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FITNESS TRADE-OFFS MEDIATED BY IMMUNOSUPPRESSION COSTS IN A SMALL MAMMAL

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Trade-offs are widespread between life-history traits, such as reproduction and survival. However, their underlying physiological and behavioral mechanisms are less clear. One proposed physiological factor involves the trade-off between investment in male reproductive effort and immunity. Based on this hypothesis, we investigated differences in fitness between artificially selected immune response bank vole groups, *Myodes glareolus*. Significant heritability of immune response was found and a correlated response in testosterone levels to selection on immune function. Male reproductive effort, reproductive success, and survival of first generation offspring were assessed and we demonstrate a relationship between laboratory measured immune parameters and fitness parameters in field enclosures. We identify a trade-off between reproductive effort and survival with immune response and parasites as mediators. However, this trade-off results in equal male fitness in natural conditions, potentially demonstrating different male signaling strategies for either reproductive effort or survival. Females gain indirect genetic benefits for either genetic disease resistance or male reproductive effort, but not both. Immune response is genetically variable, genetically linked to testosterone and may indirectly maintain genetic variation for sexually selected traits. Evidence for both a genetic and a field trade-off between reproductive effort and survival indicates an evolutionary constraint on fitness traits.

KEY WORDS: **Dominance, evolutionary constraint, parasites, reproductive costs, sexual selection, survival.**

Evolutionary theory predicts that fitness-related traits will be constrained by the existence of trade-offs between them (Stearns 1992). Experimental confirmation for the presence of fitness trade-offs has been found from the observation of natural populations, phenotypic engineering (the use of hormones to investigate the mechanistic bases of phenotypic variation) and more recently

using artificial selection (Sheldon and Verhulst 1996; Sinervo and Svensson 1998; Conner 2003; Frankino et al. 2005; Fuller et al. 2005; Mills et al. 2008). One fitness trade-off involves reproductive effort and survival, which is fundamental to life-history theory (Williams 1966; Trivers 1974). The evolution of reproductive effort is considered to be constrained when an increase in reproductive effort incurs a decrease in survival. Whilst evidence for trade-offs between fitness related traits, including that between reproductive effort and survival, is abundant, the proximate mechanisms maintaining them are less clear and evidence for a genetic link between reproductive effort and survival is lacking.

Trade-offs between fitness traits are commonly assumed to result from resource allocation strategies (Roff and Fairbairn 2007) mediated by selection on physiological mechanisms (Finch and Rose 1995; Hau 2007). Hormonal regulation via the endocrine system provides one mechanism by which organisms mediate the allocation of limited time or resources to competing functions in an optimal manner so as to maximize their fitness on a temporal scale (Ketterson and Nolan 1992). Reproductive effort has been proposed to handicap survival via its trade-off with genetic resistance to current parasites (Zahavi 1975; Hamilton and Zuk 1982). Consequently, both the endocrine and immune systems are considered to play important roles in mediating fitness trade-offs (Stearns 1989; Sinervo and Svensson 1998; Ketterson and Nolan 1999; Zera and Harshman 2001; Oksanen et al. 2002; Ricklefs and Wikelski 2002; McGlothlin et al. 2007).

Disease resistance is an important component of life history and trade-offs between reproduction and life histories correlated with the immune system are receiving increasing attention in evolutionary research (Sheldon and Verhulst 1996; Zuk and Stoehr 2002; Viney et al. 2005). Reduced immunity increases parasite infections (Nordling et al. 1998), selection for higher immunity decreases infections (Gross et al. 1980), infection recovery increases with increasing immunity (Gonzalez et al. 1999) and increased immunity increases both survival and offspring recruitment (Saino et al. 1997; Sorci et al. 1997; Gonzalez et al. 1999). However, activation of the immune system is expensive, including the processing of antigens and phagocytosis, and even immunological memory in the absence of infection has considerable energetic costs (Martin et al. 2007). The allocation of energy to the immune system is considered to be affected by variation in reproductive effort (Folstad and Karter 1992; Gustafsson et al. 1994; Wedekind and Folstad 1994), resulting in the trade-off between reproduction and survival. Recently, reproductive effort has been experimentally shown to impose costs on the immune system and decrease survival (Reed et al. 2006; Mills et al. 2009). In this article, we examine the role of disease resistance on male reproduction and survival, and we aim to demonstrate their role in mediating fitness trade-offs.

The bank vole, *Myodes* (= *Clethrionomys*) *glareolus*, is a common small mammalian species in Finland (Kallio et al. 2009) and across Europe, whose survival is compromised by diseases from ecto- and endoparasites and pathogens (Soveri et al. 2000; Kallio et al. 2007), such as intraerythrocytic infection caused by the protozoa *Babesia* transmitted through an *Ixodes* tick bite (Karbowiak and Sinski 1996) and Lyme disease carried by *Borrelia burgdorferi*-infected *Ixodes* nymphs (Tälleklint and Jaenson 1996). Up to nine helminth species that affect host mortality have been found in bank voles from Finland (Haukisalmi and Henttonen 2000) as well as coccidiosis, an intestinal infection transmitted by the endoparasite, *Eimeria cernae* (Soveri et al. 2000; Hakkarainen et al. 2007). Genetic disease resistance and immune response therefore have important consequences for survival and thus fitness in this species. Reproductive effort in male bank voles is not expressed by elaborate ornaments, but by social dominance (Hoffmeyer 1982; Oksanen et al. 1999) that is increased by exogenous testosterone (T) (Mills et al. 2009) and honesty is enforced through social costs during fights. Female bank voles also show preferences for dominant males (Horne and Ylönen 1996; Kruczek 1997) and male sexual attractants are likely to be T-dependent (Radwan et al. 2006). Directional selection acts on T (Mills et al. 2007b) and T levels are heritable (Mills et al. 2009). Experimentally elevated T increases mate searching, mobility, and male bank vole reproductive success, but has costs in terms of decreased immune function and survival (Mills et al. 2009). The bank vole is therefore a good model species in which to demonstrate the reproductive effort-survival trade-off and to test the hypothesized mechanism for its maintenance.

We employ two-way artificial selection on the bank vole to generate divergent groups for immune response (high and low) based on several immune tests challenging different components of the immune system (Norris and Evans 2000). Plasma T was measured and male reproductive effort (male–male competition for a female in estrus representing a male's effort afforded to territoriality, male–male competition, and courtship) was determined in laboratory trials. Males and females from both immune groups were then released to 15 seminatural conditions in outdoor enclosures and were monitored for number of genetic mates, reproductive success and for survival. We report on the heritability of immune response and the correlated response of T levels to selection on immune response. We also report on the relationship between immune response and a set of potentially fitness-related phenotypic traits, fitness itself, as well as the reproductive effort–survival trade-off. The proximate mechanisms, namely parasites and immune response, mediating this trade-off were also investigated. Disease-reduced environments, representing conditions under which immunosuppression by T would not

pose a survival handicap, were created. Male reproductive traits were compared between disease-reduced environments (host populations were treated with antiparasite medication to remove field ecto- and endoparasites) and disease-present environments (normal conditions).

