Juvenile immune status affects the expression of a sexually selected trait in field crickets

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Abstract

Parasite-mediated sexual selection theory presumes that variation in sexual traits reliably reflects variation in parasite resistance among available mates. One mechanism that may warrant signal honesty involves costs of immune system activation in the case of a parasitic infection. We investigated this hypothesis in male field crickets *Gryllus campestris*, whose attractiveness to females depends on characteristics of the sound-producing harp that are essentially fixed following adult eclosion. During the nymphal stage, males subjected to one of two feeding regimes were challenged with bacterial lipopolysaccharides (LPS) to investigate condition-dependent effects on harp development as compared to other adult traits. Nymphal nutritional status positively affected adult body size, condition, and harp size. However, nymphal immune status affected harp size only, with LPS-males having smaller harps than control-injected males. In addition, the harps of LPS-males showed a lesser degree of melanization, indicating an enhanced substrate use by the melanin-producing enzyme cascade of the immune system. Thus, past immune status is specifically mirrored in sexual traits, suggesting a key role for deployment costs of immunity in parasite-mediated sexual selection.

Introduction

Parasite-mediated sexual selection theory offers a functional explanation for the evolution of sexual advertisement by exaggerated secondary sexual characters or extravagant behavioural display (Hamilton & Zuk, 1982; Møller, 1990b; Zuk, 1992; Hamilton & Poulin, 1997). Presuming that sexual advertisement is costly, only individuals whose state is least impaired by the deleterious effects of parasitism could afford to invest highly in mate attraction. The mating preference for elaborate ornaments of the least-parasitized mates (Hill, 1990; Milinski & Bakker, 1990; Hill & Brawner, 1998) may enhance the fitness of the choosing sex by securing superior parental care (Korpimaki *et al.*, 1995), a reduced risk of sexually-transmitted diseases (Luong *et al.*, 2000), and/or parasite resistance genes for the offspring (Møller, 1990a; Barber *et al.*, 2001; Roulin *et al.*, 2001). In line with theoretical predictions, empirical studies have also shown that parasitic infection may reduce ornamentation (Milinski & Bakker, 1990; Møller, 1990a; Fitze & Richner, 2002).

Two major, nonmutually exclusive, mechanisms have been proposed to account for the reduced expression of sexual traits following parasitism. Firstly, the growth and proliferation of parasites deplete host resources, thereby negatively affecting the expression of condition-dependent sexual traits (Clayton & Moore, 1997). Secondly, parasites induce host defence mechanisms, such as the immune system. The nutrients and energy used to enhance immunity (Demas *et al.*, 1997; Ots *et al.*, 2001; Freitak *et al.*, 2003; Martin *et al.*, 2003) may similarly reduce host condition and thus ornament expression. To discriminate parasite-induced deployment costs of immunity, an experiment that employs an immune insult with a nonpathogenic antigen is required. Faivre *et al.* (2003), who recently performed such a study in male blackbirds *Turdus merula*, found rapid effects on the carotenoid-based bill coloration, which apparently plays a role in

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sexual selection. However, no study has investigated whether deployment costs of immunity act as a mediating factor that specifically reflects the cost of parasitism in sexually, as compared to nonsexually selected traits, such as assumed by parasite-mediated sexual selection.

We experimentally studied the long-term effects of immune system activation on the expression of a secondary sexual trait in male field crickets *Gryllus campestris*. Sexual advertisement in crickets is expressed via the calling song, which is mainly produced by the melanized harp in the modified forewings. Carrier frequency is the most important calling song component in mate choice, with females showing a preference for low carrier frequencies (Simmons & Ritchie, 1996; Scheuber *et al.*, 2004). Well-nourished nymphs produce the largest harps and thereby the lowest carrier frequencies (Scheuber *et al.*, 2003b), indicating condition during juvenile development as a key factor in sexual selection. Accordingly, parasite-induced juvenile immunity may ultimately reduce male sexual attractiveness, if resource allocation trade-offs with costly immune defence cause a reduction in harp size or structure.

