antiserum. Radioactivity is inversely related to the amount of sample plasma T, and T concentration was determined by extrapolation from the standard calibration curve.

One hundred and four fathers were mated with random females, and their T levels and those of 243 sons were measured. One hundred and four midson T values for full-sib brothers were compared to those of their fathers for heritability analyses. One hundred sons (out of 243) with the most extreme T measures were allocated to one of two T groups: low or high. Voles in each T group were then randomly assigned to one of two implant groups: empty or T filled. Prior to implantation, there was a significant difference in T levels between the high- and low-T groups of male bank voles but not between the two implant groups (tables 1, 2).

In order to monitor male T over the breeding season, blood samples were taken from all voles caught during live trappings of all 25 enclosures after 2 weeks ($n = 80$) and after 4 months ($n = 71$). Voles were released back into the enclosures at the point of capture.

Implant Treatment

Surgery consisted of anesthetizing males with isoflurane and inserting a 10-mm-long Silastic implant subcutane-

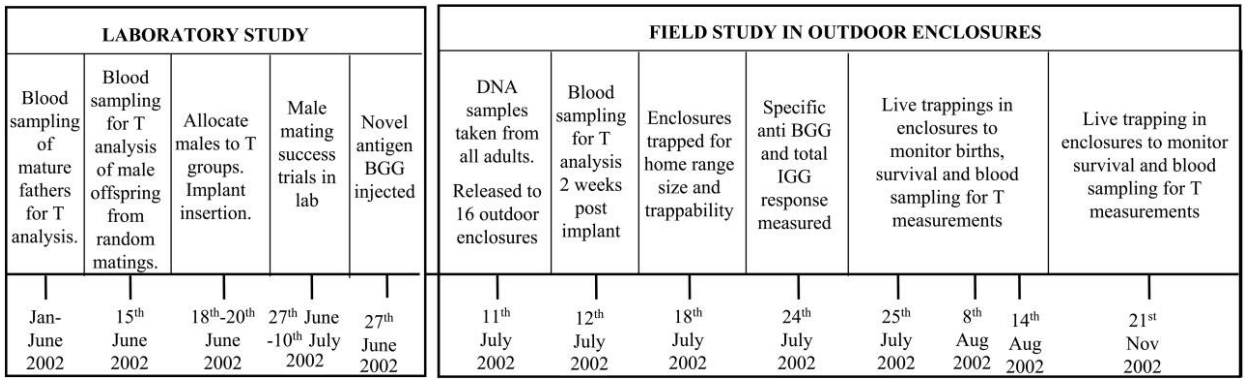

Figure 1: Time schedule of experimental study. T = testosterone; BGG = bovine gamma globulin; IGG = immunoglobulin G.

Table 1: Differences in the mean (± SE) testosterone (T) plasma level (ng mL^{-1}) of male bank voles before, 2 weeks after, and 4 months after the insertion of empty or T-filled implants

	Preimplant	*N*	2 weeks postimplant	*N*	4 months postimplant	*N*
Low-T group:						
Empty implant	2.23 ± .27	25	6.61 ± .95	20	4.51 ± 1.15	18
T-filled implant	2.30 ± .24	25	13.34 ± 1.11	20	10.64 ± 1.38	20
High-T group:						
Empty implant	9.08 ± .49	25	11.22 ± 1.43	20	5.79 ± 1.30	16
T-filled implant	8.67 ± .59	25	15.24 ± 1.54	20	9.35 ± .81	17

ously and dorsally (Konigsberg Instruments, Pasadena, CA; inner diameter: 1.47 mm; outer diameter: 1.96 mm). The implant was either empty or packed with 6.6 mg (±0.03; mean ± SE) of crystalline T (Sigma Aldrich, Helsinki; no. 86500) sealed at both ends with silicone sealant (RTV sealant, Dow Corning, Midland, MI). The sealant occupied 1 mm at each end of the tubing, so the effective length of the implant was 8 mm. Males recovered in isolation for over 1 week.