Materials and Methods

STUDY SITE AND EXPERIMENTAL DESIGN

A total of 400 males and females were wild captured from Konevesi, central Finland, (62°37′N, 26°20′E) in December 2002. The animals were maintained in laboratory conditions described in Mills et al. (2007a). Humoral adaptive immunity (antibovine gamma globulin [BGG] specific antibody response and total immunoglobulin G [IgG] level) were measured from all adults (see subsequently for methods) and plasma testosterone (T) was measured from adult males (see subsequently for methods). A total of 120 parents with the most extreme measures were paired to produce first-generation offspring. Humoral adaptive immunity and cell-mediated adaptive immunity (delayed-type hypersensitivity [DTH] test) were measured from all first-generation offspring (see subsequently for methods) and plasma T measured from all first-generation males. All litters were used in the calculations of heritability. A total of 120 male and 120 female first-generation offspring with the most extreme immune responses were selected for the experiment. DNA samples were taken and male mating success was measured in the laboratory (see subsequently for methods). Antiparasite medication or solvent control treatments were applied (see subsequently for methods) and all experimental animals were released to 15 outdoor enclosures for 9 months. Experimental animals were monitored for births, parasite abundance, survival, and parasite treatment was reapplied. At the end of winter in April 2004, the survival of remaining individuals was recorded. Paternity analyses measured male genetic mating success and male reproductive successes. Experimental schedule is shown in Figure 1.

HUMORAL ADAPTIVE IMMUNITY

Two immune responses were measured: BGG antibody production (reflecting the resources put into the production of specific antibodies in response to the novel antigen injected, BGG), and total immunoglobulin G level (IgG, 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). Methods are described in detail in (Oksanen et al. 2003; Mills et al. 2009). Individuals were injected with 0.1 mL of BGG (Sigma Chemical Co., St. Louis, MO 200 μg) and 4 weeks later, blood samples were collected (Fig. 1). Plasma levels of anti-BGG specific and total IgG antibodies were determined by micro plate enzyme-linked immunosorbent assay (ELISA). 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 Co.). P-nitrophenyl phosphate (1 mg mL^{-1}, Sigma Chemical Co.) 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 1000 artificial units per milliliter (U mL^{-1}). Immune responses were log transformed for all statistical analyses.

TESTOSTERONE ANALYSIS

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 & Co., Dietzenbach, Germany) and the methods, test for parallelism, and repeatability are fully described elsewhere (Mills et al. 2007b). Briefly, 50 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 interpolation from the standard calibration curve.

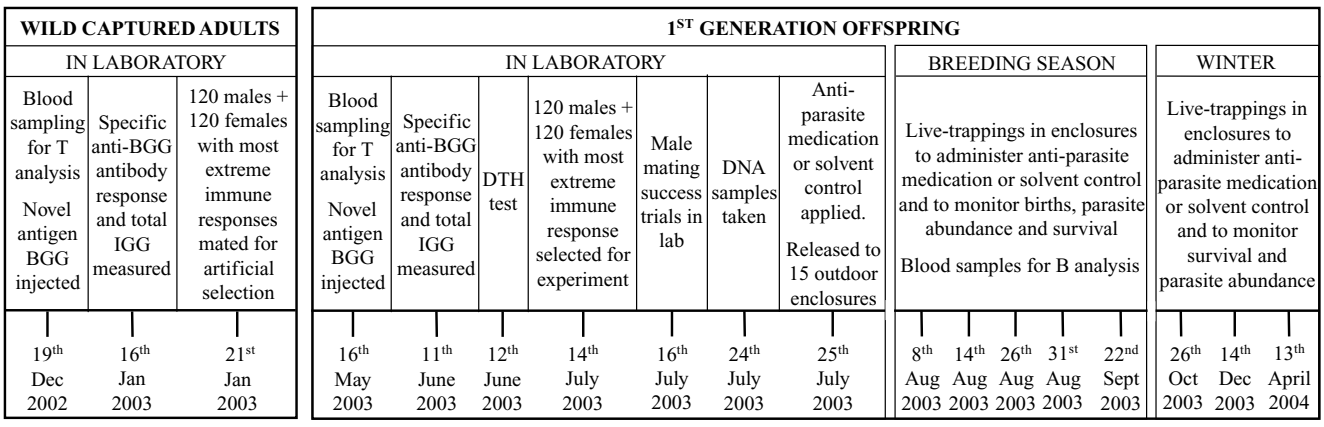

Figure 1. Time schedule of experiments. T, testosterone; BGG, bovine gamma globulin; DTH, delayed-type hypersensitivity test.

Table 1. Mean (SE) values per litter of all 93 first generation male bank voles for three measures of adaptive immune function for the low and high immune groups separately, with independent *t*-test results between immune groups.

	Low immune group		High immune group			
	Mean (SE)	*N*	Mean (SE)	*N*	*t*	*P*
Humoral immunity:						
Anti-BGG antibody (U/mL)	205,116 (34,686)	44	461,186 (54,801)	49	3.95	0.001
Total IGG level (U/mL)	899,537 (53,041)	44	1,315,550 (177,271)	49	2.15	0.034
Cell-mediated immunity:						
DTH-index (%)	32 (1)	34	38 (1)	35	3.25	0.002

ARTIFICIAL SELECTION FOR DIVERGENT IMMUNE GROUPS AND HERITABILITY

Following humoral adaptive immunity measurements, 120 parents with the most extreme measures were grouped to form two parental immune groups, high and low, and males and females within each parental group were paired. A total of 93 litters were produced totaling 460 first-generation offspring. Humoral adaptive immunity, cell-mediated immunity (see subsequently for methods), and T levels (males only) of these offspring aged 4–5 months were measured in May 2003 (Fig. 1). All measures of immunity from all 93 first-generation litters showed significant differences between the two immune groups (Table 1). Cell-mediated adaptive immunity is shown for all males measured, representing 69 litters (Table 1). A strongly significant positive correlation was found between anti-BGG antibody production and IgG concentration (Pearson's correlation coefficient [two-tailed]: $r = 0.345$, $P < 0.001$), but neither humoral response showed a significant correlation with cell-mediated adaptive immunity (anti-BGG: $r = 0.163$, $P = 0.130$; IgG: $r = 0.080$, $P = 0.461$).