We reared penultimate instar nymphs on one of two feeding regimes and investigated the consequences of a subsequent immune challenge with lipopolysaccharides (LPS) on nymphal development, adult morphology and calling song characteristics. LPS are immune elicitors that activate the humoral immune response without direct pathogenic effects (Söderhäll & Cerenius, 1998; Kimbrell & Beutler, 2001). We have shown elsewhere that this nymphal immune insult induces a long-term up-regulation of immunity (Jacot *et al.*, 2005). Here we investigated whether the costs of induced immunity are conditionally expressed in harp morphology and thereby carrier frequency.

Materials and methods

Male field crickets used in the present study were the first generation offspring of field-inseminated females. All females were caught on an uncut meadow near Hinterkappelen (Switzerland) in late June 2002, and transferred to a climate chamber for egg laying. Groups of 30 newly hatched nymphs were kept in plastic containers (l × w × h = 38 × 20 × 25 cm^3). Rearing containers contained sand substrate, egg cardboards for shelter, a water vial and a plastic tray with *ad libitum* food (i.e. fish chow, a 1 : 1 mixture of JBL Novovert and JBL Novogrand, JBL GmbH, Neuhausen, Germany). Temperature in the climate chamber was maintained at 26 °C and relative humidity at 65%. The light-dark cycle was 14 : 10 h with lights-on at 10 p.m. About 90 days after hatching, a total of 152 fourth or fifth instar male nymphs were housed individually in plastic jars (l × w × h = 13 × 9 × 18 cm^3) inside a second climate chamber under similar conditions as experienced during the first period of juvenile development.

The condition-dependent effects of immune system activation during the nymphal stage on remaining nymphal development and adult traits were investigated using a two-by-two experimental design with nymphal food treatment and nymphal immune treatment as main factors, each with two levels. The assignment of the nymphs to the experimental treatments was at random, controlling for variation in initial body size across treatment groups (food treatment: n.s.; immune treatment: n.s.; interaction term: n.s.). Nymphs were subjected to one of two feeding regimes, which started immediately upon individual housing and lasted up till adult eclosion. The daily mean food ratio of nymphs in the high-food and the low-food group was on average 0.2 and 0.015 g, respectively. We have previously shown that these food ratios reflect an *ad libitum* and restricted feeding (Scheuber *et al.*, 2003b). The food was refreshed every second or third day.

After 1 week of continuous food treatment, we elicited an immune response with an injection of lipopolysaccharides (LPS) derived from the Gram-negative bacteria *Serratia marcescens* (L6136, Sigma-Aldrich Chemie GmbH, Seelze, Germany). In this case, the immune system is activated while avoiding direct pathogenic effects from the parasite (Hoffmann *et al.*, 1996; Beutler, 2000). Prior to the injection we physically immobilized males in a small cage and cleaned the sternites with 70% EtOH. Males were injected ventrally between the third and fourth sternite using a 10 μL Hamilton syringe. Males in both food treatments were either injected with 10 μL Grace insect medium (sham-injected) or by injecting 10 μL of a Grace-based 0.1% LPS-solution (challenge). After injection, the male was immediately returned to his jar.

At adult eclosion all males were weighed and food was provided *ad libitum* to all individuals, thus restricting the manipulation of nutritional condition to the last nymphal instars only. All males were frozen 10 days after adult eclosion and structural traits were obtained at a later stage. Using image analysis software (NIH Image 1.61, National Institutes of Health, USA) we measured pronotum size, calculated as the product of length and width, and tibia length as indices of structural body size. Harp size was measured following Simmons (1995). Additionally, we assessed the degree of melanization of the right harp. We removed the whole forewing and with the aid of a stereomicroscope measured the mean grey value of the harp ranging from 0 (white) to 255 (black) with NIH Image analysis software. Body condition was determined as the residuals of the regression of body mass on body size.

On day seven after adult eclosion, we obtained a measure of calling activity by assessing calling rate over a period of 24 h. Daily calling rate was recorded using a sound sensor (DCPMicrosense Sound Level Sensor, DCP Microdevelopments, Great Ellingham, UK), which was placed in the lid of the housing container and

connected to a datalogger (LEGO Mindstorms RCX 1.0 programmable brick). At 5-minute intervals, the datalogger scored the sound intensity (in dB) inside the housing container. Due to the continuous chorus provided by other males, the background sound intensity reached peak values at 60 dB, while calls of resident males never dropped below 70 dB. Therefore, to reliably discriminate calling activity of individual males from the background chorus we only scored peaks above a threshold level of 65 dB. Daily calling rate was quantified as the proportion of time calling over a period of 24 h.