Male Dominance in the Laboratory

Male dominance mating trials were conducted at least 1 week postimplantation (fig. 1). Male dominance was defined by a set of mating trials in which two males and one female in estrus were released into an arena (1 × 1 m), and observations were made until ejaculation occurred (Oksanen et al. 1999; Mills et al. 2007*b*). Males indicate either aggressive or defensive behaviors within 5 min, and, normally, the aggressive male courted the females with successful copulation following female lordosis. The male that successfully mated was considered dominant. Eighty males were assessed three times with the other three males in their enclosure group (one at a time; six trials in total per enclosure group) and random females. Males were ranked within each enclosure group, based on their copulation success per trial. The maximum number of successful copulations per male equaled three, and males achieving this score were given the highest dominance rank of 1, whereas males achieving zero copulations were given the lowest score of 4.

Nonparametric statistics were required to analyze the ranked data based on male dominance. Therefore, the Friedman's test for randomized blocks was used to test for differences in dominance among the treatment groups (Sokal and Rohlf 1997), and the Page test for ordered alternatives (Siegel and Castellan 1988) was used to test for a trend of increasing dominance with increasing T level (represented by the four treatment groups). Due to time constraints we were unable to carry out trials for all 25 groups. We carried out a total of 120 mating success trials, six for each of 20 enclosure groups. As the sample size was considered large at 20, the z_L statistic was calculated (Siegel and Castellan 1988), and the significance of z_L, and hence the Page test statistic L, was determined from the standard normal distribution table.

Spacing Behavior and Mobility

Mate-searching activity and average distance moved were used as variables representing spacing behavior and mobility of bank voles, respectively (Jonsson et al. 2000). Mate-searching activity was determined during a trapping period when all 20 traps in an enclosure were checked at least a total of 10 times (range 10–15 times). All traps were checked three times a day for up to 5 days. Due to time constraints we were able to determine spacing behavior for males in only 14 of the 25 enclosures. The trap location was recorded for each vole captured, and all voles were immediately released at the point of capture. Mate-searching activity sizes were calculated using the 90% mononuclear probability polygon centered on arithmetic mean (Kenward 1987; Koskela et al. 1999). The average distance moved was calculated from the mean of the straight-line distances moved by each vole in consecutive trappings.

Immune Response

Two immune responses were measured: anti-BGG (bovine gamma globulin) antibody production (reflecting the resources put into the production of specific antibodies in response to the novel antigen injected, BGG) and total IgG level (a vole's first line of defense against pathogens that aims to neutralize them before a specific immune response is triggered; Greives et al. 2006). All 100 males were injected with 0.1 mL of BGG (Sigma Chemical, St. Louis; 200 μg) emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit), prior to release into the enclosures, and 4 weeks later, blood samples were collected (fig. 1). The 4-week period for mounting the response was determined from a previous laboratory experiment in which the response of adult bank vole males to BGG was analyzed 2, 4, and 6 weeks after immunization (E. Koskela, I. Jokinen, T. Mappes, and T. A. Oksanen, unpublished data).

Table 2: Generalized linear mixed model analysis of testosterone (T) values prior to implantation, 2 weeks postimplantation, and 4 months postimplantation

	T before implant			T 2 weeks postimplant			T 4 months postimplant		
Source	df (n, d)	*F*	*P*	df (n, d)	*F*	*P*	df (n, d)	*F*	*P*
Implant treatment	1, 112	.121	.729	1, 58	**20.817**	**<.001**	**1, 38**	**21.608**	**<.001**
T group	**1, 112**	**285.000**	**<.001**	1, 58	**7.666**	**.008**	1, 38	.023	.881
Implant × T group	1, 112	.480	.490	1, 58	1.326	.254	1, 38	1.697	.201

Note: Two parameters and their interaction were estimated using restricted maximum-likelihood procedures: implant treatment (empty or T-filled implants) and T group (high or low). Study enclosure was included in the model as a random factor for each T-sampling period (estimate ± residual: 0.81 ± 0.52, 5.41 ± 4.35, and 9.97 ± 6.15, respectively). df, degrees of freedom for numerator (n) and denominator (d); *F*, test statistic; *P*, probability; significant values are bold.

Plasma levels of anti-BGG-specific and total IgG antibodies were determined by microplate enzyme-linked immunosorbent assay (described in detail in Oksanen et al. 2003). Plasma samples were added to plates coated with either BGG to quantify anti-BGG-specific antibody or antimouse IgG to quantify the total IgG level. Bound bank vole immunoglobulin was detected with antimouse IgG alkaline phosphatase conjugate (A-21798; Sigma Chemical). P-nitrophenyl phosphate (1 mg mL^{-1}; Sigma Chemical) was used as the substrate, and after the enzyme reaction, the optical density was read at 405 nm. Sample concentrations were calibrated against a pool of plasmas expressed in 1,000 artificial units per milliliter (U mL^{-1}). Immune responses were log transformed for all statistical analyses.