We estimated the mother–midson (mean of sons per litter), father–midson, and midparent–midson heritability of humoral adaptive immunity for each immune group separately for all 93 litters (anti-BGG antibody production and IgG level were log transformed).

From these 93 first-generation litters, 120 male and 120 female bank voles with the most extreme immune responses of all three measures were selected for the laboratory and field experiment: low immune group: BGG = 8000–200,000 U/mL, IgG = 80,000–950,000 U/mL, DTH index = 0.15–0.34%; high immune group: BGG = 240,000–2,200,000 U/mL, IgG = 1,000,000–4,000,000 U/mL, DTH index = 0.35–0.55%.

CELL-MEDIATED ADAPTIVE IMMUNITY

Cell-mediated adaptive immunity was assessed using a delayed-type hypersensitivity (DTH) test, a standard assay in veterinary medicine (Lochmiller et al. 1993; Luster et al. 1993). Preinjection measurements of the left and right footpads were taken in triplicate with digital calipers (to the nearest 0.01 mm). The right and left front footpads were injected, respectively, with phytohemagglutinin (200 μg PHA-P, lectin from red kidney bean, *Phaseolus vulgaris* [Sigma Chemical Co.], in 50 μl sterile phosphate buffer solution [PBS]) or as a control, 50 μl of PBS only. Each footpad was re-measured in triplicate 24 h after injection. DTH-index was calculated as the difference in swelling between the left footpad (control) and the right footpad (PHA) as a percentage of mean footpad. Repeatability was calculated for footpad measurements ($n = 126$ individuals) using analyses of variance (Lessells and Boag 1987); repeatability (F ratio): preinjection: left = 0.699 (7.42); right = 0.923 (37.46); postinjection: left = 0.588 (5.17); right = 0.729 (9.31). The concentration of PHA and the period for mounting a response was determined from a previous laboratory study in which the responses of 32 adult bank voles to four concentrations (40, 100, 200, and 400 μg PHA in 50 μl PBS) were analyzed 3, 6, 9, 12, 24, 36, and 48 h post injection (SCM, TP, and IJ, unpublished data).

MALE REPRODUCTIVE EFFORT

Male reproductive effort was determined by a set of mating trials in which males competed with another male for an estrus female. These trials represent a male's effort afforded to reproductive processes including territoriality, male–male competition and courtship. The 120 males selected for the high- and low-immune groups were randomly assigned to 15 replicate populations of eight males consisting of a common and a rare immune group (either 6 high : 2 low, or 6 low : 2 high) (Fig. 2A). Trials used the two males of the rare immune group and two randomly chosen males from the common immune group from each of the original 15 replicate populations. During these trials, two males and one female in estrus were released into an arena (1 × 1 m) and observations made until ejaculation occurred (Oksanen et al. 1999; Mills et al. 2007a; Mills et al. 2009). All males tested were unfamiliar with each other being neither a relative nor a cage partner. Males indicate either aggressive or defensive behaviors within 5 min and normally, the aggressive male courts the females with successful copulation following female lordosis. A total of 60 males were assessed three times with the three other males from his replicate group (two males per trial, six trials in total per replicate group, total of 190 trials) and random unrelated, unfamiliar females. The

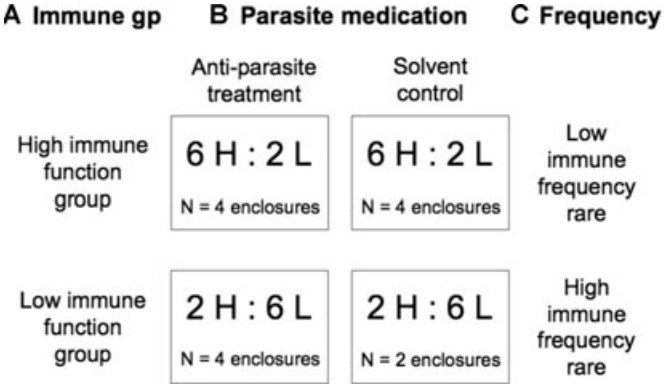

Figure 2. Experimental field design conducted in 14 × 0.2 ha outdoor enclosures containing eight male and eight female bank voles. Numbers in each box refer to the number of individuals (from each sex) from the high (H) or low (L) immune group that were released per enclosure. The experiment consisted of the following treatments: (A) immune group (H or L based on multiple immune measures), (B) parasite medication (antiparasite treatment or solvent control applied to all voles in an enclosure over five months), and (C) immune tactic frequency (ratio of the number of L and H immune group males per enclosure).

male that successfully mated was considered dominant and males were ranked within each replicate group based on their copulation success per trial (only one male copulates per trial). The maximum number of successful copulations per male after three trials equals three and males achieving this score were given the highest reproductive effort rank of 4, whereas males achieving zero copulations were given the lowest score of 1.

EXPERIMENTAL TREATMENTS AND FIELD EXPERIMENT

The 120 males and 120 females selected for the high and low immune groups were randomly assigned to 15 replicate populations at constant density: eight males and eight females. The experimental design consisted of three treatments: (1) immune group; (2) parasite medication; and (3) frequency manipulation (Fig. 2). For (1) we manipulated immune group, so that enclosures contained populations consisting mainly of either high immune group voles (6 high : 2 low) or low immune group voles (6 low : 2 high) (Fig. 2A). For (2) two parasite medication treatments were used: antiparasite medication or solvent control and all eight males and eight females in a replicate population received the same treatment (Fig. 2B). Presence and absence of endo- and ectoparasites was manipulated with the repeated application at each live trapping event over five months until mid-December of either 25 μl parasite medication, stronghold® 15 mg (selamectin) (Pfizer, Kent, England) diluted 1:10 with isopropanol (Fisher Scientific, Helsinki, Finland) or a solvent control, 25 μl of isopropanol, on the nape of the bank vole's neck. Stronghold has been shown to kill ectoparasites (this study), as well as endoparasites (McTier et al. 2000). For (3) we manipulated immune group frequency, so that enclosures contained either populations of voles in which either the low immune group was rare (2 low : 6 high) or the high immune group was rare (6 low : 2 high) (Fig. 2C).

All 15 populations were released to 0.2 ha outdoor enclosures situated in Konnevesi (described in detail by Oksanen et al. 2003) in July 2003. Unfortunately, due to adverse environmental conditions, one enclosure, containing a low immune function group (2H : 6L) in solvent control conditions, became waterlogged, thus reducing the number of replicates of this treatment from three to two (the final sample sizes are shown in Fig. 2). Enclosures were monitored between July 2003 and April 2004 by live-trappings 14–17, 20–24, 32–34, 37–40 days and 2, 3, 5, and 9 months after release to monitor births and survival. The number of fleas (Siphonaptera, Ceratophyllidae and ticks *Ixodes* spp.: Acari, Ixodidae) present was counted and removed at each live trapping event.

