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## Original Article

# Reproduction and immunity trade-offs constrain mating signals and nuptial gift size in a bushcricket

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Reproduction and immunity often require significant energetic investment and as a result, trade-offs may occur between them. Reproduction is particularly costly for males of the bushcricket *Ephippiger diurnus*: not only do they produce loud advertisement calls for long periods of time to attract mates but also they offer females a large nuptial gift, a spermatophore, that can be up to 40% of their body weight. Song traits and spermatophore size vary among males, implying that males may differ in their overall reproductive effort or in how their effort is allocated. Males are long-lived, suggesting that survival traits such as immunity may be particularly critical in fitness. Here, we tested the hypothesis that trade-offs exist between reproductive effort and immune response in *E. diurnus* and that such trade-offs may constrain male mating calls and nuptial gift size. We investigated the relationship between call syllable number, spermatophore size, and immune response in field-collected individuals. We found an inverse relationship in all pairwise comparisons of call syllable number, spermatophore size, and immune response, as well as between overall reproductive effort and immune response. Our results suggest that males exhibit different energy allocation strategies and that their trade-offs ultimately constrain signal evolution.

**Key words:** bushcricket, immune response, nuptial gift, signal evolution, trade-offs.

## INTRODUCTION

Life-history theory predicts that individuals may increase their fitness by selectively allocating resources to different reproductive and survival traits. For example, sexually selected traits tend to be energetically costly, but individuals that invest in and develop exaggerated traits may enjoy increased reproductive success (Andersson 1994). However, because individuals have a limited energy budget, they may be constrained in how much energy they can allocate to sexually selected versus naturally selected traits such as growth and defensive function (Roff 1992). Therefore, in situations where energy is in short supply, trade-offs between different sexually selected and naturally selected traits are predicted. For example, if an individual is young and can potentially reproduce multiple times throughout its life, one would predict higher investment in naturally selected traits that would increase survivorship. On the other hand, older individuals may be expected to allocate their entire energy budget to reproduction, a resource-allocation pattern known as terminal investment (Roff 1992). In general, trade-offs between survival and reproduction play an important role in determining an individual's fitness.

Among survival traits, the role of immune function has received much attention because it is energetically costly (see Rolff and Siva-Jothy 2003 on the energetics of immunity). Given that exaggerated sexual traits and an effective immune system are both costly to develop and maintain, the relationship between the two has long interested evolutionary biologists (Lawniczak et al. 2007). Initially, it was assumed that only individuals in particularly good "condition" would have the ability to invest in both a strong immune system and exaggerated sexual ornaments, which led to the proposition that sexual ornaments might function as a reliable signal of the quality of a male's immune system (Hamilton and Zuk 1982). This immunocompetence handicap hypothesis has found some empirical support, particularly in insects (Ryder 2000; Rantala et al. 2002; Rantala and Kortet 2003; Simmons et al. 2005; Pomfret and Knell 2006; Tregenza et al. 2006; Fedorka and Mousseau 2007). More recently, other studies have focused on an alternative possibility that trade-offs occur between immunity and sexually selected traits (Sheldon and Verhulst 1996; Zuk and Stoehr 2002), and some authors have found evidence for such trade-offs (Siva-Jothy 2000; McKean and Nunney 2001; Fedorka et al. 2004; Ahtiainen et al. 2005; Kerr et al. 2010; Simmons 2012; Reavey et al. 2014).

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Selective resource allocation is expected to occur not only between naturally and sexually selected traits but also among different reproductive traits, for example, between mating effort and parental effort or between precopulatory and postcopulatory reproductive effort (Peters 2002; McGlothlin et al. 2007). From a male's perspective, mating effort consists of traits such as advertisement signals, ornaments, or weapons used in disputes with other males, any one of which can increase the number of mates he obtains (Alexander and Borgia 1979). Parental effort, on the other hand, consists of resources or energy that the male donates to the female in order to increase the number or quality of his offspring from a given mating (Trivers 1972), such as by providing the female with nutrition that will be passed on to the offspring (Quinn and Sakaluk 1986). Life-history theory also predicts strategic resource allocation between mating and parental effort, and the optimal strategy may depend on various conditions such as the operational sex ratio and the availability of potential mates. For example, in populations where the risk of sperm competition is high, males should benefit from allocating more resources to mating effort (Wedell et al. 2002). On the other hand, when male–female encounter rates are low and males are unlikely to mate more than once, they should benefit from increased parental effort (Simmons 1995).

Trade-offs are usually studied by examining the relationship between 2 traits, but in many cases, multiple traits may interact (Roff and Fairbairn 2007). For example, separate inverse relationships may exist between a survival trait such as immune function and the several components of reproduction. Moreover, an inverse relationship may occur between these reproductive traits, and this interaction itself might influence the overall relationship between reproduction and immunity. By examining trade-offs between 2 traits only, one risks an incomplete understanding of the range of adjustments that an animal can make when encountering different environmental and developmental circumstances. Specifically, if there is in fact a relationship between 3 traits but one of them is omitted from the analysis, we may underestimate the extent to which adjustments in those 2 remaining traits are constrained (Pease and Bull 1988; Roff and Fairbairn 2007).

