



# When do acoustic cues matter? Perceived competition and reproductive plasticity over lifespan in a bushcricket



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Individuals often modify their behaviour in response to environmental cues and their own condition. Here we asked whether males modify ejaculates based on information from the sociosexual environment and their physiological age, and how those two factors may interact. We used two populations of the chorusing bushcricket, *Ephippiger diurnus*, to test whether males strategically adjust large, costly spermatophores they transfer to females during mating based on experience of rivals' calls, and whether males change their investment strategies with age. Males broadcast highly consistent, rhythmically repeated calls of syllables in daily choruses. Populations differ in average syllable number, an important trait under selection via female preference, and females from our two populations prefer calls with more syllables than the population mean. We reared males in one of five acoustic environments that varied in call syllable number. We then mated males twice, as young and old adults, measuring spermatophore size each time. We found that acoustic experience, age and their interaction all significantly influenced male investment, resulting in reaction norms with different slopes. Young males differentially invested in spermatophores in response to acoustic experience, whereas old males generally invested in larger spermatophores across environments. We then tested for a broad pattern of age-related investment with eight different field-collected populations, finding the majority of old males significantly increased spermatophore investment. Our findings demonstrate that both environmental context and an individual's life history state influence plasticity in reproductive investment, and such adjustments may optimize their reproductive success.

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Animals may adjust their reproductive investment in response to their condition, such as physiological age, as well as to both abiotic and biotic environmental factors (Cornwallis & Uller, 2010; Cotton, Small, & Pomiankowski, 2006; Miller & Svensson, 2014; Qvarnström, 2001). Some environmental factors, such as the sociosexual environment, are variable, and individuals are often behaviourally plastic to such cues because of their profound effect on reproductive fitness (Andersson, 1994; Kokko, Jennions, & Brooks, 2006; West-Eberhard, 1983, 2014). For example, hearing male advertisement signals influences a neighbouring male's behaviour (e.g. Kasumovic, Hall, Try, & Brooks, 2011; Rebar & Rodríguez, 2016) and also the behaviour of local females (Hebets & Sullivan-Beckers, 2010; Rodríguez, Rebar, & Fowler-Finn, 2013; Verzijden et al., 2012). In response to physiological age and social experience, individuals are thus predicted to trade off reproductive

investment across mating events when mating is costly, favouring increased investment when the benefit is high or the likelihood of a future mating event is low (Roff, 1992; Stearns, 1992; Wedell, Gage, & Parker, 2002).

In males, adjustment of ejaculate investment (e.g. spermatophores in insects) to various cues is expected. For example, males may increase investment with increasing physiological age (Clutton-Brock, 1984; Williams, 1966). Life history theory predicts that reproductive effort should reflect an individual's condition or their remaining longevity (i.e. physiological age), a notion supported by work on various aspects of chronological versus physiological age (e.g. Levine, 2013; Ligout, Munier, Marquereau, & Greenfield, 2012). Males may also modify their investment based on the social environment. These adjustments may be mediated by experience, such as the presence or absence of rival male calls during development, or to the immediate presence of rivals during mating (Bretman, Gage, & Chapman, 2011; Kasumovic & Brooks, 2011; Kelly & Jennions, 2011). Such adjustments occur in response to sperm competition, and sperm competition models offer straightforward predictions on how males will adjust

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ejaculate investment to the probability that two ejaculates compete, termed 'risk', and to the number of different males' ejaculates competing for a female's eggs, termed 'intensity'. In general, males will increase ejaculate investment when a rival is present, but decrease it with additional rivals due to increased sperm competition intensity (Engqvist & Reinhold, 2005; Parker, 1998; Parker, Ball, Stockley, & Gage, 1997; Parker & Pizzari, 2010).

Many mating behaviours, such as male signals and female mate preferences, remain flexible through adulthood (e.g. Rebar, Barbosa, & Greenfield, 2016; Rodríguez, Boughman, et al., 2013; Swanger & Zuk, 2015), and male ejaculate expenditure should similarly fluctuate in response to physiological age and the risk and intensity of sperm competition. The question remains, though, whether and how these factors interact with one another over an individual's life. Does one have priority over the other? Or is each factor more important in regulating investment at different times? Selection should favour investment by males that optimize their lifetime fitness across mating events.