PATERNITY ANALYSES

DNA samples were taken from all male and female voles prior to release in enclosures. To monitor births, gravid females live-trapped in the enclosures gave birth in the laboratory. Pups were individually marked and tissue samples 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 and likelihood-based analysis of paternity was conducted with the software Cervus version 2 (Gockel et al. 1997; Mills et al. 2007b; Rikalainen et al. 2008). All males in the same enclosure (8) were included as candidate fathers. We accepted paternity assignment for the candidate with the highest LOD score at confidence level of 95% and with no mismatches (103 of 105 assignments, 98%). Only two pups remained unassigned (2%).

Paternity analyses provided two measures of male fitness: relative male mating success (number of genetic mates) and relative reproductive success (number of offspring). Both values were made relative by dividing by the population mean and reproductive success was log $(x + 1)$ transformed to attain normality. As these two measures represent the outcome of reproductive effort and survival, they can be more accurately described as fitness (mating success/reproductive success and survival).

STATISTICAL ANALYSES

Nonparametric statistics were required to analyze the ranked data based on male reproductive effort. Therefore, the Friedman's test for randomized blocks was used to test for differences in reproductive effort 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 decreasing reproductive effort with increasing immune function and decreasing

testosterone level. We carried out a total of 190 mating success trials; six for each of 15 enclosure groups. As the sample size was considered large at 15 and $k = 4$, 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.

We used the Gail and Simon (1985) method to identify trade-offs or crossover interactions between fitness-related traits across immune groups. This method focuses on crossover between male reproductive effort and survival, which are treated as fixed effects. In our experiment, the mean trait values of fitness related traits are considered across the two immune groups. For each immune group the difference in means and sample variances of relative fitness were calculated between different fitness-related measures and these differences are classified as positive or negative. Two test statistics Q^+ and Q^- are computed for each group from the sum of squared deviations standardized by their respective sample variances (for equations see Gail and Simon 1985). To test for the significance of crossover between reproductive effort and survival, min (Q^+, Q^-) was compared with critical values from Table 1 in (Gail and Simon 1985). A large value implies that crossover occurs more frequently that can be expected by chance, given the samples variances of the means (Jia et al. 2000; Mills et al. 2007a).

Generalized linear mixed model (PROC MIXED, SAS) (Paterson and Lello 2003), which takes the potential spatial correlation of the observations into account, was used to analyze ectoparasites, testosterone, genetic male mating success, and overall fitness. Generalized linear mixed model (PROC GLIMMIX, SAS version 9.1, Cary, NC) was used to analyze male bank vole survival with a logit link function and binomial errors. Three parameters and their interactions were estimated using restricted maximum-likelihood procedures (REML): parasite medication (antiparasite medication or solvent control; categorical), immune response (high- or low-immune group; categorical), and frequency of immune group per enclosure (rare or common; categorical) were used as fixed factors. Study enclosure was included in the model as a random factor and both estimates and residuals are given. In the absence of model selection criteria, such as Akaike Information Criterion (AIC), a standard method for comparing GLMMs, we followed a step-down procedure to select the final models. We started from the full models including the main effects of previously mentioned fixed explanatory factors and their interactions, and then simplified the model by removing the nonsignificant terms (at 5% significance level, starting from interaction terms) one by one.

Results

HERITABILITY OF IMMUNE RESPONSE

Significant additive genetic components (h^2) to immune function based on mother-, father-, and midparent–midson regressions within both immune groups were found (Table 2). There is thus a detectable additive genetic basis for the variation in immune response.

DIVERGENT GROUPS FOR IMMUNE RESPONSE ON PARASITE INFECTION AND SURVIVAL

Multiple male traits were found to be affected by divergent immune group and parasite medication. Males from our high immune response group had significantly lower parasite abundance than males from the low immune group in both parasite

Table 2. Mean ($\bar{x}$) of parent $\log_{10}$ immune response, estimates of heritability (h^2) and variance components for bank vole immune response (anti-BGG antibody production) based on mother–midson, father–midson, and midparent–midson values for the low and high immune response groups separately. V_A, additive genetic variance (twice the parent–midson covariance); V_P, phenotypic variance; V_E, environmental variance; CV_A, coefficient of additive genetic variation ($CV_A = 100\bar{x}^{-1}\sqrt{V_A}$); CV_P, coefficient of phenotypic variation; CV_R, coefficient of residual variation ($CV_R = 100\bar{x}^{-1}\sqrt{V_P - V_A}$) (Houle 1992). Heritabilities were calculated from the slope of the linear parent–midson regression for each immune group separately. Mother–midson and father–midson covariances estimate only half of the additive genetic variance, therefore, they were doubled to obtain heritability estimates (Falconer and Mackay 1996). Number of all parent–midson pairs=44 and 49 for low- and high-immune response groups, respectively. *P<0.05.

	Immune group	$\bar{x}\pm$SE	$h^2\pm$SE (95% CI)	V_A	V_P	V_E	CV_A	CV_P	CV_R
Mother:	Low	4.93±0.08	0.43*±0.2 (0.04–0.59)	0.14	0.32	0.18	6.89	24.41	18.43
	High	5.78±0.08	0.25*±0.2 (0.01–0.31)	0.08	0.32	0.24	5.25	24.37	21.11
Father:	Low	5.12±0.05	0.31*±0.2 (0.04–0.39)	0.09	0.28	0.19	5.44	22.69	18.85
	High	6.06±0.06	0.32*±0.1 (0.01–0.24)	0.38	1.18	0.80	11.41	46.83	38.62
Midparent:	Low	5.02±0.06	0.30*±0.1 (0.07–0.53)	0.11	0.37	0.26	6.21	26.33	22.02
	High	5.92±0.06	0.19*±0.1 (0.04–0.34)	0.07	0.37	0.29	4.91	26.15	23.54

Table 3. Generalized linear mixed model (PROC MIXED, SAS) was used to analyze a) the number of ectoparasites (fleas: Siphonaptera, Ceratophyllidae, and ticks *Ixodes* spp.: Acari, Ixodidae) in male bank voles during their breeding season. Generalized linear mixed model (PROC GLIMMIX, SAS) was used to analyze male bank vole survival (dead or alive) b) at the end of the breeding season and c) at the end of the winter, 9 months after the start of the field experiment, with a logit link function and binomial errors. Estimate and residual for study enclosure were: a) 0.234 and 0.528, *N*=54 and (estimate ± SE): b) 0.228±0.031, *N*=112; c) 0.761 ± 0.838; *N*=112. *df*, degrees of freedom for numerator (*n*) and denominator (*d*); *F*, test statistic; *P*, probability. Significant values are highlighted in bold text.