Here, we studied the potential trade-offs between 3 important and energetically costly fitness-related traits in the bushcricket *Ephippiger diurnus* (Orthoptera: Tettigoniidae: Bradyporinae), an acoustically communicating species found in the Mediterranean region of southern Europe. Previous studies have documented that both precopulatory (Busnel et al. 1956) and postcopulatory reproductive effort (Busnel and Dumortier 1954; Duijm et al. 1983) are conspicuous in males: they broadcast an intense advertisement song and transfer a very large spermatophore, a nuptial gift, to the female. Song and nuptial gift production are energetically expensive, and they account for a considerable portion of a male's reproductive effort in this species: males do not actively search for mates, defend territories or engage in combat, and calling appears to be the only behavior they perform to encounter mates. They also do not provide their mate or offspring with any care or resources other than the nuptial gift. Moreover, *E. diurnus* males have an extended adult longevity during which they can mate multiple times (Jarrige et al. 2013; Barbosa F, Rebar D, Greenfield M, personal communication), suggesting that an effective immune function is likely to be a major component of survival effort. Thus, *E. diurnus* presented a unique opportunity to investigate trade-offs between 3 traits that are likely to be under strong selection and therefore represent key elements of life-history strategy. Because we could measure these

traits under controlled laboratory conditions, we were also able to gain a perspective on intrinsic variation in the trade-offs that individual males engage in.

Our study investigated the specific relationships between 1) nuptial gift size and call syllable number; 2) nuptial gift size and immune response; and 3) call syllable number and immune response. We predicted negative relationships between these traits and, in particular, that such trade-offs involving the immune response may constrain male mating signals and nuptial gift size. We also examined the overall interaction between these 3 traits, testing the hypothesis that there is a general trade-off between reproduction and survival in *E. diurnus*. Our findings offer a rare analysis of the several factors that may influence signal evolution in acoustic animal species.

## METHODS

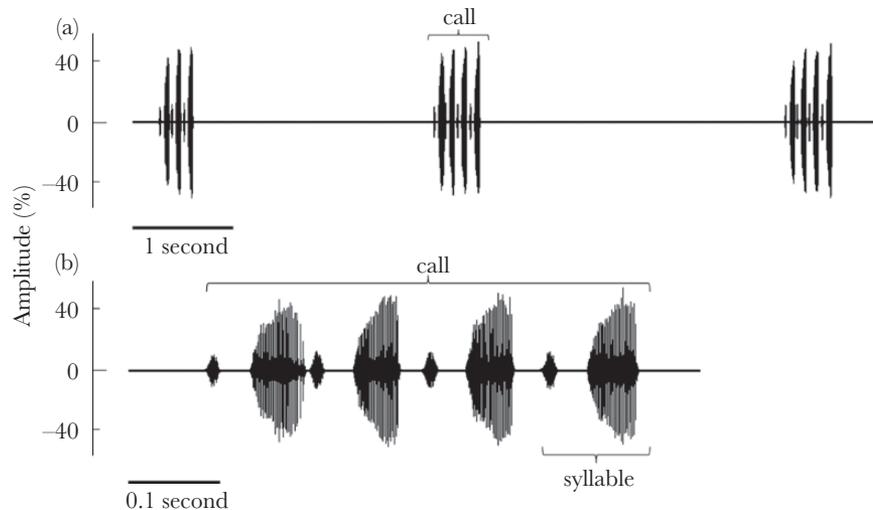
### Study species

*Ephippiger diurnus* is a flightless bushcricket distributed in small, isolated populations throughout southern France and northeast Spain (Spooner and Ritchie 2006). Males broadcast an advertisement song for many hours each day that consists of intense (90 dB at 1 m; Greenfield et al. 2004), rhythmically repeated calls (Figure 1; Busnel et al. 1955, 1956). The general energetic expense that acoustic insects incur when producing regularly repeated calls (Prestwich 1994; Reinhold et al. 1998; Gerhardt and Huber 2002) suggests that signal production may be limited by a male's energy budget. Males also produce and transfer an unusually large nuptial gift to females during copulation: a spermatophore weighing up to 40% of their body mass (Busnel and Dumortier 1954; Duijm et al. 1983). The spermatophore consists of a sperm-filled ampulla surrounded by a large spermatophylax, which is composed mostly of protein and water (Gwynne 2001), and is consumed by the female after mating is completed (Busnel and Dumortier 1954; Wedell 1994). Mating appears to be energetically costly for males: after transferring a spermatophore, they undergo a refractory period of 48–72 h during which they neither sing nor pair with females (Busnel et al. 1956; Wedell 1993, 1994). Evidence from comparative studies suggests that spermatophores function as sperm protection devices in *E. diurnus*: females take longer to consume larger spermatophylaxes, which results in longer ampulla attachment periods and consequently the transfer of more ejaculate (Wedell 1991). That is, males may enjoy higher fertilization success by virtue of transferring more sperm and by increasing the female's remating interval (Wedell 1993, 1994).