On day eight, we audio recorded the calling song to obtain a qualitative measure of calling behaviour. Recordings were done with a microphone (SONY ECM-16), which was located in the lid of the housing container and connected to a digital tape recorder (SONY TCD-D100). For data analysis, we digitized a 30-second sequence taken at least 2 min after the start of calling activity. Using Canary software (Charif *et al.*, 1995) we evaluated chirp rate (No. of chirps/s), chirp duration (ms), interchirp duration (ms), chirp intensity (fW/m^2) and carrier frequency (kHz) according to standard criteria (Scheuber *et al.*, 2003a). All eclosed males survived until day eight, on which the daily calling activity was recorded. Among the 112 eclosed adults, 15 males did not show any calling activity over the 24-h period, which was independent of the experimental treatments. Due to problems with the recording system, nine additional males had to be excluded from the analyses. Two immune-treated males with deformed forewings were also excluded. The analyis of calling song characters was based on the remaining 86 males.

Data analyses were performed with the JMP IN statistical package (Sall & Lehmann, 1996). Nymph survival until adult eclosion and calling probability were analysed with logistic regression using log-likelihood ratio tests (McCullagh & Nelder, 1989). To analyse the experimental variation in nymphal development and morphological traits, body mass, body condition and calling song characters of adults, we used general linear models with food and immune treatment as fixed factors. When analysing morphological characteristics of adult crickets and nymphal development we statistically controlled further for variation in initial nymphal weight, which also largely accounts for differences in development between nymphs. We used a stepwise backward procedure in all analyses and tested the significance of predictor variables in a hierarchical fashion, retaining lower order effect in the analyses in case a higher order term was significant.

Results

Effects on nymphal development

The food treatment had a positive effect on body mass gain during the first 7 days (repeated measures ANOVA:

Table 1 Measures (means ± SE) of nymphal development in relation to food availability and immune treatment. Δ weight indicates the weight difference between the 7th day of the experiment and adult molt.

Variable	*Ad libitum* food		Food-restricted	
	Ringer	LPS	Ringer	LPS
Survival probability (%)	87.5 ± 5.3	75.7 ± 7.2	65.8 ± 7.8	64.9 ± 8.0
Time to adult eclosion (days)	23.66 ± 2.06	23.07 ± 1.93	27.36 ± 2.41	21.71 ± 2.36
Δ weight (mg)	28.7 ± 28.0	0.9 ± 28.0	−15 ± 19.5	−62.8 ± 32.4

$F_{1,125} = 15.2$, $P < 0.001$) prior to the immune treatment. Both food treatment groups increased in body mass, but the increase among *ad libitum* fed nymphs was steeper than in food-restricted nymphs.

Among a total of 152 nymphs, 40 individuals did not reach the adult stage. Overall survival was 74%, with 66 and 88% of the males surviving in the food-restricted and *ad libitum* fed group, respectively (Table 1). The probability to reach adulthood was significantly affected by the food treatment ($\chi^2_1 = 5.18$, $P < 0.05$), while there was no additional effect of the immune treatment ($\chi^2_1 = 0.84$, n.s.; interaction term: $\chi^2_1 = 0.97$, n.s.) or initial weight ($\chi^2_1 = 2.06$, n.s.).

Controlling for initial weight ($F_{1,108} = 13.17$, $P < 0.001$), the immune treatment ($F_{1,107} = 1.25$, n.s.) had no effect on remaining nymphal period, while the relationship between initial weight and time until adult eclosion was affected by the food treatment (interaction term: food treatment × initial weight: $F_{1,106} = 6.02$, $P < 0.05$) (Table 1). The negative relationship between initial weight and remaining nymphal period was steeper in food-restricted males compared to males reared with *ad libitum* food. In addition, body mass change from the day of the immune challenge until adult eclosion was marginally affected by the food treatment ($F_{1,88} = 3.51$, $P = 0.06$) while there was no effect of the immune treatment ($F_{1,87} = 1.58$, n.s.; interaction term: $F_{1,86} = 0.12$, n.s.) (Table 1).

