

COSTS OF AN INDUCED IMMUNE RESPONSE ON SEXUAL DISPLAY AND LONGEVITY IN FIELD CRICKETS

ALAIN JACOT,¹ HANNES SCHEUBER, AND MARTIN W. G. BRINKHOF²
*University of Bern, Zoological Institute, Division of Evolutionary Ecology, Wohlenstrasse 50a,
CH-3032 Hinterkappelen, Switzerland*

Abstract.—Immune system activation may benefit hosts by generating resistance to parasites. However, natural resources are usually limited, causing a trade-off between the investment in immunity and that in other life-history or sexually selected traits. Despite its importance for the evolution of host defense, state-dependent fitness costs of immunity received little attention under natural conditions. In a field experiment we manipulated the nutritional condition of male field crickets *Gryllus campestris* and subsequently investigated the effect of an induced immune response through inoculation of bacterial lipopolysaccharides. Immune system activation caused a condition-dependent reduction in body condition, which was proportional to the condition-gain during the preceding food-supplementation period. Independent of nutritional condition, the immune insult induced an enduring reduction in daily calling rate, whereas control-injected males fully regained their baseline level of sexual signaling following a temporary decline. Since daily calling rate affects female mate choice under natural conditions, this suggests a decline in male mating success as a cost of induced immunity. Food supplementation enhanced male life span, whereas the immune insult reduced longevity, independent of nutritional status. Thus, immune system activation ultimately curtails male fitness due to a combined decline in sexual display and life span. Our field study thus indicates a key role for fitness costs of induced immunity in the evolution of host defense. In particular, costs expressed in sexually selected traits might warrant the honest advertisement of male health status, thus representing an important mechanism in parasite-mediated sexual selection.

Key words.—Acoustic signaling, costs of immunity, *Gryllus campestris*, life-history trade-offs, longevity, nutritional condition, sexual selection.

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Life-history theory assumes that variation in host immune defense reflects an optimal balance between the benefits and costs of parasite resistance (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Schmid-Hempel 2003). Parasites and pathogens are predisposed to reduce host fitness by decreasing their reproductive output, longevity or both (Moore 2002), and an immune response may effectively reduce the potential cost of parasitism (Iwanaga et al. 1995; Roitt et al. 1996; Wakelin and Apanius 1997). However, the investment in immune defense presumably comes with a cost, because resource limitation would cause a trade-off that reduces the expression and fitness value of other life-history traits (Sheldon and Verhulst 1996). Costs of immunity thus play a central role in the evolution of host resistance to parasites.

The expenditure for immunity basically involves evolutionary costs and deployment costs (Rolff and Siva-Jothy 2003; Schmid-Hempel 2003). Evolutionary costs are based on genetic trade-offs, as revealed in the negative genetic covariance between resistance traits and other fitness components following artificial selection experiments on immune function (Verhulst et al. 1999) or parasite resistance (Lenski 1988; Kraaijeveld and Godfray 1997; Kraaijeveld et al. 2001). Deployment costs involve the expenses for maintenance of the immune system and the implementation of an immune response in case of infection. Evidence for deploy-

ment costs on various life-history traits comes from experiments in which an antigenic insult activates the immune system while excluding direct costs of parasitism associated with an actual infection (Demas et al. 1997; Ilmonen et al. 2000; Råberg et al. 2000; Ahmed et al. 2002; Armitage et al. 2003). An immune response may specifically evoke a reduction in the expression of sexually selected traits, such as recently shown in male blackbirds *Turdus merula* (Faivre et al. 2003). Costs of immune system activation might thus represent an important mechanism to parasite-mediated sexual selection theory, which predicts that secondary sexual characters honestly advertise the signaler's health.

It is largely unknown how variations in ecological factors or individual state affect the trade-off between immune defense and other life-history traits under natural conditions. Life-history theory predicts that such trade-offs are state dependent, for instance related to variation in nutritional condition, which concerns the pool of resources through food availability and body reserves. First evidence for state-dependent fitness costs comes from a laboratory study with bumblebees (Moret and Schmid-Hempel 2000) that suffered an immediate survival cost to an immune insult when subjected to food deprivation, but not with unlimited food supply. Consequently, costs of immune system activation might be hidden under natural conditions, if individuals in superior nutritional condition compensate the additional demand by expending extra resources at modest costs to other life-history traits. Moreover, field studies so far have focused on short-term effects on physiology, behavior, or reproductive performance (Schmid-Hempel 2003) while ignoring deferred effects, such as on longevity, that strongly influence lifetime fitness.