Source	a) Number of ectoparasites			b) End of breeding season survival			c) End of winter survival		
	df n, *d*	*F*	*P*	*df n*, *d*	*F*	*P*	*df n*, *d*	*F*	*P*
Parasite medication	**1, 11.9**	**17.26**	**0.001**	**1, 107**	**9.71**	**0.002**	1, 16.8	0.31	0.585
Immune response	**1, 46.7**	**7.56**	**0.009**	1, 107	0.26	0.611	**1, 107**	**5.13**	**0.026**
Frequency	1, 41.6	2.78	0.103	1, 107	1.01	0.316	1, 107	0.80	0.372
Parasite medication ∗ immune response	1, 47.7	0.27	0.603	**1, 107**	**4.80**	**0.031**	1, 107	0.44	0.509

medication treatments ($P = 0.009$; Table 3a; Fig. 3A). Furthermore, the immunological advantages of a high immune response translate into higher male bank vole end of breeding season survival in solvent control treatments ($P = 0.031$; Table 3b; Fig. 3B) and overwintering survival in both parasite medication treatments ($P = 0.026$; Table 3c; Fig. 3C).

A comparison of solvent control with antiparasite-medicated males revealed that antiparasite medication significantly reduced ectoparasite infection of males from both immune groups ($P = 0.001$; Table 3a; Fig. 3A). Antiparasite medication significantly increased end of breeding season survival ($P = 0.002$; Table 3b), but only for low-immune group males ($P = 0.031$; Table 3b; Fig. 3B). It is not surprising that antiparasite medication did not affect overwintering survival ($P = 0.585$; Table 3c; Fig. 3C) as treatment ceased mid-winter in December, whereas survival was recorded in the following April.

GENETIC TRADE-OFF BETWEEN IMMUNE RESPONSE AND TESTOSTERONE

We compared T levels in premedication treatment males in the laboratory with immune response. Males from the low-immune group had higher plasma T levels than those from the high-immune group (Independent samples *t*-test: $t = 2.247$, $df = 120$, $P = 0.027$; Fig. 4A). The significantly higher T levels of males from the low-immune group continued into the breeding season ($P = 0.027$; Table 4a; Fig. 4C). In addition, we found a significant negative correlation between T and $\log_{10}$ IgG level (Pearson's correlation coefficients [two-tailed]: $r = -0.291$, $P = 0.006$, $n = 90$), and a nonsignificant trend for a negative correlation between T and $\log_{10}$ anti-BGG antibody response ($r = -0.202$, $P = 0.06$, $n = 90$), further confirming an immune response-T genetic trade-off.

TRADE-OFF BETWEEN IMMUNE RESPONSE AND REPRODUCTIVE EFFORT

Male reproductive effort, the combined reproductive effort against another male and courtship effort towards a female, was also significantly higher in low-immune group males (Friedman's test, rank order based on the number of matings: $X_r^2 = 16.06$, $df = 14$, $P < 0.01$; Fig. 4B). Page's test revealed a significant increase in reproductive effort with decreasing immune function ($z_L = 3.22$, $P = 0.0007$), confirming the presence of a trade-off between immune response and reproductive effort. Page's test also revealed a significant increase in reproductive effort with increasing plasma T level ($z_L = 2.37$, $P = 0.0089$) confirming the important role of T in male bank vole reproductive effort in agreement with previous studies (Mills et al. 2007b, 2009).

IMMUNOSUPPRESSION COSTS CONSTRAIN MALE REPRODUCTIVE SUCCESS

We found a significant interaction between immune group and parasite medication on both measures of male fitness (mating and reproductive successes: $P = 0.039$ and $P = 0.029$, respectively; Tables 4b,c). In solvent control conditions, no difference in either measure of fitness was found between the immune groups (Figs. 4D,E), yet in antiparasite-treated populations, low-immune group males had substantially higher fitness than high-immune group males (Figs. 4D,E), highlighting the presence of considerable immunosuppression costs on reproductive traits. We repeated the model controlling for life span as a covariate and the interaction between immune group and parasite medication on fitness (reproductive success) was still significant ($P = 0.035$).

In agreement with previous studies in bank voles (Mills et al. 2007b, 2009), a significantly positive regression demonstrates that males with higher T levels at the height of the breeding season

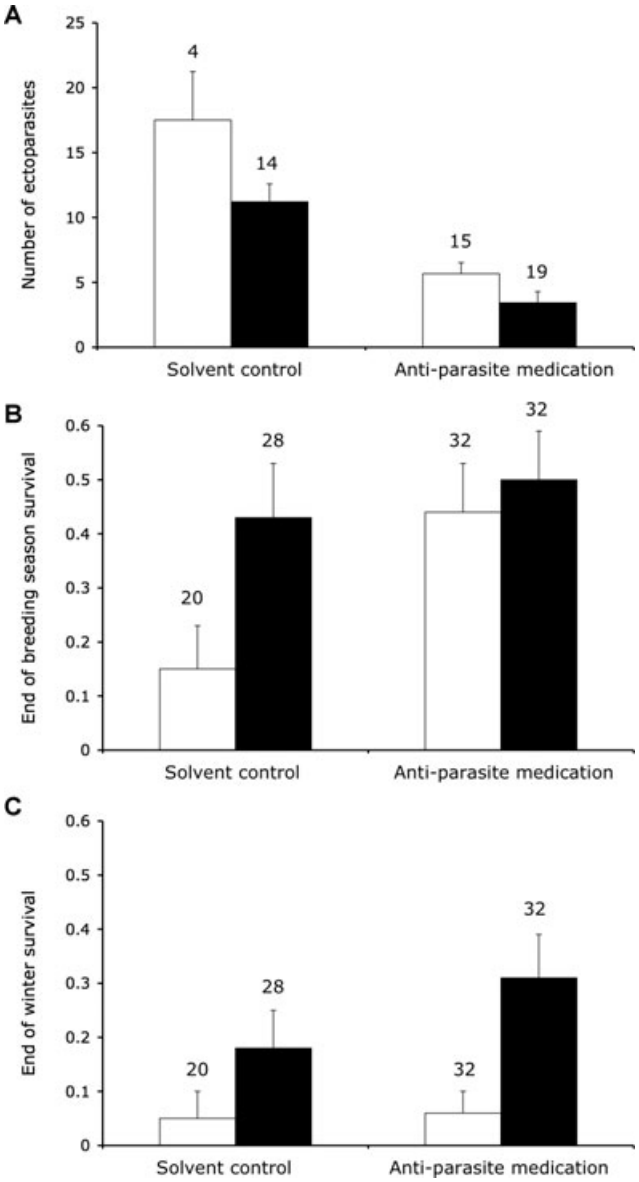

Figure 3. **Mean (± 1 SE) (A) total number of ectoparasites (fleas and *Ixodes* ticks) observed during the breeding season, (B) end of breeding season survival (October 2003), and (C) end of winter survival (April 2004) of male bank voles after release to outdoor enclosures shown for solvent control treated and antiparasite medicated males separately. Numbers above bars represent sample sizes: (A) only males live-trapped from the 14 enclosures for which ectoparasites could be counted (n = 52); (B) and (C) all males from all 14 enclosure groups (n = 112). □, low immune group and ■, high immune group.**

had higher reproductive success ($F_{1,45} = 8.23$, $r^2 = 0.16$, $y = 0.514x + 0.569$, $P = 0.006$).