In species in which males use advertisement signals to attract mates, males can use rivals' signals to assess the social environment, and ultimately sperm competition. Numerous male behaviours are sensitive to acoustic cues, including male ejaculate expenditure to sperm competition risk (Bretman, Gage, et al., 2011; Bretman, Westmancoat, Gage, & Chapman, 2011; Gray & Simmons, 2013; Kasumovic & Brooks, 2011; Kasumovic et al., 2011). Studies on ejaculate investment have predominantly centred on the presence or absence of cues, but the range of adjustments males could make to acoustic cues is much broader. For instance, males could adjust ejaculate investment based on the quality of perceived rivals, as is the case for precopulatory behaviours to rival cues (Kasumovic et al., 2011; Lane et al., 2015; Yoshikawa, Ohkubo, Karino, & Hasegawa, 2016). Or males could adjust ejaculate investment based on how females adjust their behaviour, such as mate preference or remating rate, to acoustic cues. Male adjustments to female adjustments have been documented in signalling behaviours (Bertram, Harrison, Thomson, & Fitzsimmons, 2013; Kahn, Dolstra, Jennions, & Backwell, 2013). For example, male fiddler crabs adjust their courtship effort to match the shift in female mate preferences across a mating period (Kahn et al., 2013). Acoustic signals can thus provide information about the quantity and quality of rivals and about how females will adjust their mating behaviour. Males may thus be selected to respond to what these cues say about likely sperm competition risk and intensity.

Here we used a long-lived, multiply mating chorusing insect, the flightless bushcricket, *Ephippiger diurnus* (Orthoptera: Tettigoniidae) (Hockham, Graves, & Ritchie, 2004), to test how male age and acoustic experience may interact to influence the size of male spermatophores. Males were tested shortly after attaining reproductive maturity and approximately 10 days later, an age interval that is expected to affect their physiological condition. Acoustic experience refers to long-term exposure to acoustic treatments to which individuals were subjected in the laboratory from late juveniles through adulthood, except at the moment of mating. The species is distributed in geographically isolated populations that tend to be genetically, morphologically and behaviourally differentiated (Party, Streiff, Marin-Cudraz, & Greenfield, 2015; Spooner & Ritchie, 2006). Males call in daily choruses for up to 6 h per day to attract mates (Duijm, 1990; Ritchie, 1991, 1992). Each call is composed of syllables, and the average number of syllables is population specific (Ritchie, 1992). There is some variation between individuals in each population, but syllable number within individuals is highly repeatable (Barbosa, Rebar, & Greenfield, 2016b; Rebar et al., 2016). Females consistently favour calls with more syllables than the population mean in our two study populations (Barbosa, Rebar, & Greenfield, 2016a), even with call experience

(Rebar et al., 2016). Upon mating, males produce large nuptial gifts, spermatophores composed of a sperm-filled ampulla and nutritional spermatophylax that are up to 40% of their body weight (Busnel & Dumortier, 1954; Wedell, 1994a). This investment is costly, as evidenced by an average postmating refractory period of 4 days before males can mate again (Vahed, 2007; Wedell, 1993). Whether spermatophore and ejaculate investment reflects parental investment (i.e. nutrition) or mating investment (i.e. sperm protection or increased female refractory period) remains unknown for many bushcricket species (Lehmann, 2012; see Quinn & Sakaluk, 1986 for a general introduction to the alternative functions). For instance, nuptial gift consumption by females in another bushcricket species decreased then increased feeding over the short term (24 h), but not over the long term (4.5 days; Lehmann & Lehmann, 2016). In other words, nutritional gifts such as spermatophores can be complex, and determining their specific consequences to females can be challenging. Nevertheless, both hypotheses predict that larger spermatophores would result in delayed female remating and thus yield increased fitness returns for the male. Therefore, repeatable male songs, known female preferences and large spermatophores make *Ephippiger* an ideal species for investigating whether condition and environment influence a male's investment, and if so, how these two influences interact.

Previously, we experimentally manipulated male experience of rivals' calls from late juveniles through adulthood by rearing males in one of five acoustic environments that varied in call syllable number. We found that young *Ephippiger* males adjusted spermatophores in response to the acoustic environment, but that this response was not simple (see Rebar et al., 2016). Young males increased spermatophore size when exposed to some song compared to no song, but decreased spermatophore size as the song stimuli became more attractive or varied.