In this study we investigated condition-dependent costs of

¹ Present address: Max Planck Institute for Ornithology, Postfach 1564, D-82305 Starnberg (Seewiesen), Germany; E-mail: jacot@orn.mpg.de.

² Present address: University of Bern, Department of Social and Preventive Medicine, Division of Epidemiology and Biostatistics, Finkenhubelweg 11, CH-3012 Bern, Switzerland; E-mail: brinkhof@ispm.unibe.ch.

immune system activation in male field crickets (*Gryllus campestris*) under field conditions. Free-living males are generally food limited, since food supplementation elevates their body condition, daily calling rate, and thereby number of females attracted (Holzer et al. 2003; present study). Male attractiveness is further determined by the quality of the calling song. Females show a directional preference for low carrier frequencies and high chirp rates (Scheuber et al. 2004), which indicate a superior past (Scheuber et al. 2003a) and current nutritional condition (Scheuber et al. 2003b), respectively. We manipulated nutritional condition and then subjected males to an immune insult using a standardized dose of bacterial lipopolysaccharides (LPS). LPS are surface molecules of gram-negative bacteria. In this case we injected LPS from the common insect pathogen *Serratia marcescens*, which is potentially lethal to field crickets (Adamo et al. 2001). LPS itself is a nonpathogenic, nonliving elicitor that induces several pathways of the invertebrate immune response (Imler et al. 2000; Kimbrell and Beutler 2001; Lavine and Strand 2002). We thus assessed short- and long-term costs of immunity regarding key parameters of male fitness, which included body condition, daily calling rate, calling song characteristics, and longevity.

MATERIALS AND METHODS

The study was performed on an uncut meadow located on a south-facing slope near Bern, Switzerland, throughout the 2002 reproductive season of the field crickets, from the end of April until the start of July. Field crickets *Gryllus campestris* overwinter as penultimate or ultimate instar nymphs. Following emergence and the final molt into adulthood, males start calling from a platform cleared of vegetation in front of their burrows to attract females for mating (Rost and Honegger 1987). Males are reproductively active throughout their adult life, mating multiply with the same as well as with different females, indicating longevity as an important life-history trait (Zuk 1987; Simmons and Zuk 1992). For a more detailed account of the *G. campestris* life history see Rost and Honegger (1987).

Daily and seasonal variations in biotic and abiotic factors, such as food availability and weather conditions, are bound to affect male body condition, calling behavior and survival under field conditions. Since these sources of variation are hard to assess and to control statistically, we implemented a grouped experimental design, in which groups of up to 10 individuals and involving the different treatments entered the experiment on successive days throughout the season. Thus, males within groups experienced similar environmental conditions during the experimental period, and by controlling for variation among groups we maximized the likelihood of detecting treatment effects. Moreover, given that virtually all males in the population reached adulthood in a period of one week, group effects essentially controlled for age-related variation in trait values as well.

Experimental individuals were randomly selected among the calling males on the meadow, captured, and weighed to the nearest 0.01 g using an electronic balance (Sartorius PT150, Sartorius AG, Goettingen, Germany). The males were then transferred to a 50-cm-high tentlike cage of fine mesh

(mosquito) netting, which completely enclosed a one square meter area on a novel site of the meadow. Experimental sites further contained an artificial burrow and a small feeding dish that was located near the burrow entrance. Males were randomly assigned with respect to body mass to one of four experimental treatments, and experimental treatments were randomly assigned to experimental sites.