The various *E. diurnus* populations differ in several morphological and behavioral traits, most notably the number of syllables in male advertisement calls and nuptial gift size. We studied a population found at an elevation of 1700 m on the Col de Mantet, Department Pyrénées Orientales, France (42°28'N, 2°18'E). Males in this population produce a mean of 4.3 syllables per call (see Figure 1), and nuptial gifts average 33% of their body weight. Both of these values are higher than those found in most other *E. diurnus* populations (Duijm 1989).

### Collection and rearing

Study animals were collected in August 2013 and 2014 as either last-instar nymphs or recently emerged adults ( $N = 23$  males and 22 females in 2013, 15 males and 15 females in 2014) and brought to the laboratory where they were kept in climate-controlled chambers maintained at 25 °C and an L:D 16:8 h photoperiod. We kept



**Figure 1**

Oscillogram of (a) a series of 3 advertisement calls of an *Ephippiger diurnus* male from the Col de Mantet population and (b) a detailed view of a 4-syllable call, showing the syllable structure. The short and long components of a syllable correspond to the opening and closing of a male's forewings, respectively.

males and females in separate chambers to limit female exposure to male calls. Animals were kept in individual plastic containers (146-mm height, 117-mm diameter) and fed cabbage, pollen, and fish flakes ad libitum. We misted individuals with water daily.

### Advertisement song

Males were recorded with an electret condenser microphone (LinearX M51, LinearX Systems, Tualatin, OR; corrected frequency response =  $\pm 1$  dB 10 Hz to 40 kHz) while held in plastic cages with acoustically transparent mesh covers in a laboratory room at 25 °C. We separated males by placing 12 cm of 25 kg/m<sup>3</sup> acoustic insulation foam between each male (Flexolan, Dierdorf, Germany), which attenuated the calls of neighbors by 25 dB. Although they could still hear other males, this dampening ensured that males did not adjust their own call rate to alternate calls with another male. At the same time, our test males perceived a background chorus that influenced them to begin and sustain singing. We recorded each male for three 60-s intervals distributed throughout the morning in an attempt to capture a male's call variation. We later analyzed the recorded calls using digital sound processing software (Adobe Audition 3.0, Adobe Systems Incorporated, San Jose, CA). We counted the total number of calls given and the number of syllables per call and then calculated each male's calling rate (calls/minute) and average number of syllables per call (Figure 1). We then randomly chose 3 calls from each recording to measure peak frequency (Hertz) and syllable period.

Although several song traits may affect the energetic costs associated with a male's signal, we focused on syllable number because females in the Col de Mantet population that we studied, as well as in most other populations (Ritchie 1996; Party et al. 2014), consistently prefer a higher syllable number than the mean value. Females also prefer a faster than average call rate in some *E. diurnus* populations, but this preference was not found at Col de Mantet (Party et al. 2014). Moreover, call rate is difficult to measure systematically in this species because males often do not call regularly when alone. On the other hand, the number of syllables per call is affected little by the presence of other calling males.

Producing songs with a higher syllable number per call may not be more energetically expensive if males then sing at a slower rate.

To investigate whether this is the case, we examined the relationship between call syllable number and call rate, as well as between call syllable number and "call investment," for those males for whom we were able to measure rate. We estimated male call investment as the total number of syllables produced per minute, and we assumed that it is proportional to the energy expended on advertisement (Hoback and Wagner 1997). Finally, we calculated the repeatability of call syllable number to estimate the upper limit of its heritability and determine whether it may be subject to sexual selection pressure. Repeatability of syllable number was equated with the intraclass correlation coefficient (Lessells and Boag 1987), calculated as  $S_A^2 / (S^2 + S_A^2)$ , where  $S^2$  is the within-group variance component (error mean square [MS]),  $S_A^2$  is the among-group variance component (group MS – error MS)/ $n$ , and  $n$  is the number of repeated measures of syllable number per male. We used 10 calls from each male.

### Nuptial gifts

We paired each male with a randomly chosen female in order to facilitate a mating and to then measure his spermatophore if one was transferred. To avoid disrupting the mating process and interfering with spermatophore production, we measured spermatophore size indirectly. We weighed males and females just prior to pairing them in a plastic container with mesh walls and a perch. Pairs remained together until they mated, after which we immediately separated them and weighed each individual. We estimated the size of the spermatophores by calculating the weight difference before and after mating for both males and females, and averaged the two.