Reproductive Success in the Field

DNA samples were taken from all male and female voles prior to release in enclosures. In order to monitor births, enclosures were live trapped every week until the end of September, coinciding with the end of the breeding season. However, due to time constraints, reproductive success was monitored in only 13 of the 25 enclosures. Gravid females were brought to the laboratory to give birth. The pups were individually marked, and tissue samples were stored at −70°C for paternity analyses. Immediately after birth, all voles were released back into the enclosures at their point of capture. Individuals were genotyped at six microsatellite loci (Gockel et al. 1997; Rikalainen et al. 2008), and likelihood-based analysis of paternity was conducted with the software Cervus, version 2, using the "One parent known" option to assign paternity (Mills et al. 2007*a*). All males in the same enclosure (4) were included as candidate fathers. The following simulation parameters were used: 10,000 cycles, 100% of candidate parents sampled, 100% of loci typed, and a genotyping error rate of 1%. We accepted paternity assignment for the candidate with the highest log of likelihood ratios score at a confidence level of 95% and with no mismatches (244 of 245 assignments; 99.6%). Only one pup remained unassigned (0.4%).

Survival in the Field

In order to measure survival, all 25 enclosures were live trapped after 1, 3, 4, 8, and 16 weeks. Immediately after we identified individuals, all voles were released back into the enclosures at their point of capture.

Statistical Methods

Generalized linear mixed models were used to analyze the data. This allows the use of dependent variables as nested data points by controlling for study enclosure as a random blocking factor in the model (Paterson and Lello 2003). Heritabilities were calculated from the slope of the linear father-midson regression. Father-midson covariances estimate only half of the additive genetic variance; therefore, they were doubled to obtain heritability estimates (Falconer and Mackay 1996).

Results

Heritability of Testosterone (T)

We found significant heritability between paternal and midson plasma T levels (measured from 243 sons representing 104 litters; table 3; fig. 2). After males had been assigned to high- and low-T groups, the heritability of T remained statistically significant for each T group separately (table 3).

T Levels

After 2 weeks the T levels of voles inserted with T-filled implants were significantly higher than those of voles inserted with empty implants (tables 1, 2). Furthermore, voles from the high-T group still had significantly higher T levels than those from the low-T group, and the interaction term was not significant (table 2). These results represent all voles that were captured 2 weeks after release into the enclosures.

Of the voles captured after 4 months, those that received T-filled implants still had significantly higher T levels than

Table 3: Mean ($\overline{X}$) of father testosterone (T), estimate of heritability (h^2), and variance components for bank vole T based on father-midson values for all males and for the low- and high-T groups separately

	$\overline{X} \pm$ SE	$h^2 \pm$ SE (95% CI)	V_A	V_P	V_E	CV_A	CV_P	CV_R
All fathers	4.98 ± .35	.58* ± .16 (.26 –.90)	7.15	12.33	5.18	53.69	157.3	101.9
Low T	4.86 ± .81	.46* ± .10 (.26 –.64)	6.56	14.27	7.70	52.85	171.6	126.1
High T	5.56 ± .66	1.06* ± .28 (.48 –1.64)	16.04	16.71	.67	72.00	173.3	34.6

Note: V_A, additive genetic variance (twice the father-midson covariance); V_P, phenotypic variance; V_E, environmental variance; CV_A, coefficient of additive genetic variation ($CV_A = 100\overline{X}^{-1}(V_A)^{1/2}$); CV_P, coefficient of phenotypic variation; CV_R, coefficient of residual variation ($CV_R = 100\overline{X}^{-1}(V_P - V_A)^{1/2}$; Houle 1992); CI, confidence interval. Number of father-midson pairs = 104 for all males and 31 and 23 for low- and high-T groups, respectively.

* $P < .001$.

those voles receiving empty implants, but there was no longer a difference in T level between the original T groups nor a significant interaction term (tables 1, 2).