Effects on adult body condition and morphology

The food treatment had a positive effect on body mass ($F_{1,101} = 32.14$, $P < 0.001$) and body condition ($F_{1,99} = 9.09$, $P < 0.05$, Fig. 1) at adult eclosion, while controlling for variation in initial weight ($F_{1,101} = 67.64$, $P < 0.001$) and body condition ($F_{1,98} = 0.75$, n.s.), respectively. The immune treatment did not affect body mass ($F_{1,100} = 0.58$, n.s.; interaction term: $F_{1,99} = 0.44$, n.s.) or body condition ($F_{1,98} = 0.07$, n.s.; interaction term: $F_{1,97} = 1.50$, n.s., Fig. 1). Males with *ad libitum* food were heavier and in better condition compared to food-restricted males.
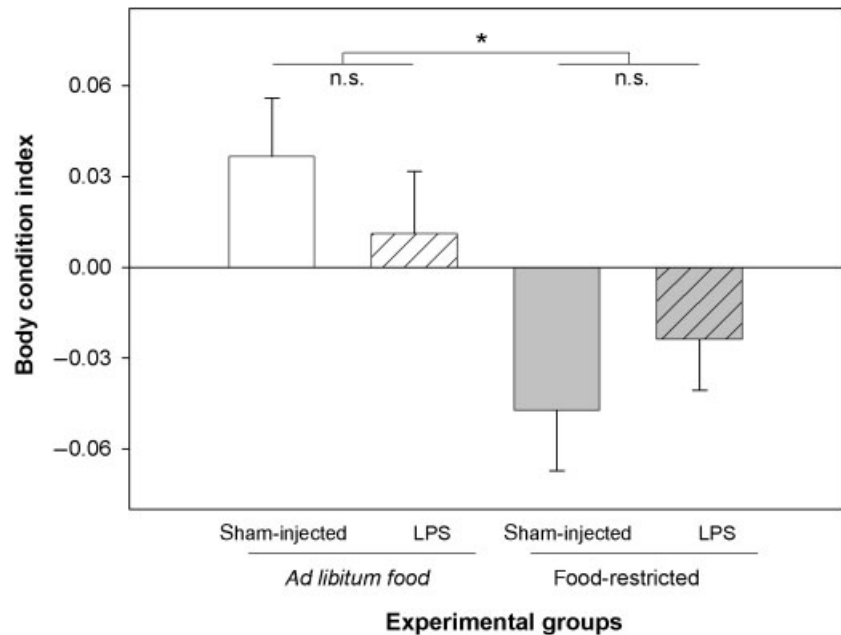
Fig. 1 Effect of food treatment and immune treatment on adult body condition (mean ± SE) measured on day of eclosion. *Ad libitum* fed males were in better body condition compared to food-restricted males, independent of immune treatment.

Pronotum size ($F_{1,109} = 22.26$, $P < 0.001$, Fig. 2a) and tibia length ($F_{1,109} = 17.66$, $P < 0.001$, Fig. 2b) were positively affected by the food treatment. The immune treatment did not affect the size of the pronotum ($F_{1,108} = 1.72$, n.s.; interaction term: $F_{1,108} = 0.01$, n.s.) or the length of the tibia ($F_{1,108} = 0.73$, n.s.; interaction term: $F_{1,107} = 1.00$, n.s.). All analyses were controlled for initial weight (pronotum: $F_{1,109} = 93.03$, $P < 0.001$; tibia: $F_{1,109} = 41.21$, $P < 0.001$).

Effects on harp size and melanization

Controlling for initial weight ($F_{1,107} = 32.92$, $P < 0.001$), harp size was affected by the food treatment ($F_{1,107} = 38.69$, $P < 0.001$) and by the immune treatment ($F_{1,107} = 11.69$, $P < 0.001$), while there was no interaction ($F_{1,106} = 0.23$, n.s.) (Fig. 3a). The harp was about 7–9 % larger in *ad libitum* fed crickets compared to food-restricted males. The immune challenge induced a 4–5% reduction of harp size.