### Immune response

We conducted an encapsulation assay to measure a male's ability to detect and neutralize a foreign object inserted into his body cavity (Gillespie et al. 1997; Cerenius and Söderhäll 2004). Encapsulation assays consist of measuring the darkness of an implant inserted into an individual's body for a period of time: the higher an individual's encapsulation response, the darker the implant will become due to the attached melanized hemocytes (Fedorka et al. 2004; Rantala and Kortet 2004; Baer et al. 2006; Bailey and Zuk 2008;

Bailey et al. 2011). We performed encapsulation assays on males the day after their second mating, following standard protocols for Orthoptera (Bailey et al. 2008, 2011). We used a sterile needle to make a small hole between the second and third abdominal segments on the ventral side of a male. In this hole, we inserted a sterile implant consisting of a 3-mm length of monofilament (0.4-mm diameter) that had been abraded with fine sandpaper to facilitate hemocyte adhesion. We sacrificed the males 24 h later by freezing them. We then removed the implants and photographed them to measure darkness. Implants were photographed over a white background and under standardized light conditions using a Leica IC80HD camera mounted on a Leica MZ6 microscope under  $\times 2.5$ . Camera and microscope settings were kept constant across all photographs. We used Image J software (Rasband 2014) to quantify implant melanization. The software calculates a mean value for the darkness of the implant, ranging from 0 (darkest) to 255 (lightest). To make the results easier to interpret, we calculated male implant darkness score by subtracting 255 from the mean darkness value calculated by the software, and multiplied the result by  $-1$ . Therefore, a higher implant darkness score corresponds to a stronger immune response.

### Trade-offs

In 2013, we recorded calls from 23 males at approximately 12 days past the adult molt and then measured their spermatophores within 48 h after their singing was recorded. Thus, we determined the relationship between male call syllable number (precopulatory reproductive effort) and spermatophore size (postcopulatory reproductive effort). After a minimum of 10 days had passed following a male's first mating, we paired him again with a randomly chosen female, except that no male was paired with the same female as in his first mating. We determined the weight of the spermatophore that each male transferred during his second mating and performed the encapsulation assay 24 h after the spermatophore transfer. Here, we determined the relationship between spermatophore weight and immune response level (potential survival). Fifteen of 23 males mated and transferred a spermatophore in this second session. Owing to logistical constraints, we were unable to record advertisement songs from the males during the relevant time interval in the test of nuptial gifts and immune response or to measure immune response in the test of nuptial gifts and advertisement song.

We collected additional insects in 2014 and repeated these methods, to verify if the pattern we observed in 2013 would be repeated in a different year. Additionally, we wanted to explore the relationship between song and immune response, which we were unable to do with the 2013 individuals. Therefore, we performed the test of immune response and advertisement song on the males collected in 2014. We applied the procedures described above, pairing males for a second mating after having recorded their song during the preceding 3 days. We then measured the level of the male's immune response and evaluated the relationship between a male's call syllable number (mating effort) and the level of his encapsulation response (potential survival). All 3 bivariate relationships were evaluated by calculating the Pearson product-moment correlation. To account for a potential effect of male body size on the measured traits, we also examined all 3 bivariate relationships by conducting 3 analysis of covariances (ANCOVAs; JMP 7; SAS Institute Inc., Cary, NC) using male size as a covariate.

We examined the relationship between spermatophore weight and song, as well as spermatophore size and immune response, in the 2014 individuals, and compared these results with those

obtained in 2013. To verify whether the same pattern occurred in both years for the 2 relationships, we conducted 2 ANCOVAs (JMP 7; SAS Institute Inc., Cary, NC). In one, we used year as a fixed effect, spermatophore size as a covariate, and call syllable number as the dependent variable. In the second, we used year as a fixed effect, spermatophore size as a covariate, and immune response as the dependent variable.

Previous work demonstrated that in some circumstances, female weight can influence spermatophore size in *E. diurnus*: older males transfer larger spermatophores to larger females in their second mating, but female weight has no effect on spermatophore size on a male's first mating or on young males (Jarrige et al. 2013). We verified if female weight influenced spermatophore size in our study by calculating the Pearson product-moment correlation between female weight and relative spermatophore size for first and second copulations.

We were also interested in determining the relationship between overall reproductive effort and survival effort, a comparison that would require that all 3 traits be measured in the same individuals. Thus, when we evaluated the relationship between song and immune response in the insects collected in 2014, we also determined spermatophore weight. We analyzed the potential influence of overall reproductive effort on survival effort via a generalized linear model (GLM; R Development Core Team 2014) using call syllable number and relative spermatophore weight as predictors of immune response.