Male Spacing Behavior and Mobility

Males implanted with T had significantly higher mate-searching activity than those inserted with empty implants (table 4; fig. 3*A*). The T implants increased the average distance moved by low-T-group males but not by high-T-group males (table 4; fig. 3*B*).

Male Dominance in the Laboratory

Of the 120 trials, 80 involved competition between males from each of the divergent T groups, and high-T males successfully mated with the female in 54 of these trials. Furthermore, males from the high-T + T-filled-implant treatment were most successful in mating, achieving copulations in 45 of the 60 trials in which they were involved, whereas males from the low-T + empty-implant treatment were least successful, mating only 15 times in their 60 trials. Friedman's tests confirm that there were significant differences in dominance among the four male treatment groups (rank order based on the number of matings: $\chi_r^2 = 9.65$, df = 19, $P = .02$; fig. 3*C*), and Page's test revealed a significant trend of increasing dominance with increasing T level represented by the four male treatment groups shown in figure 3*C* ($z_L = 3.06$, $P = .001$).

Male Reproductive Success in the Field

In the 13 enclosures monitored, male bank voles implanted with T sired a significantly greater number of pups in outdoor enclosures than those inserted with empty implants (table 4; fig. 3*D*). There was no significant effect of T group on male reproductive success (table 4).

Immune Function

We found a significant interaction between implant treatment and T group on anti-BGG antibody production (table 5; fig. 3*E*). The T-filled implants reduced antibody production of high-T-group males but had no significant effect on antibody production of low-T-group males. There was no effect of either T group or implant treatment on IgG levels (table 5; fig. 3*F*).

Survival

A marginally significant effect of T group was found on survival, whereby high-T-group males had reduced survival (table 4; fig. 3*G*). Furthermore, a nonsignificant trend for an interaction between T group and implant treatment was found on survival. Exogenous T did not affect males from the low-T group, whereas T reduced survival in males from the high-T group.

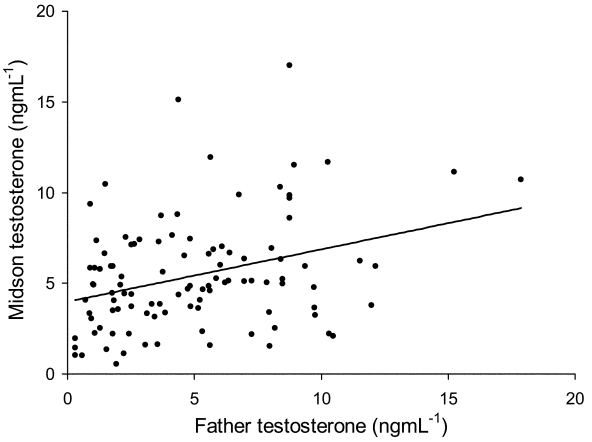

Figure 2: Relationship between father-midson testosterone levels for all original 104 litters (simple linear regression: $y = 3.982 + 0.289x$; $R^2 = 0.11$; $F = 12.76$, df = 1, 104, $P = .001$).

Table 4: Generalized linear mixed model analysis of mate-searching activity (ha), mobility (average distance moved), reproductive success, and survival over 12 weeks

	Mate-searching activity			Mobility			Reproductive success			Survival		
Source	df (n, d)	*F*	*P*	df (n, d)	*F*	*P*	df (n, d)	*F*	*P*	df (n, d)	*F*	*P*
Implant treatment	**1, 28**	**5.207**	**.030**	1, 23	.276	.604	**1, 47**	**7.416**	**.009**	1, 77	.269	.606
T group	1, 24	.007	.935	1, 22	.016	.902	1, 47	1.750	.192	1, 77	3.744	.057
Implant × T group	1, 26	.131	.721	1, 24	4.017	.056	1, 47	.146	.704	1, 77	3.204	.077

Note: Two parameters and their interaction were estimated using restricted maximum-likelihood procedures: implant treatment (empty or T-filled implants) and T group (high or low). Trappability (the proportion of times a bank vole was caught in a trap) was included as a covariate in the model for (A) mate-searching activity ($F = 45.76$, df $= 1, 15$, $P < .001$). Study enclosure was included in all models as a random factor for each male trait (estimate ± residual: $-1.65 \times 10^{-6} \pm 1.16 \times 10^{-4}$, -0.11 ± 12.1, $9.03 \times 10^{-2} \pm 0.0$, and 0.627 ± 1.67, respectively). df, degrees of freedom for numerator (n) and denominator (d); *F*, test statistic; *P*, probability; T, testosterone; significant values are bold.