We further analysed the allometric relationship between structural size and harp size, i.e. the size of the harp given body size. Food treatment ($F_{1,107} = 15.03$, $P < 0.001$) and immune treatment ($F_{1,107} = 11.26$, $P < 0.001$; interaction term: $F_{1,106} = 0.54$, n.s.) had a significant effect on relative harp size (Fig. 3b), independent of initial weight ($F_{1,106} = 1.81$, n.s.). Given body size, males reared under superior nutritional conditions had relatively larger harps, while immune challenged males had relatively smaller harps.

Melanization of the harp was significantly affected by the immune treatment ($F_{1,99} = 14.82$, $P < 0.001$), while controlling for initial weight ($F_{1,99} = 4.09$, $P < 0.05$) and harp size ($F_{1,97} = 0.11$, n.s.). Control males had signifi-

cantly darker harps compared to LPS-males (Fig. 4). Furthermore, the nymphal food treatment had no effect on harp melanization (food treatment: $F_{1,98} = 0.52$, n.s.; interaction term: $F_{1,97} = 0.45$, n.s.).

Effects on calling song

Daily calling rate was not affected by food treatment ($F_{1,106} = 0.05$, n.s.), or the immune treatment ($F_{1,106} = 0.04$, n.s.; interaction term: $F_{1,104} = 0.06$, n.s.).

The probability of recording a male's calling song for the analysis of calling song characters was independent of our experimental treatments (food treatment: $\chi^2_1 = 0.09$, n.s.; immune treatment: $\chi^2_1 = 0.01$, n.s.; interaction term: $\chi^2_1 = 0.003$, n.s.). Nymphal food treatment had a significant effect on peak frequency ($F_{1,84} = 4.13$, $P < 0.05$), independent of immune treatment ($F_{1,83} = 0.06$, n.s.; interaction term: $F_{1,82} = 2.20$, n.s.). Since harp size determines carrier frequency ($F_{1,82} = 10.86$, $P < 0.05$), we investigated whether the effect of the food treatment on carrier frequency is due to experimentally induced variation on harp size. The food treatment effect was no longer significant ($F_{1,81} = 0.35$, n.s.) when we included harp size as covariate in our analyses. All other calling song characters were unaffected by the experimental treatments (Table 2) or harp melanization (carrier frequency: $F_{1,78} = 0.016$, n.s.; chirp intensity: $F_{1,79} = 0.87$, n.s.).

Discussion

Parasite-mediated sexual selection implicitly assumes that parasitic infection reduces the expression of sexually selected traits, thereby allowing females to maximize
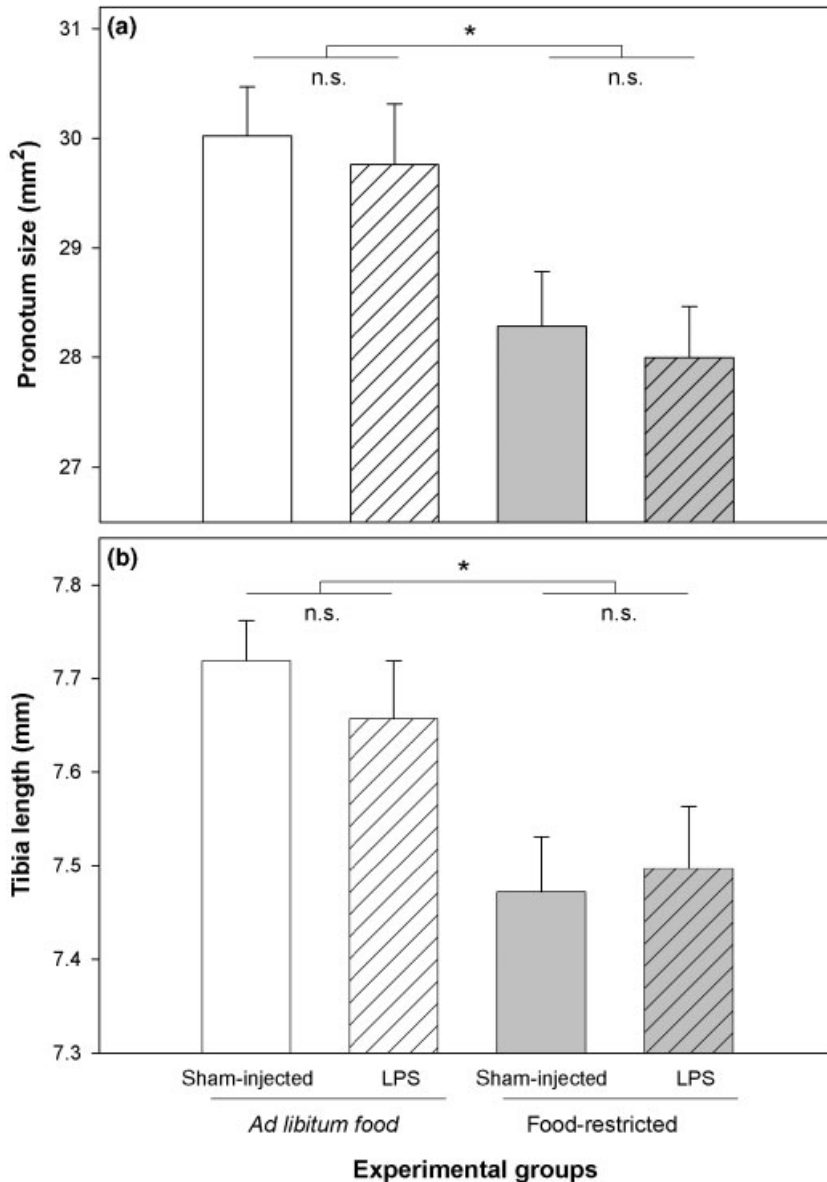
Fig. 2 Effect of food treatment and immune treatment on adult structural size. Adult pronotum size (mean ± SE) (a) and tibia length (mean ± SE) (b) were affected by food availability of juvenile crickets independent of immune treatment.