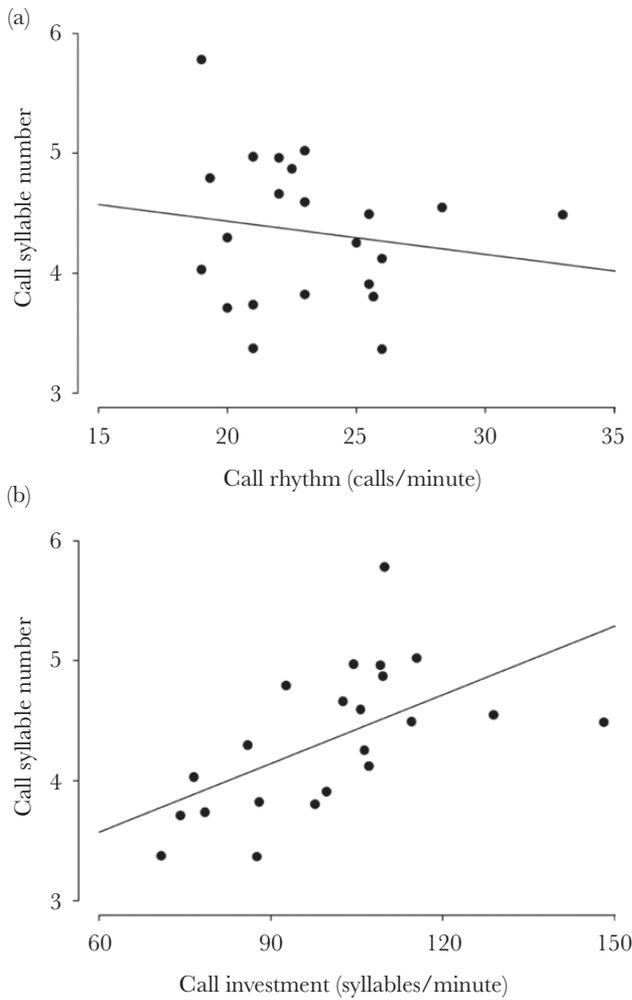
## RESULTS

### Reproductive traits

Calls from males collected in 2013 had an average of  $4.32 \pm 0.09$  syllables (mean  $\pm$  standard deviation [SD]). We were able to measure the call rate of 16 males, and they sang with an average rate of  $23.2 \pm 1.4$  calls/minute. We found no relationship between call rate and call syllable number ( $r^2 = -0.02$ ,  $P = 0.48$ ) (Figure 2a), implying that the relationship between call investment and syllable number was positive ( $r^2 = 0.34$ ,  $P < 0.01$ ) (Figure 2b). Call syllable number was highly repeatable at 0.71, with calls ranging from 3 to 7 syllables ( $F_{(22,217)} = 24.5$ ,  $P < 0.0001$ ). Males collected in 2014 sang with a mean rate of  $21.87 \pm 2.53$  calls/minute ( $n = 8$ ) and broadcast an average of  $5.03 \pm 0.36$  syllables per call (mean  $\pm$  SD). Males produced nuptial gifts corresponding to  $33.96 \pm 4.1\%$  and  $33.59 \pm 3.3\%$  of their body weight (mean  $\pm$  SD) in 2013 and 2014, respectively.

### Trade-offs

We found an inverse relationship in all 3 pairwise interactions investigated: call syllable number, nuptial gift size, and immune response. The pattern was consistent in the data from 2013 and 2014 (Figure 3). There was a negative relationship between call syllable number and nuptial gift size (measured as the percentage of body mass a male donated as a spermatophore) in 2013 and 2014 (Pearson product-moment correlation,  $n = 23$ ,  $r = -0.33$ ,  $P < 0.01$  in 2013;  $n = 15$ ,  $r^2 = -0.23$ ,  $P = 0.08$  in 2014). There was also a negative relationship between immune response strength and nuptial gift size in 2013 and 2014 (Pearson product-moment correlation,  $n = 15$ ,  $r^2 = -0.51$ ,  $P < 0.01$  in 2013;  $n = 15$ ,  $r^2 = -0.53$ ,  $P < 0.01$  in 2014). Finally, we found a negative relationship between immune response strength and call syllable number in 2014 (Pearson product-moment correlation,  $n = 15$ ,  $r^2 = -0.41$ ,