IMMUNE RESPONSE-MEDIATING REPRODUCTIVE EFFORT–SURVIVAL TRADE-OFF

To test that immune response mediates the trade-off between reproductive effort (laboratory trials) and survival, we regressed standardized relative values of these fitness-related traits on the two immune groups. We identified immune response-mediated trade-offs between male reproductive effort and survival with significant crossover interactions (no parasites: min $[Q^+, Q^-] = 7.35$, $I = 2$, $P < 0.025$, Fig. 5A; parasites present: min $[Q^+, Q^-] = 7.65$, $I = 2$, $P < 0.025$, Fig. 5B; Gail and Simon 1985).

We also examined fitness that represents the outcome of survival on reproductive success. In a parasite-reduced environment (Fig. 5A), low immune function does not appear to be evolutionarily disadvantageous. In fact, low immune function males treated with antiparasite medication survived as well as high immune function males, in spite of their greater reproductive effort leading to higher overall fitness (Fig. 5A). In the presence of parasites (Fig. 5B), low-immune males survived less well, nevertheless, their overall fitness was equal to that of high-immune function males. Our graphical representation in Figure 5 shows that only the overall fitness of low-immune group males is reduced from parasite-reduced (Fig. 5A) to parasite-present environments (Fig. 5B), highlighting that only males with low genetic disease resistance show a survival handicap and immunosuppression costs.

FREQUENCY DEPENDENT SELECTION

There was no effect of immune group frequency (Fig. 2C) on either measure of survival ($P = 0.316$, $P = 0.372$; Tables 3b,c), or on any of its two- or three-way interactions, indicating that bank vole survival is not affected by the immune phenotype of other individuals in the population. Similarly, we found no frequency-dependent effect of immune group on either measure of fitness ($P = 0.821$, $P = 0.841$; Tables 4b,c), suggesting that the frequency of reproductive tactics measured here (2:6, 6:2) had no effect on the reproductive success of males with other strategies.

Discussion

Our study provides evidence for the heritability of immune function and shows that selection on immune response results in correlated responses on T levels. This research supports the relevance of immune function for overall fitness by demonstrating strong relationships between immune parameters measured in the laboratory and fitness parameters in seminatural field enclosures. Our study confirms the trade-off between survival and reproductive effort and highlights immune response as the proximate mediator. Our experimental parasite manipulation compares life-history traits between individuals in the presence and absence of disease and demonstrates that for males with low genetic disease resistance, the costs of mounting an immune response reduce their fitness.

ARTIFICIAL SELECTION AND PARASITE MEDICATION

Our divergent groups for immune response, even after only one generation, were clearly successful in regulating parasite infection