fitness by preferentially mating the least-parasitized males. By excluding direct detrimental effects, we revealed immune system activation as a key-mediating factor for the proposed costs of parasitism. Independent of nutritional condition, an immune insult on male field cricket nymphs specifically affected the morphology of the sound-producing harp, while the structural adult size was affected by the food treatment only. Since harp morphology is lastingly determined during the last nymphal stage and the final moult into adulthood, this suggests a specific resource allocation trade-off between immunity and sexual trait expression.

The effects of the food treatment and immune treatment on harp size were only partly reflected in the carrier frequency of the calling song. As in the investigation by Scheuber and colleagues (2003b) of the sole effect of nymphal nutrition on sexually selected traits, food-restricted males showed smaller harp sizes and calling songs of higher carrier frequency as adults than males with *ad libitum* food supply. This may translate into lower sexual attractiveness, since females show a directional preference for lower carrier frequencies (Scheuber *et al.*, 2004). Although we also found an overall negative correlation between harp size and carrier frequency, the negative effect of immune system activation on harp size was statistically not reflected in the carrier frequency. We presume that this discrepancy is at least partly caused by the lower power to detect a treatment effect in carrier
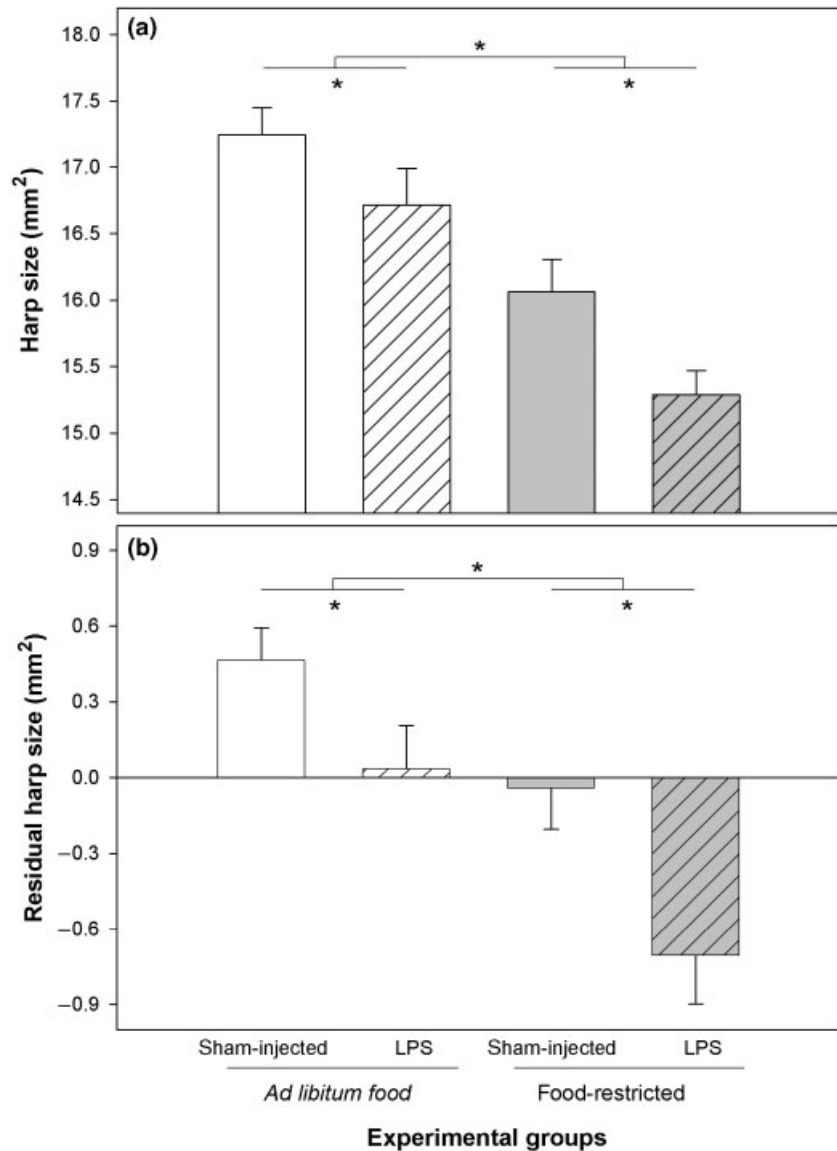
Fig. 3 Adult harp size (mean ± SE) (a) and residual harp size (mean ± SE) controlled for adult pronotum size (b) were affected by food and immune treatment of juvenile crickets.