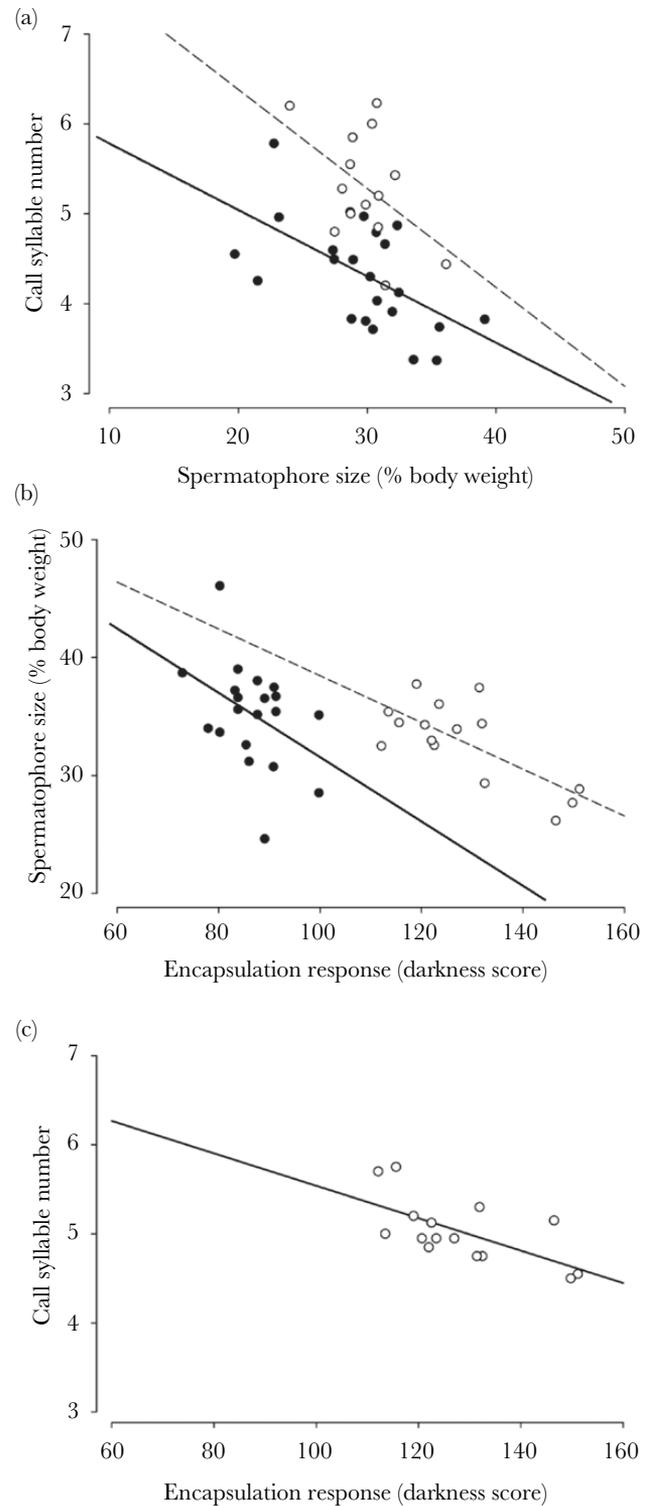
**Figure 2**

(a) Relationship between call syllable number and call rate (measured as calls/minute). Pearson product-moment correlation,  $n = 16$ ,  $r^2 = -0.02$ ,  $P = 0.48$ . (b) Relationship between call syllable number and call investment (measured as syllables/minute). Pearson product-moment correlation,  $n = 16$ ,  $r^2 = 0.34$ ,  $P < 0.01$ .

$P < 0.05$ ). ANCOVA did not detect an effect of male size in any of the relationships investigated (Table 1). We also did not detect an effect of female weight on relative spermatophore size, neither for first nor for second copulations (Pearson product-moment correlation,  $n = 36$ ,  $r^2 = 0.02$ ,  $P = 0.35$  for first copulation;  $n = 28$ ,  $r^2 = 0.03$ ,  $P = 0.33$  for second copulation).

ANCOVA did not detect significant differences between the 2013 and 2014 least squares linear regression models for call syllable number versus nuptial gift size ( $F_{(1,33)} = 0.38$ ,  $P = 0.54$  for the year  $\times$  nuptial gift size interaction term) or for nuptial gift size versus immune response ( $F_{(1,25)} = 0.52$ ,  $P = 0.48$  for the year  $\times$  nuptial gift size interaction term). These results indicate the existence of trade-offs between immune response and call syllable number, between immune response and nuptial gift, and between calling and nuptial gift in *E. diurnus*, and that these trade-offs are not likely to be spurious.

Does a trade-off also exist between the immune response and overall reproductive effort, as represented by call syllable number and nuptial gift size? The GLM fitted to our data indicated that both relative spermatophore size and call syllable number account



**Figure 3**

Pairwise trade-offs between call syllable number, nuptial gift size, and immune response. Axes reflect the phenotypic range of sampled males for each trait. (a) Relationship between call syllable number and nuptial gift size (measured as the percentage of body mass a male donated as a spermatophore) in 2013 (solid circles, solid line) and 2014 (open circles, dashed line). (b) Relationship between immune response strength and nuptial gift size in 2013 (solid circles, solid line) and 2014 (open circles, dashed line). (c) Relationship between immune response strength and call syllable number in 2014.

**Table 1**  
ANCOVA on spermatophore size and immune response from the 2013 and 2014 individuals

Dependent variable	Source of variation	df	F	P
Spermatophore size (2013)	Syllable number	1	6.3810	<b>0.02</b>
	Male weight	1	0.0137	0.91
	Syllable number × male weight	1	0.0313	0.86
Spermatophore size (2014)	Syllable number	1	3.3395	0.09
	Male weight	1	3.0244	0.11
	Syllable number × male weight	1	0.8082	0.39
Immune response (2013)	Spermatophore size	1	13.5399	<b>&lt;0.005</b>
	Male weight	1	1.4225	0.26
	Spermatophore size × male weight	1	0.0039	0.95
Immune response (2014)	Spermatophore size	1	12.3602	<b>&lt;0.005</b>
	Male weight	1	0.9795	0.34
	Spermatophore size × male weight	1	5.1868	0.43
Immune response (2014)	Syllable number	1	6.6470	<b>0.02</b>
	Male weight	1	1.4757	0.25
	Syllable number × male weight	1	0.0177	0.89

Significant values are in bold ( $P < 0.05$ ). df = degrees of freedom.