To assess condition-dependent costs of immune system activation we implemented a two-by-two factorial design with food supplementation and immune challenge as main factors. Male nutritional condition was manipulated through food supplementation over the first eight days following the confinement, and immune status was altered on day three through the inoculation of a nonpathogenic antigen. To study the combined effect of the food and immune treatments, body condition and calling song parameters on day three thus served as a reference or baseline. Similar to Holzer et al. (2003), food-supplemented males had ad libitum access to fish food (NovoBel Aquarium fish food, JBL GmbH, Neuhofen, Germany), whereas the feeding dish of control individuals remained empty. All feeding dishes were exchanged daily. To activate the immune system in half of the males in each food treatment group, we injected on day three a Grace's insect medium (Sigma, G8142 Sigma-Aldrich Chemie GmbH, Seelze, Germany) solution containing LPS derived from the gram-negative bacteria *Serratia marcescens* (Sigma, L6136). Prior to the injection we immobilized the male in a small cage and carefully cleaned the ventral sternites with a small piece of cotton wool that was drenched in a 70% ethanol solution. Using a 10- μ l Hamilton syringe we then ventrally injected between the third and fourth sternite 10 μ l of a 0.1% LPS solution (corresponding to 10 μ g LPS/g cricket) in experimental males (immune-challenged group), whereas control males received 10 μ l of Grace's insect medium only (sham-injected group). Our dosage of LPS is about one-tenth that used in other species of field cricket (Adamo 1999), but has been shown to induce a long-term upregulation of immune function (i.e., prophenoloxidase and lysozyme-like activity) in *G. campestris* (Jacot et al. 2004). Following injection, males were immediately returned to their cage. Male body mass was measured on days 3 (defined as the baseline body mass), 4, and 6.

To evaluate treatment effects on male sexual display quantitatively and qualitatively, we assessed their variation in daily calling rate and calling song characteristics. Daily calling rate was measured automatically, over a 23–24 hour period prior to inoculation (defined as the baseline daily calling rate) and over the 72 hours thereafter, using a sound level sensor (DCP Microsense, DCP Microdevelopments Ltd, Norfolk, U.K.), which was fixed at 15 cm above the platform in front of the burrow and connected to a data logger (Mindstorms RCX 1.0 programmable brick, LEGO, Baar, Switzerland). Thus, loudness (dB) was sampled every 3 min, resulting in 480 datapoints over a 24-hour period. To reliably discriminate background noise from male calling, which on average exceeds environmental noise by 49 (± 3.28) dB, we only scored datapoints of 25 dB above the background noise as "calling". For use in the present analyses we calculated the mean calling rate over the recording period. Using a digital audio tape recorder (Sony TCD-D100 Sony, Tokyo) and

a stereo microphone (Sony ECM-MS957), we further sampled a calling song, shortly prior to the immune treatment (i.e., baseline calling song) and 24 hours thereafter. To minimize the variance in calling song characters due to differences in body temperature (Martin et al. 2000; Hedrick et al. 2002), all recordings were made in late afternoon (between 17:00 and 20:00 h) when the whole meadow was in the shade. The analysis of calling song characters was performed using Canary software (Charif et al. 1995). We measured chirp rate (n chirps/sec), chirp duration (msec), pulse number (n pulses/chirp), and carrier frequency (kHz) using standard criteria (Scheuber et al. 2003b). Each individual was recorded for a 30 sec period at least 2 min after the start of continuous calling activity. All calling song characteristics were calculated from the first 10 sec of the digitized 30-sec time period. Repeatability analyses have shown that 10 sec represent a reliable time period for calling song analyses (Holzer et al. 2003; Scheuber et al. 2003b).

On day 8 we terminated the food treatments and transferred the males to a new cage within the field site to assess their longevity. Mortality was checked daily. Dead individuals were collected to obtain digital images of structural size traits, which were taken using a video camera that was mounted on a dissecting microscope, and analyzed using image analysis software (NIH Image 1.61, National Institutes of Health, Bethesda, MD). We measured the length of the left and right hind tibia, pronotum size, and harp size (i.e., an important sound-producing structure in the modified forewing; following Simmons (1995).

The dataset comprises of 11 groups, which entered the experiment on consecutive days throughout the reproductive season. Group size varied between eight and 10 individuals, adding up to an initial total of 98 experimental males. Six males were lost within the first three days, leaving 92 males for analysis. Controlling for variation across groups, males subjected to different treatments (control, sham-injected, $n = 23$; control, LPS, $n = 24$; supplemented, sham-injected, $n = 24$, supplemented, LPS, $n = 21$) did not differ in body mass ($F_{3,88} = 0.101$, $P = 0.90$), condition ($F_{3,47} = 0.609$, $P = 0.548$), or structural size (pronotum: $F_{3,47} = 0.791$, $P = 0.459$, tibia: $F_{3,38} = 0.395$, $P = 0.676$, harp size: $F_{3,46} = 0.185$, $P = 0.832$). Initial body mass and pronotum size were highly correlated ($r = 0.782$). Sample sizes for structural traits were smaller, because some dead crickets were partially devoured by ants upon recovery. To maximize the dataset for the analysis we therefore took the change in body mass as an indicator of change in body condition, given that structural size traits are fixed in adults.