Relationship between Immune Response and Survival

A significant positive relationship was found between specific anti-BGG antibody response and survival for bank voles that received empty implants (fig. 4). However, there was no significant relationship between anti-BGG antibody response and survival for bank voles treated with T-filled implants (linear regression: $y = 9.69 - 0.80x$; $R^2 = 0.02$; $F = 0.408$, df $= 1, 24$, $P = .529$).

Discussion

We assessed the activational role of T on physiological and behavioral fitness-related traits in the male bank vole. We found that significant heritability of circulating levels of T and exogenous T, administered via implants, had different impacts on males with naturally high and low T levels. The T implants enhanced dominance in the lab, spacing behavior, mobility, and reproductive success in the field. However, these benefits of elevated T came at a cost to antibody production and survival.

Behavior and Fitness

In microtine rodents, male reproductive success is largely determined by active searching and direct competition for sexually receptive females (Madison 1980; Kawata 1985; Ostfeld 1985). Our results show that the following behaviors increased with elevated T: mate-searching activity in the field (fig. 3*A*) and social status (laboratory dominance; fig. 3*C*). The relative number of pups sired by male bank voles has been found to covary with natural plasma T level in the field (Mills et al. 2007*a*). This study revealed that exogenous treatment with T not only increased plasma T levels but also had concomitant effects on field reproductive success (fig. 3*D*). There is a general consensus that plasma T has positive effects on male sexual behavior (e.g., Marler et al. 1995; Salvador et al. 1996; Enstrom et al. 1997; Briganti et al. 2003), while the evidence is more ambiguous for sexual ornaments. This article further demonstrates that T not only modulates fitness-related behaviors that are important for both intra- and intersexual selection in the bank vole but also ultimately regulates reproductive success.

Behavioral Strategies

We found differences in male dominance and mobility between bank vole groups with naturally high and low T levels. Furthermore, the impact of experimentally elevating T also had different effects on immune response and survival between the two groups. Combined, these results suggest the presence of different individual strategies that are likely to have evolved via balancing selection due to correlated responses of T on multiple fitness-related traits (Gil and Faure 2007; Sellers et al. 2007). The origin of these different specific phenotypes may be both genetic and environmental during early development, a hypothesis already proposed to explain the ecological crossover of male bank vole dominance across different environments (Mills et al. 2007*b*).

Heritability of T

The evolution of exaggerated male traits is an integral part of sexual selection theory, and heritable genetic variation is an essential requirement for a trait to respond to selection. Yet, despite the knowledge that T influences the development of many sexually selected traits, few studies have measured heritable genetic variation of T. Individual variation of T is considered to reflect dichotomous patterns: either a fixed individual strategy with heritable genetic variation shaped by intrinsic factors, such as additive or nonadditive genetic effects, maternal effects, or early developmental environment, or a flexible strategy responding to extrinsic factors, such as social context, recent history, and environmental changes (Kempenaers et al. 2008). In this study we found that male bank vole circulating levels of T are heritable, which has recently been confirmed by a separate quantitative genetic study on bank