frequency, since the calling song could only be recorded for a subset of individuals. Therefore we focused on treatment effects on harp morphology.

Harp size and the degree of melanization of the harp were both reduced in previously immune challenged individuals. Energy resources or nutrients used for the melanization and sclerotisation process of the harp are most probably limited in immune treated males. The development of harp size and investment in melanization might ultimately be based on the availability of the substrate for the prophenoloxidase (proPO)-cascade, and it is here where the costs for any trade-off may lie. The proPO-cascade is a key pathway for innate immune responses in invertebrates (for review see Söderhäll & Cerenius, 1998). Besides the importance

in melanotic encapsulation and nodule formation, melanin synthesis is also relevant in cuticular sclerotisation and wing pigmentation. In damselflies, wing spot size correlates with measures of immunity (Rantala et al., 2000) and the ability to resist a parasite (Siva-Jothy, 2000). The positive phenotypic correlation between wing spot size and immunocompetence measurements among individuals suggests that melanin-based sexual traits in invertebrates may play a key role in parasite-mediated sexual selection (Siva-Jothy, 1999). Assuming that melanized sexual traits and the immune system are competing for the same nutrients, we expect physiological trade-offs between the melanin-producing enzyme cascade of the immune system and the expression of secondary sexual traits. In this paper
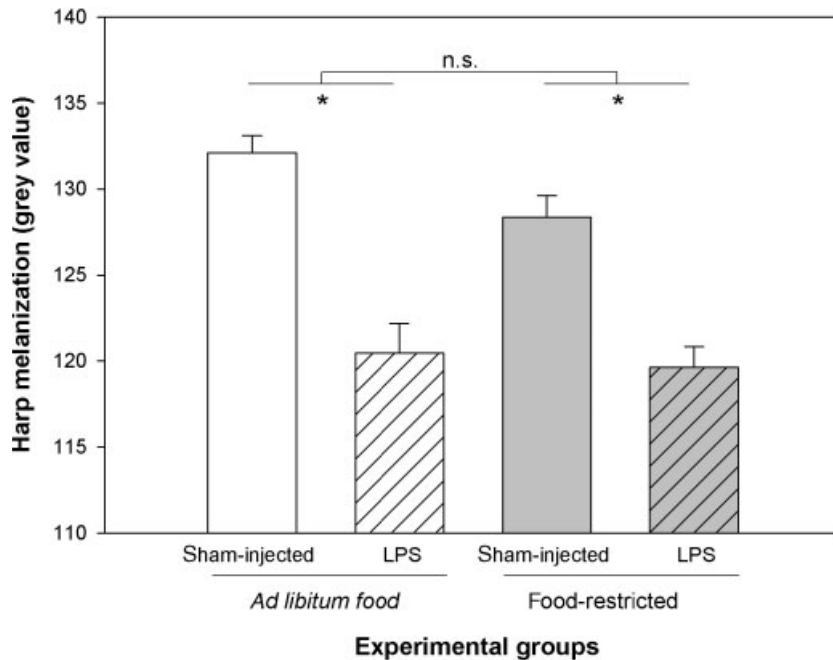
Fig. 4 Effect of food treatment and immune treatment on harp melanization (mean ± SE). LPS-males had less melanized wings than sham-injected males, independent of food treatment.