Here we extended on that previous work in several ways. First, we continued to investigate the complex relationship between male investment and acoustic environments. The previous data came from one population and in 1 year; the relationship needed to be confirmed to rule out the possibility of a spurious effect. We note that the inverted U-shaped relationship observed in the preliminary experiment (Rebar et al., 2016) matches predictions from sperm competition risk and intensity. We thus undertook the current study to obtain sufficient data to address these predictions across populations and years. Second, we tested whether males modified spermatophores as they aged. We used population level reaction norms (Foster, 2013) as a graphical device to highlight the changes in the slopes of the mean phenotypes expressed in each environment across mating events, noting that this does not necessarily imply any genotype-specific effects. Third, we addressed the interplay between age and perceived sperm competition by comparing investment patterns across acoustic environments as males age.

We predicted that young males would adjust spermatophore size in a manner consistent with sperm competition risk and intensity, increasing spermatophore size when some calls were present due to increased sperm competition risk (e.g. Bretman, Gage, et al., 2011; Gray & Simmons, 2013). However, we predicted that males would decrease spermatophore size as call attractiveness increased. Previous work on female preference for male syllable number evaluated in single-stimulus tests shows that females walk longer distances towards a high-syllable number song than towards a song composed of fewer syllables (Barbosa et al., 2016a; Rebar et al., 2016). The distance travelled in these single-stimulus tests reflects a female's attraction towards a particular call type and her 'motivation' to mate with a male broadcasting that song. In the field, females would arrive in the vicinity of such males more often than those producing less attractive songs. We inferred that

when many attractive males are found in an area, local females would be more likely to remate than in an area where such males are rare or absent. This increased likelihood of female remating is expected to increase sperm competition intensity. This decreased investment would also occur in a variable call environment because attractive rivals were still present. This response is predicted because males may evaluate peak calls rather than means (cf. Limousin & Greenfield, 2009), and females would still be more likely to remate because of the presence of attractive males.

We then predicted that old males would invest a higher proportion of their available resources into spermatophores, regardless of acoustic cues, as they would be unlikely to have another mating opportunity. This prediction is consistent with terminal investment (Clutton-Brock, 1984; Williams, 1966), and old males would thus have an equal response across acoustic environments. However, differential investment in spermatophores by young males would yield significant differences in the slopes of the reaction norms between environments. This predicted interaction between acoustic environments and age prompted us to separately test for an overall effect of age; we measured the size of spermatophores produced by males when young and old from eight different populations, maintaining a similar acoustic environment for the populations.

## METHODS

### Study Species

*Ephippiger diurnus* is a complex of geographically isolated populations found in central and southern France and northern Spain. The various populations cluster into different clades, with shared geographical location and male call characteristics between populations in each clade (Party et al., 2015; Spooner & Ritchie, 2006). We studied two populations from different clades to capture the larger genetic structure of *E. diurnus* to test for patterns of male reproductive plasticity. The first population was collected from Vias, France (43°18'N, 3°22'E; elevation 27 m) and the second from Font Romeu, France (42°29'N, 2°00'E; elevation 1620 m). Vias males produced an average of 2.1 syllables per call in 2013, and individual males could average one to three syllables per call (Greenfield, Esquer-Garrigos, Streiff, & Party, 2016). Font Romeu males produced an average of 3.5 syllables per call in 2013, and individual males averaged two to eight syllables per call (Rebar et al., 2016). Females from both populations prefer calls with more syllables than the population mean (Barbosa et al., 2016a; Rebar et al., 2016).

We collected animals from Vias in May 2014 and May 2015 as young nymphs up to the penultimate instar ( $N = 52$  and  $53$  males, and  $55$  and  $50$  females in 2014 and 2015, respectively) and from Font Romeu in July 2014 at the same developmental range ( $N = 105$  males and females) and brought them to the laboratory. We housed them in individual plastic containers (107 mm in diameter and 119 mm high) with acoustically transparent plastic mesh replacing one-third of the sidewall. The nonoverlapping development of the two populations allowed us to rear both populations sequentially in the same climate-controlled chamber maintained at 25 °C and with a photoperiod of 16:8 h light:dark, and we used the same chamber the following year for the second group of Vias males. Individuals were fed cabbage, pollen and fish flakes ad libitum, and misted daily with water.