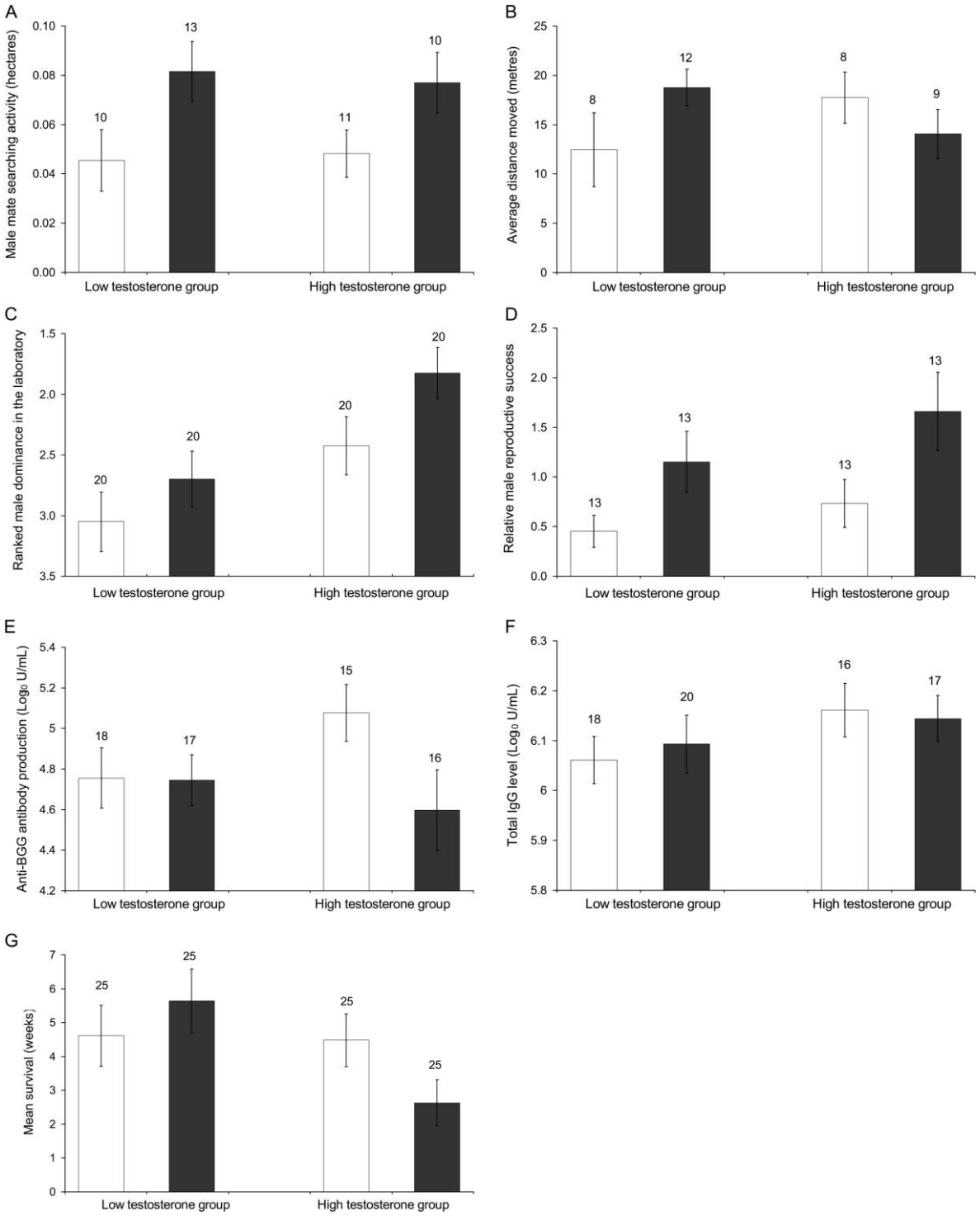

Figure 3: Mean (± 1 SE) *A*, mate-searching activity (ha) calculated as 90% mononuclear probability polygons; *B*; mobility calculated as the average distance moved between consecutive trappings (m); *C*, ranked data on mating success based on the number of matings by each male within each enclosure group (1: highest mating success, 4: lowest mating success); *D*, reproductive success made relative by dividing by the enclosure mean; *E*, anti-BGG antibody production; *F*, total immunoglobulin G level; and *G*, survival over 12 weeks in outdoor enclosures of male bank voles from the groups divergent for testosterone that had received either empty (*open bars*) or testosterone-filled (*filled bars*) implants. Sample sizes are given above the bars.

Table 5: Generalized linear mixed model analysis of anti-BGG antibody production (U mL^{-1}) and total IgG level (U mL^{-1})

	Anti-BGG antibody			Total IgG level		
Source	df (n, d)	*F*	*P*	df (n, d)	*F*	*P*
Implant treatment	1, 50	.930	.340	1, 28	.141	.711
Plasma T level	1, 50	.026	.873	1, 28	.332	.569
Implant × plasma T level	**1, 50**	**5.242**	**.026**	1, 28	.149	.703

Note: Two parameters and their interaction were estimated using restricted maximum-likelihood procedures: implant treatment (empty or T-filled implants) and initial plasma T level (ng L^{-1}; continuous). Study enclosure was included in the model as a random factor for each male immune measure (estimate ± residual: $1.12 \times 10^{-2} \pm 2.92 \times 10^{-2}$ and $9.67 \times 10^{-4} \pm 7.78 \times 10^{-3}$, respectively). df, degrees of freedom for numerator (n) and denominator (d); *F*, test statistic; *P*, probability; T, testosterone; BGG, bovine gamma globulin; IgG, immunoglobulin G; significant values are bold.

vole T levels (E. Schroderus et al., unpublished manuscript). These results support sexual selection theory, hypothesizing that direct selection on morphological or behavioral traits will result in evolutionary changes via indirect selection on heritable circulating levels of T (Adkins-Regan 2008).