**Table 2**  
GLM of immune response with call syllable number and relative spermatophore size as predictor variables

	Estimate	Standard error	Chi square	P
Intercept	6.117	0.321	352.054	<0.0001
Call syllable number	-0.179	0.053	11.648	<0.001
Relative spermatophore size	-1.092	0.541	4.041	0.044

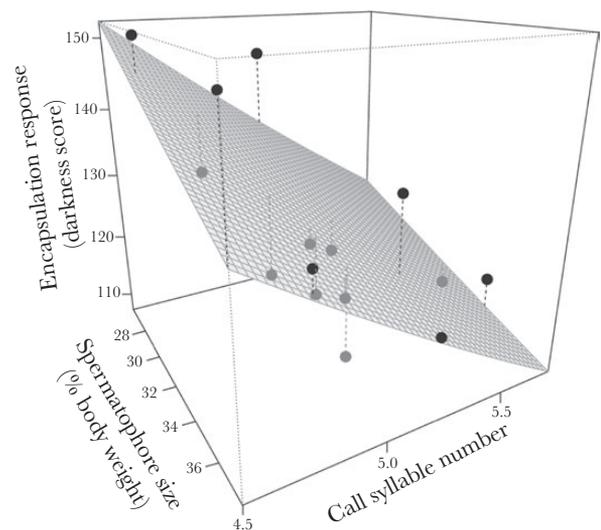
The model is depicted graphically in Figure 4.

for a significant proportion of variation in male immune response (Table 2, Figure 4). Each individual predictor had a significant effect on immune response, with syllable number being stronger. These results demonstrate that a general trade-off between reproduction and potential survival is present in *E. diurnus*.

## DISCUSSION

Our results support the hypothesis that trade-offs exist between reproduction and survival in *E. diurnus* males. Specifically, we document a consistent pattern of trade-offs between precopulatory and postcopulatory reproductive efforts and immune response in both 2013 and 2014. These trade-offs underlie a more general relationship between naturally and sexually selected traits: individuals invest in either immunity or overall reproductive effort. Individuals also vary in how they allocate their reproductive effort, as a trade-off is consistently observed between the 2 components of reproductive effort.

Previously, various studies have explored the relationship between immunity and sexually selected traits. Some of those studies focused on arthropods because their immune response is relatively less complex but nonetheless costly to activate and maintain. Several of these studies have shown that male song may function as an indicator of immunocompetence in orthopterans (Ryder 2000; Rantala and Kortet 2003; Simmons et al. 2005; Fedorka and Mousseau 2007), whereas others have documented trade-offs between immunity and either parental or mating effort (McKean and Nunney 2001; Leman et al. 2009; Kerr et al. 2010; Simmons 2012). Here, we continue beyond these efforts and reveal a complex interaction between all 3 traits. The trade-offs we report are consistent with findings in previous studies in other tettigoniids. First, a recent study investigating whether male signals are an indicator of



**Figure 4**  
Relationship between immune response, call syllable number, and relative spermatophore size. The plane corresponds to the GLM of immune response with call syllable number and relative spermatophore size as predictors (see Table 2). Dots are the observed values for each male, and vertical lines are the residuals.

nuptial gift quality in *E. diurnus* had also found a negative relationship between call syllable number and absolute nuptial gift size in the first mating of young males (Jarrige et al. 2013). Second, a comparative study of many tettigoniid species had identified a trade-off between acoustic signals and nuptial gifts (Del Castillo and Gwynne 2007). In that study the authors assumed that higher frequency calls are more energetically costly, and after controlling for phylogeny and body size, they showed that species with higher frequency calls produce smaller spermatophores.

One limitation of our study is that it provides only correlational evidence. As such, although it seems likely that the trade-offs we reported are a result of males being energetically limited, we cannot affirm that this is the case. There may be alternative explanations to the cause of the patterns observed. For instance, it is possible that syllable number is a fixed male trait and that males that can only produce calls with a low syllable number compensate for their

less attractive signals by producing larger spermatophores. Another possibility is that males that make larger spermatophores produce calls with fewer syllables not due to energy limitations but as a behavioral strategy that may come into play under conditions that promote sex-role reversal. Sex roles can be reversed in *E. diurnus* under poor food conditions. When this is the case, females compete for males, whereas males often reject mates and call less frequently (Ritchie et al. 1998). It is unlikely that roles were reversed in our study because individuals were fed ad libitum. Still, further work will be necessary to demonstrate that the trade-offs between immunity and reproductive traits are due to energy limitation. It should also be noted that several previous studies have used correlational evidence to explore the relationship between immune response and sexually selected traits (Ryder 2000; Rantala and Kortet 2003; Simmons et al. 2005; Drayton et al. 2012), showing that this type of evidence can be very informative to address questions about both trade-offs and the immunocompetence handicap hypothesis.