### Acoustic Environments

We used the same experimental set-up described in detail elsewhere (Rebar et al., 2016). Briefly, we used five identical

100 × 100 cm and 80 cm high boxes lined with 6 cm of 35 kg/m<sup>3</sup> acoustic isolation foam (Flexolan, Dierdorf, Germany). One side was left open. On that side, we placed a sun-simulating bulb (Solar Glo PT2193; Rolf C. Hagen Inc., Mansfield, MA, U.S.A.) and a single loudspeaker (FT17H; Fostex Corp., Tokyo, Japan) pointing into the box. The loudspeaker covered most of the energy contained in *E. diurnus* calls (10–50 kHz), and we compensated for the spread of sound energy by rotating individual containers daily. The boxes partitioned the climate-controlled chamber into five environments. Because of space limitations, we were unable to replicate boxes. We therefore undertook several measures to limit differences between environments other than the acoustic treatment itself. First, we verified that the abiotic conditions in each box were similar. Second, we ensured that the environments were acoustically isolated from one another. Third, we rotated the positions of the acoustic environment within the chamber between the two populations in 2014, and again between the 2 years of the Vias population. Minimal differences between populations or between years within the one population strongly support the notion that our environments were similar except for the effect of interest, the acoustic environment itself.

We randomly assigned the collected nymphs to one of the five acoustic environments, but introduced them to that environment upon reaching the penultimate instar to standardize acoustic exposure ( $N = 10$ – $11$  males and females per environment for Vias in 2014 and 2015;  $N = 21$  males and females per environment for Font Romeu; see below). Males and females were reared in individual containers, and we positioned each container so that the mesh sidewall always faced the loudspeaker, and rotated individual cages within each environment daily to homogenize biotic and abiotic experience. We waxed the elytra of adult males together to prevent them from calling, thus restricting acoustic experience to the stimuli we presented. All individuals remained in their respective acoustic environment throughout the experiment except while mating.

We broadcast acoustic stimuli to each environment for 6 h per day beginning 2 h after daylight through the loudspeaker that was centred in the open side of each box. The peak amplitude of the stimuli presented was calibrated to that of a male calling at 1 m distance (90 dB; Greenfield, Siegfried, & Snedden, 2004; Party, Brunel-Pons, & Greenfield, 2014) with a sound level meter (CEL-430/2; Casella Cel, Kempston, U.K.).

Individuals were reared in one of five acoustic environments: Silent, Low, Mid, High or Mixed. In the Silent environment, individuals were never exposed to any acoustic stimuli. The other four acoustic environments varied in the number of syllables per call, and the variation in syllables was centred on the most common call syllable number (i.e. mode) for each population (two syllables for Vias; three syllables for Font Romeu). The Low environment presented calls that were one syllable less than a common call, the Mid environment consisted of common calls and the High environment of calls one syllable more than a common call. The Mixed environment consisted of Low, Mid and High calls presented in equal amounts. Females from these populations prefer calls with more syllables than the population mean. We used this known preference to characterize the attractiveness of rivals in the environment. That is, Low contained unattractive competitors whereas High contained attractive competitors.

We constructed four calls of different amplitude (relative dB: 0 dB, –4 dB, –8 dB, –12 dB) for the Low, Mid and High environments. Because calls were broadcast from a single location rather than spatially separated for each environment, the amplitude adjustments simulated distance between calls and thus the perception of a chorus. We presented calls using randomization without replacement within each block of four calls, and spaced calls at 2 s

intervals. This spacing reflects the natural manner of alternating calls between neighbours in the field (Brunel, 2012; Party et al., 2014). We constructed 20 randomized blocks of the four calls of different amplitudes for each acoustic environment, and then looped the blocks of calls to broadcast for 6 h daily, thus ensuring that all call models were broadcast in equal amounts.

### Male Reproductive Investment

#### First mating

We randomly paired a young virgin male (mean  $\pm$  SD =  $12.2 \pm 1.1$  days, range 10–14 days) with a 14-day-old virgin female from the same acoustic environment. We paired females at the youngest age at which they are reliably sexually mature in the studied populations. Pairs were mated in a different room from where they were reared that was also maintained at the standard 25 °C (range 24–25.5 °C). Male elytra were still waxed together, limiting the acoustic information that a female could receive from a male during pair formation. However, females are stimulated to mate in the presence of acoustic cues, and so this room contained an active chorus of males from various populations whose calls extended beyond the range that males from either population would naturally experience. We maintained the chorus at a minimum of 5 m from a mating pair to only facilitate mating. We note, however, that males produce spermatophores during mating rather than beforehand such that males could have adjusted spermatophore size in 'real time' due to the chorus. Our interest was in acoustic experience, or the long-term influence of the acoustic treatments, rather than any momentary adjustments to the chorus used for facilitating mating. We thus controlled for this potential confounding effect by subjecting all mating pairs across all acoustic treatments to the same chorus during mating.