Statistics were performed using JMP IN (Sall and Lehmann 1996) software. Statistical modeling implicated general linear models (normal error), and significance testing of predictor variables was hierarchical using a stepwise backward procedure. The sole effect of the food treatment prior to the immune insult on baseline (day 3) body mass, daily calling rate, and calling song characters was analyzed with multiple regression, using group and food treatment as predictor variables. Initial body mass at the start of the experiment was added as a covariate in the body mass analysis. To investigate the subsequent, combined effect of the food treatment and immune treatment on body mass and daily calling rate, we

took the deviation from baseline on days 4, 5, and 6 as repeats in a repeated-measures ANOVA. This analysis separates among-individual from within-individual effects. Multiple regression was used to analyze the change in calling song characters and longevity. In testing the relative effects of the two experimental treatments we used group, food treatment, immune treatment, and their mutual interaction as fixed factors. Baseline body mass was further included as a covariate in analyzing longevity.

RESULTS

Effects on Body Mass

Controlling for significant variation among successive groups ($F_{10,74} = 4.45$, $P < 0.001$) and in initial body mass ($F_{1,74} = 173.83$, $P < 0.001$), the food treatment significantly affected body mass on day three ($F_{1,74} = 19.89$, $P < 0.001$). Although both food-treatment groups increased in body mass, food-supplemented males were heavier than control males on day 3 (Fig. 1A).

Males in each treatment group on average decreased in body mass over the first three days following the immune treatment. However, body mass loss was larger following the inoculation of LPS than of Grace's insect medium in both the food-supplemented (mean mass loss \pm SE in g, LPS vs. sham-injected: -0.042 ± 0.019 vs. -0.034 ± 0.013) and control groups (-0.021 ± 0.016 vs. -0.002 ± 0.010). We specifically investigated the change in body mass after inoculation in relation to the prior change in body mass among individuals. The interaction between immune treatment and change in body mass prior to the immune treatment had a significant effect on change in body mass following the immune treatment (interaction immune treatment \times body mass change day 0–3: $F_{1,73} = 5.23$, $P = 0.025$; controlled for variation between groups, $F_{10,56} = 4.80$, $P = 0.007$). For LPS males, the greater the pretreatment gain in mass, the greater was the post-treatment loss in mass. For sham-injected males, there was little effect of pretreatment change in mass on post-treatment change in mass (Fig. 1B).

Effects on Daily Calling Rate

Controlling for variation among groups ($F_{10,80} = 4.22$, $P < 0.001$), the baseline daily calling rate prior to the immune treatment was significantly affected by the food treatment ($F_{1,80} = 17.72$, $P < 0.001$). Food-supplemented males called significantly more often than control males (Fig. 2A).

The subsequent overall change in daily calling rate varied between groups ($F_{10,73} = 6.56$, $P < 0.001$) and was affected by the immune treatment ($F_{2,73} = 7.05$, $P = 0.01$), whereas there was no additional effect of the food treatment ($F_{1,72} = 0.10$, $P = 0.756$; interaction term: $F_{1,71} = 1.23$, $P = 0.27$). There were no significant within-individual treatment effects. In response to the immune treatment, all males initially reduced their calling rate (day 4), but the decrease was larger in LPS-injected than in sham-injected individuals. Subsequently, all males increased in daily calling rate, but only sham-injected males returned to their baseline level (Fig. 2B).