Our findings raise the question of under which circumstances one should expect such trade-offs to occur. Although the production of energetically costly acoustic calls in conjunction with spermatophores is a common feature of many orthopterans (Wedell 1994; Gwynne 1995; Vahed and Gilbert 1996; Lehmann GUC and Lehmann AW 2007), should such a pattern of trade-offs be widespread? *Ephippiger diurnus* stands out among these species because of the large size of its spermatophores as well as its regular calling for many hours each day. Because trade-offs are often the result of limited energy budgets, one would predict that they are more likely to occur in species with costly reproductive traits. Within *E. diurnus*, populations differ in the number of syllables per call and nuptial gift size (Ritchie 1991, 1992). Calls range from monosyllabic (in central France), to an average of 2–4 syllables in the Pyrenees, and from 2 to 6 syllables along the Mediterranean Coast (Duijm 1989). With the exception of some coastal populations, the Col de Mantet population has the highest syllable number among studied populations (Duijm 1989), as well as one of the largest spermatophores relative to male body size (Barbosa F, Rebar D, Greenfield M, unpublished data). Our observations of the population since 2008 show that its density is generally high, with 10 or more adults often found in the same 1-m<sup>2</sup> shrub (Barbosa F, Rebar D, Greenfield M, personal communication). Under such densities, a strong immune response may be a particularly critical survival trait. Combined, these features may account for the pronounced trade-offs reported here.

Our results imply that male *E. diurnus* operate under a limited energy budget in which investment in one particular trait will come at the expense of another. As a result, such trade-offs may constrain the evolution of the traits discussed here, traits which could otherwise be under directional selection. Evidence suggests that this is true for male song: previous work has reported that females from different *E. diurnus* populations, including Col de Mantet, prefer male calls with higher syllable numbers than their population's average (Ritchie 1996; Party et al. 2014). Our results suggest that males that produce higher syllable calls are indeed spending more overall energy on calling: the lack of a relationship between call rate and syllable number shows that males do not compensate for a higher number of syllables by slowing their call rate. The mismatch between male trait and female preference for call syllable number may reflect the fact that acoustic signals are constrained by energy, which males may be spending on nuptial gifts or immune response. Female preference should exert directional selection on call syllable number, but it appears that trade-offs with other traits are constraining male songs in this population.

Another implication of these trade-offs is that males may differ in how they allocate their energy budget: some males may invest more heavily in immunity rather than reproduction, whereas others might invest more in song than in nuptial gifts, and so forth. What factors affect a male's strategy? The optimal energy allocation strategy may depend on within-population dynamics. For example, because the potential for disease transmission increases with population density, males may benefit from investing more heavily in immunity when population density is high. But at the same time, the payoff of investing in nuptial gifts is likely decreased in this case: Female remating rate will likely be high, leading to intense sperm competition that will decrease the payoff of investing in sperm protection (Parker and Pizzari 2010). Furthermore, the social environment experienced by a male during development and adulthood may also contribute to male investment in immunity, mate attraction, or nuptial gift production. The perceived quality and quantity of competition for mates, as mediated by male calls, may influence the patterns of investment by males in those environments.

Although social environments play important roles in trait development (Kasumovic et al. 2011; Verzijden et al. 2012), our experimental males showed variation in the trade-offs investigated here despite similar rearing conditions as adults. Might variation in male strategy arise from the effects of juvenile development? Although environmental differences during development may have contributed to generating trade-off variants, because individuals were collected at the same developmental stage from the same population and reared as adults under similar conditions, the trade-offs among males likely reflect underlying genetic variation. The high repeatability estimate of call syllable number indicates the strong possibility of a genetic basis for variation in at least one of the traits studied. Yet it is also possible that genetic variation exists not only for each individual trait but also for the trade-offs themselves. That is, the observed phenotypic correlation may reflect genetic correlations between the traits studied here, perhaps as a result of pleiotropic effects or linkage disequilibrium (Roff and Fairbairn 2007). If there is not only one optimal strategy, but different combinations of trait values yield equivalent fitness peaks, then genetic variation in trade-offs may be maintained (Mangel and Stamps 2001). This process could account for similar observations made in other species of variable trade-offs in the absence of environmental differences (Billerbeck et al. 2000; Roff et al. 2002; Lee et al. 2003; Roff et al. 2003). In *E. diurnus*, the existence of different male strategies is expected because these insects live in a variable environment with fluctuating population densities, and the trade-offs between reproductive and survival traits in *E. diurnus* are bound to play a significant role in the evolution of sexually selected traits in this species.

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