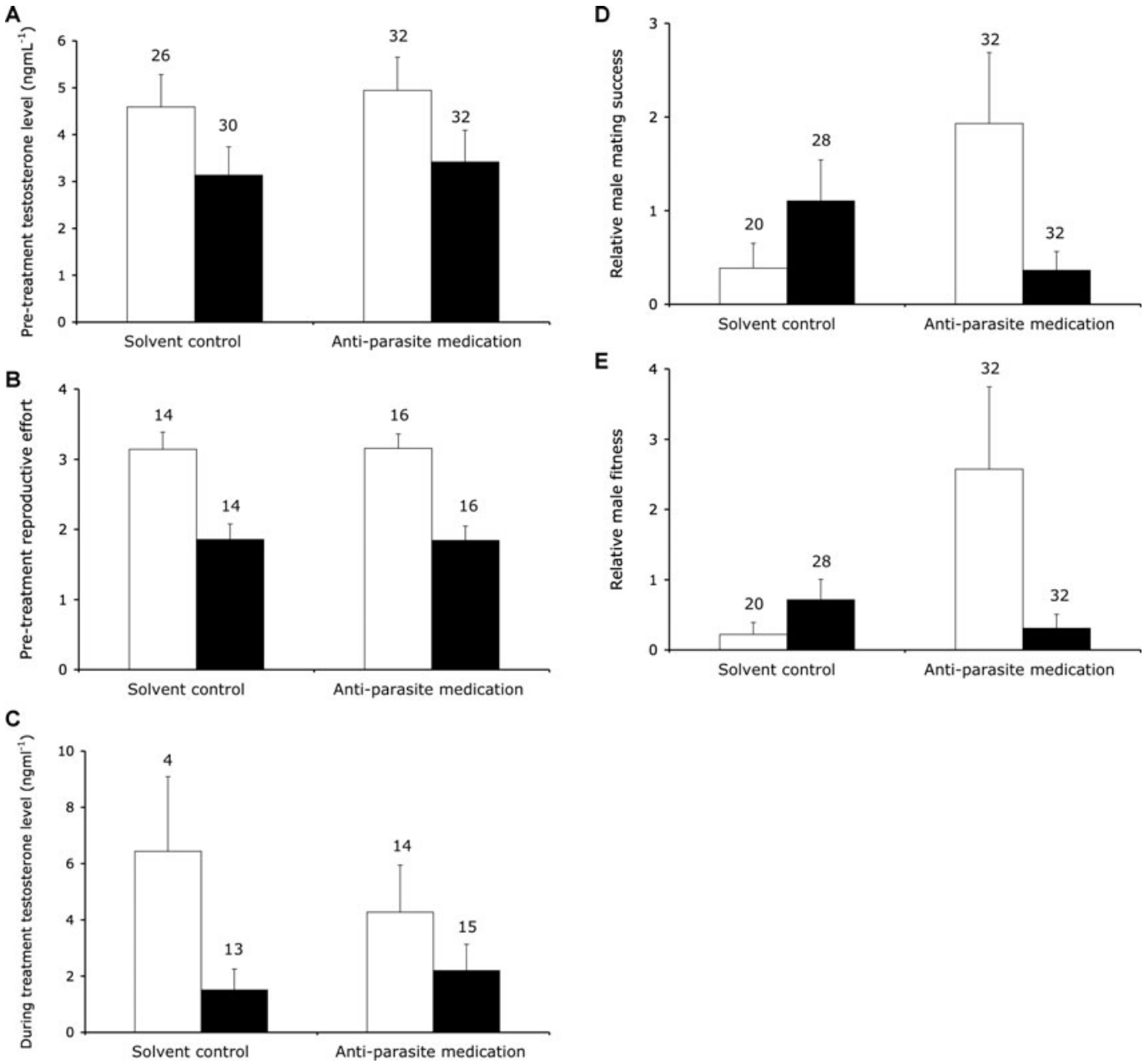

Figure 4. Mean (± 1 SE) measurements in the laboratory prior to medication treatment for (A) testosterone level (ng mL^{-1}) and (B) reproductive effort of four randomly chosen males (two low and two high immune group males) per enclosure group. Reproductive effort is based on ranked dominance data determined from the number of successful matings by each male in laboratory trials (4: highest dominance, 1: lowest dominance). Mean (± 1 SE) (C) testosterone level (ng mL^{-1}) during the breeding season, (D) relative male mating success (number of genetic mates) and (E) relative fitness (reproductive success of all males, alive or dead) of male bank voles after release to outdoor enclosures. All data are shown for the solvent control treated and antiparasite medicated males separately. LSD comparisons of relative male mating success and relative fitness between low and high immune groups in (E) for males treated with either solvent control: $P = 0.417$ or antiparasite medication: $P = 0.025$ and (F) for males treated with either solvent control: $P = 0.400$ or antiparasite medication: $P = 0.016$. Mean mating success and fitness were made relative by dividing by the population mean. Numbers above bars represent sample sizes: a) all males from 15 enclosure groups ($n = 120$); (B) four males from 15 enclosure group ($n = 60$); c) only the males live-trapped from the 14 enclosures for which blood could be sampled ($n = 46$); d) and e) all males from 14 enclosure groups ($n = 112$). □, low immune group and ■, low immune group.

Table 4. Generalized linear mixed model (PROC MIXED, SAS) was used to analyze a) plasma testosterone level in male bank voles during their breeding season, b) male bank vole relative mating success in outdoor enclosures (number of genetic mates), and c) male bank vole relative fitness in outdoor enclosures (measure of reproductive success that also incorporates mortality, log-transformed). Estimates and residuals for study enclosure were: a) 19.587 and 5.053, N=46; b) 0.485 and 0.070, N=112 and c) 0.0±0.091, N=112. df, degrees of freedom for numerator (n) and denominator (d); F, test statistic; P, probability. Significant values are highlighted in bold text.

Source	a) Testosterone during the breeding season			b) Relative male mating success			c) Relative fitness		
	df n, d	*F*	*P*	*df n, d*	*F*	*P*	*df n, d*	*F*	*P*
Parasite medication	1, 12.9	0.08	0.777	1, 11.0	0.55	0.473	1, 107	1.19	0.277
Immune response	**1, 40.3**	**5.28**	**0.027**	1, 91.4	0.71	0.402	1, 107	0.85	0.360
Frequency	1, 35.4	0.05	0.824	1, 96.3	0.05	0.821	1, 107	0.04	0.841
Parasite medication ∗ immune response	1, 40.6	0.82	0.370	**1, 91.4**	**4.37**	**0.039**	**1, 107**	**4.90**	**0.029**

in seminatural field conditions (Fig. 3A). Furthermore, even in the presence of antiparasite medication, these divergent groups still regulated parasite abundance, as males from the high-immune group showed no survival advantage after receiving antiparasite medication (Fig. 3B). As our three measures of immune function differed significantly between groups (Table 1), significant heritability was found for both measures of humoral immunity (Table 2), and our divergent immune groups were successful in overcoming parasite-induced mortality, it is safe to assume that our artificial selection experiment successfully manipulated immune function between our groups. Antiparasite medication had a considerable effect in reducing parasite numbers (Fig. 3A), thus validating our use of this medication for our field experiment.

HERITABILITY, TRADE-OFF, AND SEXUAL SELECTION

Our study provides evidence for the heritability of immune response (Table 2), which adds to the previously found heritability of T level in bank voles (Mills et al. 2007b), suggesting that selection by females for either trait will be passed on to her offspring according to "good genes" theory of sexual selection. However, not only did we find a genetic trade-off between immune response and T level, but also a trade-off between immune response and laboratory reproductive effort. What are the consequences of these trade-offs for sexual selection theory? Dominant males that fully express their T-dependent behavioral traits are hypothesized to be signaling honestly indicating both genetic disease resistance (alleles that provide resistance against current pathogens) and an ability to acquire abundant resources (investment into reproductive effort with sufficient resources remaining to also invest in immune defense, two metabolically costly traits) (Fisher 1930). Previous studies have confirmed that female bank voles prefer to mate with dominant males (Mills et al. 2009), yet this study suggests that dominant males do not signal disease resistance, so why do females continue to prefer these dominant males? Recent sexual selection models predict that the genetic benefits of mate choice for either mating success or survival are equal, and a negative relationship between male attractiveness and survival is plausible (Kokko et al. 2002). Our results provide such evidence, as males from the two divergent immune groups have equal fitness in field conditions with natural parasite pressure (Fig. 5B). Therefore, these fitness benefits, and those of their offspring, open up the possibility of evolutionary stable signaling (ESS). The presence of an evolutionary stable strategy suggests that if sexual selection runaway processes drive dominant males to have lower viability, then higher immunity is not necessarily expected (Emerson 2000; Hall et al. 2000; Aoki et al. 2001) as we have found in bank voles. Therefore, females mating with dominant males will gain indirect benefits for reproductive success and signal honesty is maintained.

Female bank voles mating with one male will gain indirect genetic fitness benefits for either genetic disease resistance or male reproductive effort, but not both. However, female bank voles are known to be polyandrous (Mills et al. 2007b) and, as already proposed (Mills et al. 2007a), females may gain genetic benefits through bet hedging, with the possibility of gaining indirect genetic fitness benefits for both genetic disease resistance and male reproductive effort within a litter, but not within an individual offspring.