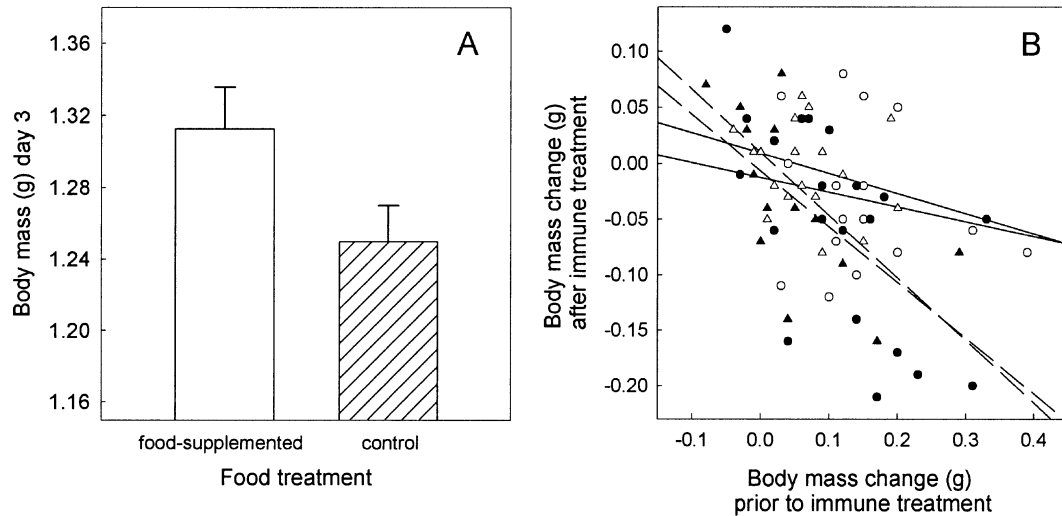


FIG. 1. (A) The effect of food treatment on baseline body mass (mean \pm SE), as taken on day 3. (B) Change in body mass following immune treatment (days 3–6) as a function of the body mass change during food supplementation (days 0–3). Food-supplemented treatments are represented by circular symbols and control treatments with triangular symbols. Open symbols and solid regression lines indicate sham-injected males; solid symbols and broken lines, LPS-injected males.

Effects on Calling Song Characters

Food-supplemented males showed a higher baseline chirp rate than control males ($F_{1,44} = 4.15, P = 0.048$), independent of variation among groups ($F_{8,44} = 7.98, P < 0.001$). Other calling song characters were not affected by the food treatment (peak frequency: $F_{1,45} = 0.84, P = 0.36$; chirp length: $F_{1,44} = 0.25, P = 0.62$; pulse number: $F_{1,44} = 1.40, P = 0.24$).

None of the calling song characters deviated significantly from the baseline values following the immune treatment on day 3. All P -values for food treatment, immune treatment, and their mutual interaction exceeded 0.25.

Effects on Longevity

Controlling for variation among groups ($F_{10,60} = 14.95, P < 0.001$) and baseline body mass ($F_{1,60} = 5.54, P = 0.022$),

longevity was significantly affected by the immune treatment ($F_{1,60} = 17.85, P < 0.001$) and the food treatment ($F_{1,60} = 5.01, P = 0.029$). The effect of immune treatment was independent of food treatment ($F_{1,59} = 0.98, P = 0.33$). The variation over successive groups was partly explained by a progressive decline in longevity with season ($n = 11, r = -0.97, P < 0.001$). Food-supplemented males lived on average 30.5 (± 3.2) days (sham-injected) and 25.9 (± 2.8) days (LPS), whereas control males lived on average for 29.8 (± 2.7) days (sham-injected) and 22.1 (± 2.1) days (LPS). Independent of the positive effect of food-supplementation on longevity, LPS males had a shorter life span than sham-injected males (Fig. 3).

DISCUSSION

We here showed under field conditions that an immune insult causes a lasting reduction in sexual display as well as