Immune Response

The immune system is composed of a large number of interrelated components that have different developmental and use costs (Lee 2006). Of the two immune measures used in this study, adaptive defense (anti-BGG production) has high developmental and low use costs, whereas innate defense (total IgG level) has low developmental but high use costs. Induced adaptive responses require substantial time and resources during early development and are thus reduced in faster-reproducing species in favor of innate defenses (Lee 2006). In terms of innate defense, constitutive immune defense, such as natural antibodies, is favored in fast-living species in order to minimize costly inflammatory responses. Bank voles are fast-living species with a short development time, high reproduction, and low survival, and we found that elevated T decreased the production of anti-BGG antibodies for high-T males but not total IgG level for either high- or low-T males.

One explanation for the differential effect of T on different immune components may be due to the high developmental cost of anti-BGG antibodies compared to those for IgG, such that a reduction in their production during periods of high reproductive effort would be an adaptive response to relieve some of the demands on an individual's resources, as suggested by the immunocompetence handicap hypothesis (Folstad and Karter 1992; Wedekind and Folstad 1994). A recent quantitative genetic study has revealed tight linkage between T and immune response (E. Schroderus et al., unpublished manuscript), and consequences of reduced immune response include increased infection with parasites (S. C. Mills et al., unpublished manuscript). We did not measure parasite infection in this study, but exogenous T decreased survival in males from the high-T group, and immune response covaried positively with survival. All these results combined suggest the presence of a trade-off between T and both disease resistance and survival. Despite the presence of trade-offs associated with male sexual signals, if a female receives higher total fitness returns from mating with a male with an exaggerated signal compared to a male with a less exaggerated trait, sexual selection is still expected to explain the evolution of exaggerated male signals (Kokko 2001). A trade-off between T and disease resistance implies that male sexual signals will be constrained by a survival handicap, ensuring the honesty of the signal to females in terms of male viability quality (Zahavi 1975; Grafen 1990). The continually faster evolution of disease organisms compared to that of their hosts ensures that female selection

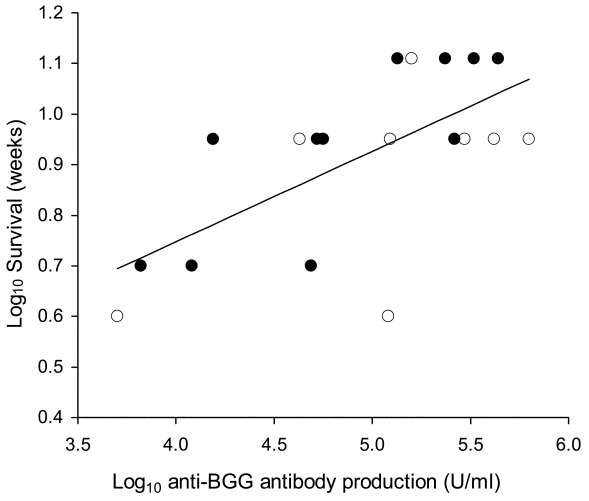

Figure 4: Relationship between anti-BGG antibody production and bank vole survival (only those inserted with empty implants) over 12 weeks in outdoor enclosures (simple linear regression: $y = -6.683 + 2.87x$; $R^2 = 0.22$; $F = 5.76$, df = 1, 22, $P = .026$) for males that were from the low-testosterone group (*open circles*) or high-testosterone (*filled circles*) group.

is never in the same direction long enough to drive any disease-resistant genes to fixation, thus maintaining variation in male traits and the process of sexual selection. One explanation for our results may be that T-mediated trade-offs maintain the honesty of male signals.