Table 2 Means (±SE) and test statistics for daily calling rate and calling song characters in relation to nymphal food and immune treatment.

| Variable | *Ad libitum* food | | Food-restricted | | Statistics | | |
	Sham-injected	LPS	Sham-injected	LPS	Food	Immune	Food × Immune
Daily calling rate (%)	20.17 ± 2.80	20.29 ± 3.27	20.24 ± 2.74	18.83 ± 3.10	$P = 0.83$	$P = 0.81$	$P = 0.85$
Carrier frequency (kHz)	4.86 ± 0.04	4.81 ± 0.03	4.88 ± 0.05	4.96 ± 0.04	$P = 0.05$	$P = 0.81$	$P = 0.14$
Chirp rate (chirps/s)	34.4 ± 0.87	35 ± 1.27	36.5 ± 1.28	36 ± 0.92	$P = 0.16$	$P = 0.90$	$P = 0.62$
Chirp duration (ms)	109.4 ± 2.7	105.1 ± 2.8	102.7 ± 2.8	102.9 ± 2.5	$P = 0.10$	$P = 0.40$	$P = 0.41$
Interchirp duration (ms)	184.7 ± 8.2	192.1 ± 17.3	177.5 ± 11.3	177.3 ± 7.8	$P = 0.69$	$P = 0.59$	$P = 0.64$
Chirp intensity (fW/m^2)	29.8 ± 3.7	30.4 ± 3.2	30.3 ± 4	26.7 ± 2.9	$P = 0.67$	$P = 0.70$	$P = 0.56$

we demonstrate that immune system activation causes a reduction in melanization of a sexual trait and thereby is an indicator of juvenile health status.

In the long term, harp melanization may affect a male's sexual attractiveness via different sexually selected routes. The degree of melanization is a major determinant of wing sturdiness, although unpigmented sclerotization may additionally reduce its wear-and-tear (Cohen, 1991). Singing field crickets stridulate their modified forewings for several hours every day and the abrasion of the sclerotised toothed file and/or the scraper in the harp may lastingly alter song characteristics and thereby sexual attractiveness (Hartley & Stephen, 1989; Ritchie *et al.*, 1995). In line with this hypothesis, melanin content of bird feathers influences their durability (Bergman, 1982; Bonser, 1995), which has important implications for flight performance and female mate choice (Merilä & Hemberg, 2000). Secondly, darker, more strongly melanized wings absorb more sunlight and facilitate thermoregulation, especially during cold tem-

peratures. Inter- and intraspecific variation in basking behaviour in *Colias* butterflies is mainly due to wing pigmentation (Ellers & Boggs, 2003). Male crickets with darker harps may benefit from an elevated body temperature under adverse weather conditions, which may enhance calling song quality and quantity (Doherty, 1985; Hedrick *et al.*, 2002; Martin *et al.*, 2000) and thereby sexual attractiveness.

To conclude, adult harp morphology is an integration of a variety of selection pressures that juvenile crickets experience during their nymphal period. While harp melanization was solely affected by the immune treatment, harp size was altered by nymphal food availability and immune status in an additional way. Under natural field conditions, parasitism often reduces body condition (Moore, 2002), either directly or via resource allocation trade-offs. Together with the costs of an immune response, this will increase the difference in scaling relationship towards smaller wings at any given body size and therefore reduce male attractiveness and fitness.

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