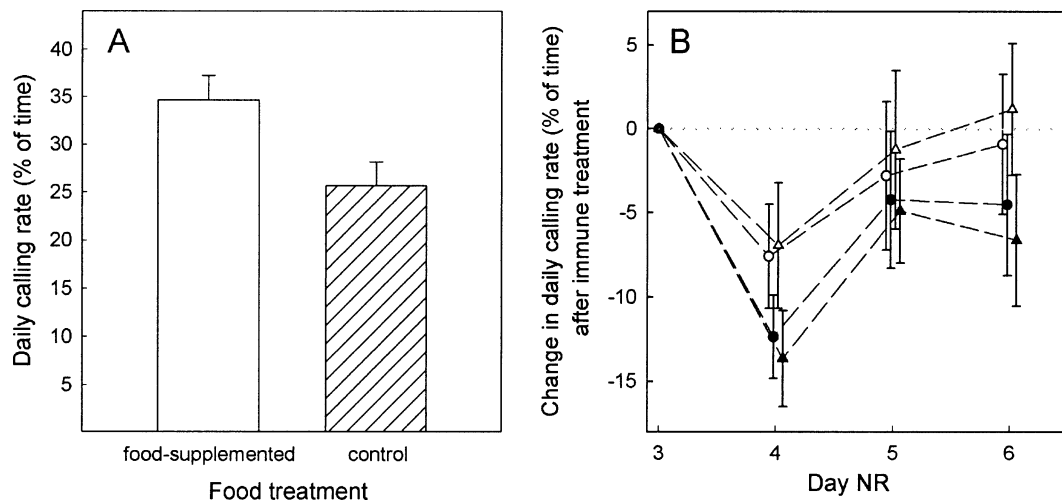


FIG. 2. (A) Effect of the food treatment on baseline daily calling rate (mean \pm SE), taken on day 3. (B) Immune treatment induced change in daily calling rate from baseline (day 4 to day 6). Symbols are as in Figure 1.

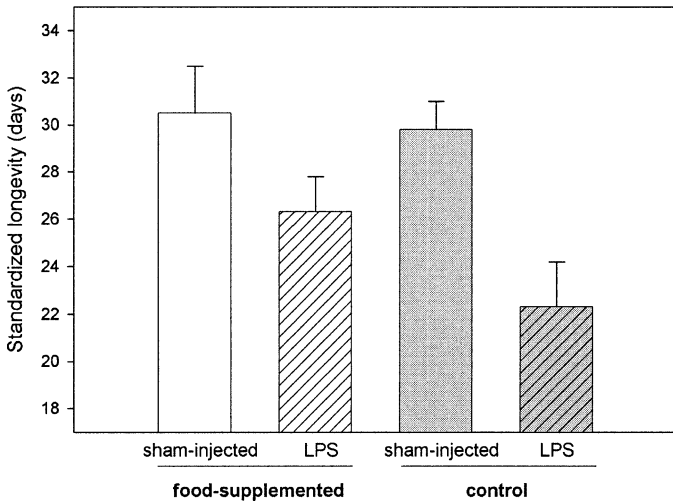


FIG. 3. Longevity (mean \pm SE) in relation to food treatment and immune treatment, standardized to the mean longevity over groups.

longevity, two key components determining lifetime fitness of a male field cricket. Although food supplementation improved sexual advertisement and longevity, this cost of immunity was independent of nutritional condition.

The LPS-induced decline in daily calling rate is likely to reduce male conspicuousness to receptive females and thereby mating success (Gerhardt and Huber 2002). Studying the exclusive effect of nutritional condition, Holzer et al. (2003) previously showed that the superior calling rate of food-supplemented males, a result confirmed in the present study, enhanced the number of attracted females under field conditions. It is unfortunately unknown whether female field crickets incorporate a discrete comparison of the calling activity of resident males in their mate choice decisions. Thus, similar to chorus attendance in frogs (Murphy 1998), time spent calling might influence male mating opportunities, and the mechanism of sexual selection may involve neither female choice nor male-male competition. Calling song characteristics such as chirp rate, which determine male attractiveness to females, also indicated no effect of the LPS on calling song quality. We consider it unlikely that the latter result was basically due to the masking effect of uncontrolled, environmentally induced variation in operational body temperature between treatment groups. By investigating successive groups that included males of all experimental treatments we controlled for the overall effects of the season, but the randomized temporal recording of calling songs under shaded conditions minimized the between-subject variation in operational body temperature within groups. As such, variation in the frequency of sexual advertisement may have a larger effect on male mating success than qualitative variation in secondary sexual traits (Gerhardt and Huber 2002). Thus, the decline in male display following immune system activation in field crickets suggests an evolutionary trade-off between the investment in immunity and the quantity of sexual display. Moreover, costs of immunity on sexually selected traits, which include display frequency if assessed by females, might at least partly account for the cost of parasitism envisioned by parasite-mediating selection theory (Hamilton

and Zuk 1982; Faivre et al. 2003). The reduction in sexual display following an induced immune response would allow females to discriminate infected males from parasite-free or resistant males in mate choice decisions.