PARASITES AND IMMUNE RESPONSE-MEDIATING REPRODUCTIVE EFFORT–SURVIVAL TRADE-OFF

Zahavi's (1975) and Hamilton and Zuk's (1982) original hypotheses proposed that genetic disease resistance mediates the trade-off between male reproductive effort and survival and our study provides evidence for this hypothesis (Figs. 5A,B). Males with high immune response show high survival (Figs. 3B,C, and 5), but low reproductive effort (Figs. 4B and 5), whereas males with low immune response show high reproductive effort, but low survival. The survival handicap of reproductive effort is hypothesized to act via the allocation of resources to immune response, such

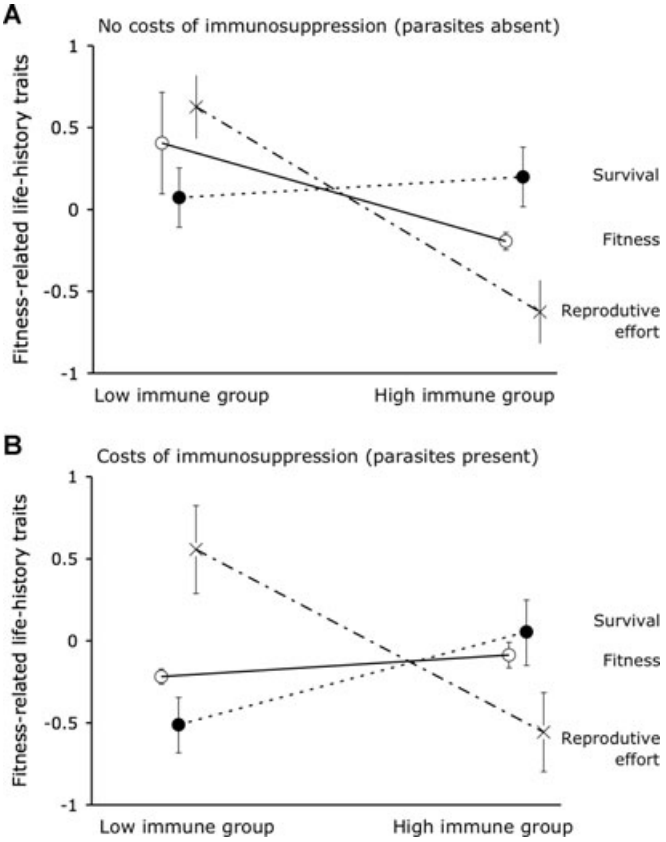

Figure 5. Relationship between three fitness-related life-history traits across immune groups. Fitness represents the reproductive success of males, which also incorporates mortality. Survival represents end of breeding season survival. Reproductive effort represents the ranked dominance of males measured in sterile laboratory conditions prior to the field experiment. All traits were made relative by dividing by the population mean and standardized to have a mean of zero and standard deviation of 1. Sample sizes for fitness and survival: all males from all 14 enclosure groups (n = 112) and for reproductive effort: four males from all 14 enclosure groups (n = 56). —○—, fitness (field reproductive success, which also incorporates mortality), ···●···, survival (alive or dead at the end of the breeding season), ---X---, reproductive effort (high to low dominance ranked 4 to 1).

that males allocating their resources to reproductive effort will be handicapped by a suppressed immune response (Grossman 1985; Folstad and Karter 1992; Wedekind and Folstad 1994). To demonstrate immunity as the mechanism behind this trade-off we created environments in which a suppressed immune system should not be a handicap, that is, disease-free environments. We therefore removed parasites by treating bank vole enclosure populations with antiparasite medication, thus removing ecto- and endoparasites and the diseases they carry (McTier et al. 2000). In the absence of immunosuppression costs (no parasites; Fig. 5A) survival is equal between high- and low-immune group males (Table 3b). Conversely, in the presence of immunosuppression costs (parasites present; Fig. 5B), low-immune males do show a considerable survival handicap compared to high-immune males (Table 3b) and this is likely to be due to their allocation of resources to reproductive effort. Our study demonstrates that males with low genetic resistance to disease only show a handicap from reproductive effort when parasites, and thus diseases, are present, indicating that the reproductive effort–survival trade-off depends on the presence of parasites and/or diseases they carry in accordance with original hypotheses (Zahavi 1975; Hamilton and Zuk 1982).

GENETIC TRADE-OFF AND EVOLUTIONARY CONSTRAINTS

Our study also highlights a correlated response of T levels to selection on immune function. Selection for high-immune response results in indirect selection for low T level both in the laboratory (Fig. 4A) and under seminatural field conditions (Fig. 4C), as well as low reproductive effort (Fig. 4B). Selection for low-immune response results in the opposite trend. A genetic basis for the immunosuppressive cost of sexual trait development has been found (Verhulst et al. 1999), but to our knowledge, this is the first study to demonstrate a genetic trade-off between immune response and T levels.

The survival handicap of reproductive effort suggests that low immune males are constrained by their immune system from fully expressing their sexual traits, as our evidence shows (Figs. 4D,E). On the other hand, the fitness of males from the high-immune group was not affected by either the presence/absence of parasites (Fig. 4E), suggesting that they are at their maximum competitive capability to acquire mates. These results, combined with those showing a genetic trade-off between immune response and T levels, demonstrate that the fitness related traits, reproductive effort, and survival are constrained from evolving in accordance with evolutionary theory (Stearns 1992).

FREQUENCY-DEPENDENT SELECTION

Finally, our experimental design enables us to test whether the success of one immune tactic depends on its frequency in the population in a negative frequency dependent manner (Ayala and Campbell 1974; Gromko 1977; Mappes et al. 2008). Our results indicate that neither bank vole survival nor reproductive success is affected by the immune phenotype of other individuals in their enclosure population in the ratios tested here. Rare genotypes for immune function that provide resistance against an invading disease are expected to increase in frequency in the next generation and mate choice would have augmented such an effect if the individuals carrying alleles providing the highest resistance were also selected as mates. However, our immune groups were selected for general immune parameters rather than specific parasite resistant genotypes, therefore, it is not necessarily surprising that we did

not find a significant effect. Furthermore, replicates of one frequency ratio were lost; therefore, further experimentation should be carried out before drawing any solid conclusions.

Conclusion

This study confirmed the trade-off between reproductive effort and survival and demonstrated that parasites and immune response mediate the trade-off. Males with divergent immune responses were found to have equal fitness that may demonstrate the presence of evolutionary stable signaling strategies for reproductive effort and survival. As both immune response (this study) and T (Mills et al. 2009) are heritable in bank voles, females will benefit in terms of indirect genetic benefits for either reproductive effort or survival for her offspring, but not both. High genetic variation for male bank vole dominance has been found (Horne and Ylönen 1998; Oksanen et al. 1999; Mills et al. 2007a), and a previous study proposed that genotype by environment interactions in the presence of environmental variation may be one mechanism underlying its maintenance (Mills et al. 2007a). The genetic trade-off between immune response and T levels found in this study provides evidence that the evolution of male reproductive traits are also constrained by a survival handicap, in accordance with evolutionary theory, based on genetic disease resistance to parasites, which themselves fluctuate temporally. Therefore, the reproductive effort-survival trade off may represent another mechanism maintaining genetic variation for multiple life-history traits.

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