Alternatively, males with naturally high levels of T may represent a high-quality male behavioral strategy and thus have sufficient resources to invest in reproduction and immunity. Indeed, if we focused on natural levels of empty-implant males, we found higher anti-BGG levels in the high-T group, and the immunosuppression and survival costs of elevated T were observed only in males with naturally high levels of T. These differences may further represent the individual strategies differentiating the two groups. Males from the high-T group may favor distinct types of immune response from those favored by low-T males. Such different responses to elevated T may be modulated by differences in either the availability, density, or affinity of appropriate receptors on or in cells responding to T or the levels of plasma-binding protein levels in the blood that alter hormone availability, as it is the unbound hormone that is biologically active (Ball and Balthazart 2008).

Neither group showed a change in total IgG response to elevated T. Differential sensitivity to steroid by various components of the immune system has already been found in Siberian hamsters and mice (Bilbo and Nelson 2001; Brown et al. 2007).

Immune Response and Survival

Immune response shows a positive relationship with survival (fig. 4), and we found that male mortality was highest in high-T males with artificially elevated T (fig. 3*G*), suggesting that the immunosuppression cost of high T has important implications for male bank vole fitness. Although a recent study linked immune response with parasite infection and revealed higher mortality of voles with low immune response (S. C. Mills et al., unpublished manuscript), disease was not measured in this study, and therefore we have no evidence that immune response was the proximate mediator of death in bank voles. Instead, those voles that produced a higher immune response may have been of higher genetic quality or in better condition, and a third variable may have caused the correlation between survival and antibody response.

Hypothalamus-Pituitary-Gonadal Axis and Plasticity

Endocrine steroid release is controlled by the hypothalamus-pituitary-gonadal axis, wherein the hypothalamus secretes gonadotropin-releasing hormone, which in turn regulates gonadotropin secretion by the anterior pituitary (Squires 2003). The gonadotropins' luteinizing hormone (LH) and follicle-stimulating hormone (FSH) regulate gonadal function and stimulate T production; T is known to exert both direct (mediated by androgen receptors) and indirect (mediated by its aromatization to estradiol) negative gonadotropin feedback on the hypothalamus and anterior pituitary in human males (Finkelstein et al. 1991; Bagatell et al. 1994; Hayes et al. 2001; Rochira et al. 2006). The exogenous T received by bank voles in this study was therefore likely to have engaged this negative feedback system and downregulated gonadotropin release. While our study demonstrates the activational effects of exogenous T on fitness-related traits, we do not know how the concomitant downregulation of endogenous gonadotropins affected these traits, how their effects compare to males with naturally elevated T, or whether they would have acted synergistically or additively with T. Nevertheless, exogenous T appears to regulate physiological and behavioral traits in a similar manner to endogenous T, as phenotypically engineered males continued to show male-male competition, courtship, and the production of functional sperm. Although experiments on male trade-offs focus mostly on T, recent work in the side-blotched lizard *Uta stansburiana* has revealed that LH and FSH may provide a clearer understanding of the relationship among traits (Mills et al. 2008), and focusing on LH and FSH removes the problem of negative feedback faced by T.

In conclusion, our study suggests that bank voles may show different reproductive strategies as a result of the correlated responses of T on male behavior and immune responses. Male signals are required to show a handicap to honestly reflect male viability, and although this study shows immune response and survival costs to artificially elevated levels of T, these results were found for only one group of males with naturally high T levels, and hypotheses other than the immunosuppression by T may explain these findings. Nevertheless, male bank voles do appear to be optimizing their T levels according to their quality, and this study provides evidence for a genetic basis to male plasma T level (table 3; fig. 2). Therefore, females that mate with dominant males will acquire good genes in terms of siring success, emphasizing the role of T in ensuring the honesty of male signals.

Acknowledgments

We wish to thank P. Keskinen, T. Solismaa, and E. Virtanen for their invaluable laboratory help; R. Närä and T. Savolainen for vole husbandry and fieldwork; Konnevesi Research Station for providing fieldwork facilities; B. Trainor for his advice on testosterone implants; L. Gibson for veterinary advice; and the associate editor and referees for their valuable comments. The study was financially sup-

ported by the Academy of Finland (grants 103508 and 108566 to S.C.M.; 118603, 109165, and 204284 to T.M.; 115961 and 119200 to E.K.; and 104568 and 108955 to T.A.O.) and the Centre of Excellence in Evolutionary Research of the Academy of Finland.

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Associate Editor: Ellen D. Ketterson
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