The trade-off between the investment in immunity and male sexual display most likely concerns limited energy reserves. Immune responses in insects are energetically costly, such as recently shown in cabbage butterflies (*Pieris brassicae* L.), which raised their standard metabolic rate in response to an implanted nylon filament (Freitak et al. 2003). Given that the average metabolic rate of calling field cricket males is nearly three times that of noncalling males (Hoback and Wagner 1997), the reduction in sexual display might indicate a physiological trade-off with the investment in immune function. Metabolic costs were also indicated by the decline in body condition following the immune insult, which was proportional to the mass gain during the preceding period of food supplementation. Field cricket body condition, as expressed in mass residuals, appropriately reflects energetic fat reserves under controlled feeding conditions (Gray and Eckhardt 2001). The production of several compounds against bacteria and fungi by the insect immune system further relies on fat body reserves (Hetru et al. 1998; Miller et al. 1999). Furthermore, a fat-reserve-dependent investment in immune function might explain why the reduction in sexual display showed no relation to nutritional condition, if immune defense obtains primacy in the allocation of resources following the immune insult. Such an allocation rule appears adaptive, given the potential lethal nature of unrestrained genuine bacterial infections, such as shown in house crickets (*Acheta domesticus*) exposed to *Serratia marcescens* (Adamo 1998). In addition to eliciting enhanced synthesis of lysozymelike molecules or hemocytes, LPS inoculation may also involve a "malaise syndrome" characterized by decreased feeding and growth, such as shown following peptidoglycan injection in larvae of the tobacco hornworm (*Manduca sexta*; Dunn et al. 1994). The decrease in singing may thus have been due to both an energy decline caused by this illness-induced anorexia and/or due to a cytokine-induced suppression (Dantzer 2001) that may occur regardless of the animal's energy stores. The latter hypothesis would specifically account for the similar reduction in calling rate in control and food-supplemented crickets.

Male longevity in field crickets was reduced by the immune insult, whereas food supplementation independently prolonged the life span. Two previous laboratory studies in insects similarly indicated survival costs to an induced immune response. Bumblebees (*Bombus terrestris*) showed a higher mortality over the 72 hours following immune system activation, yet under starved conditions only. This starvation paradigm appears of lesser importance to species such as field crickets, which face less extreme nutritional conditions, but unfortunately the longevity of nonstarved workers was not assessed (Moret and Schmid-Hempel 2000). Under ad libitum feeding conditions mealworm beetles (*Tenebrio molitor*) indeed showed a longevity cost to an induced encapsulation response (Armitage et al. 2003). Thus, in line with our findings in field crickets, immune system activation may even shorten the life span under nonstressed or improved nutritional conditions.

Interestingly, the reduction in longevity was not caused by an instant increase in mortality upon LPS inoculation. LPS is usually cleared from the insect hemolymph in the first few hours following injection (Kato et al. 1994), and immune-challenged males survived on average three to four weeks after the immune insult. Although we cannot exclude a retarded effect of metabolic expenses associated to a short-term immune response (Freitak et al. 2003), the reduced longevity more likely reflects costs associated with a systemic upregulation of immune function. Moret and Siva-Jothy (2003) recently showed that prechallenged larvae of mealworm beetles produced a long-lasting antimicrobial response, which functionally improved survival under exposure to a fungal infection. Adult male field crickets similarly showed an enduring elevation of immune function following nymphal LPS-inoculation but at the cost of a lasting decline in metabolic condition as indicated by their reduced hemolymphic protein load (Jacot et al. 2004). This long-term decline in metabolic condition might represent an important mechanism underlying the presently revealed reduction in male longevity (or even daily calling rate on the shorter term). Alternatively, upregulation of immune function might have increased the incidence of immunopathology (Zuk and Stoehr 2002; Schmid-Hempel 2003) and thereby reduced the life span.

In conclusion, we here showed under natural condition that immune system activation causes a decline in male fitness, resulting from the combined effect of a reduction in sexual display and reproductive life span. Given that parasites are an omnipresent natural selection pressure, the cost of immune defense on reproductive performance and survival thus plays a key role in the life history of animals. The evolutionary optimal investment in immunity will ultimately depend on variation in the risk and impact of parasitism, as well as on the fitness costs and benefits of immunological parasite resistance.

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