We measured male spermatophore investment indirectly so as not to interfere with the mating process. We weighed males and females immediately before and after mating to the nearest mg, calculating spermatophore mass as the average of the weight differential for each individual. Pairs were mated in a new plastic container without transparent sidewalls to further attenuate the chorus above and beyond the 5 m distance. Males were returned to their rearing container and back to their respective acoustic environment immediately following mating.

#### Second mating

We followed the same protocol as in the first mating. Males were mated 8–12 days after the first mating such that all males were 21–26 days post adult moult (mean  $\pm$  SD =  $23.4 \pm 1.3$  days). We used the same pool of females as in the first mating, all of which had mated once, and ensured that males did not pair with the same female as before. This approach was undertaken for several reasons. First, the small size of *E. diurnus* populations limited our ability to sample them, and we thus matched the number of males and females collected. Second, their phenology did not allow us to collect females that spanned a large age range such that we could control female age across matings. Third, any older males in the field would encounter older females, nearly all of which have previously mated. Our approach thus reflects what males would do in a natural situation. We also note that a previous study on a related population found that older males did not treat virgin or mated, or old and young, females differently in terms of the spermatophore size that they produced (Jarrige, Greenfield, & Goubault, 2013). Not all males mated successfully in the allotted mating interval or survived until the second mating, particularly for Font Romeu, and this resulted in smaller sample sizes.

### Statistical analyses

We used a linear mixed model with relative spermatophore size (percentage of body weight donated to the female) as the dependent variable to test for adjustments in reproductive investment due to acoustic experience and age, and tested each population separately. We divided spermatophore weight by male pre-mating weight to measure reproductive investment relative to the size of each male. Our models included terms for acoustic experience and age as fixed effects, male identity nested within acoustic experience as a random effect, and an acoustic experience\*age interaction term. The model for Vias also included a year term nested within environment as a random effect because we collected individuals in 2014 and 2015. While year did not contribute to explaining variation (see Results), we retained it in the model as a point of replication. We did remove the term from all subsequent post hoc analyses and from the figures for clarity. We also included female nested within acoustic experience as a random effect and female weight as a fixed effect, but removed both terms from each model due to their negligible contribution (all  $P > 0.3$ ). We also ran each model with absolute spermatophore size as the dependent variable and male pre-mating weight as a covariate. These models produced nearly identical results, and we present models and figures with relative spermatophore size for ease of interpretation.

Significant acoustic experience\*age interaction terms (see Results) prompted us to determine when acoustic experience influenced male investment in spermatophores. We compared the spermatophores produced by males between the five acoustic environments when males were young and when they were old (10 pairwise comparisons for each age). We thus performed a total of 20 post hoc pairwise comparisons, and we used the Benjamini–Hochberg procedure to control for the false discovery rate (FDR) of all 20 comparisons for each model (Benjamini & Hochberg, 1995). We also calculated effect sizes as the difference of two means for acoustic experience pairs to quantify differences and account for our small sample sizes. We first calculated effect size as Hedges'  $g$  to minimize bias in our estimates from the small sample sizes, and then converted that to the standardized correlation  $r$  to express effect size in terms of small ( $r < 0.2$ ), medium ( $0.2 \leq r < 0.5$ ) or large ( $r \geq 0.5$ ) effects (Grissom & Kim, 2005; Nakagawa & Cuthill, 2007).

In total, 90 and 63 males from Font Romeu and 98 and 90 males from Vias mated successfully in the first and second matings, respectively, and were thus included in the analysis. We carried out all statistical analyses with JMP 10 (SAS Institute Inc., Cary, NC, U.S.A.).

### Age-related Investment

We first looked at the age terms and the acoustic experience\*age interaction terms from the linear mixed models to establish whether males adjusted investment as they aged. We then verified these results by inspecting the reaction norms across acoustic environments. However, any age-related effect may have been, in part, due to strategic resource allocation when young in response to the acoustic environment. This strategy predicts that, in response to acoustic cues, males that invested more when young would invest less when older than males that invested less when young. We therefore followed up our acoustic environment experiment by testing for a general pattern of age-related investment across populations of *E. diurnus*, rearing all males in a common acoustic environment to restrict any interaction between acoustic cues and spermatophore investment. We predicted that males would invest in larger spermatophores as they aged because of the reduced likelihood of future mating opportunities.

We tested for a broad pattern of age-related investment by collecting males and females as late-instar nymphs or immature adults from eight populations in either July 2014 or July 2015, including one experimental population again. These populations are distributed across the range of *E. diurnus* and map onto the different clades: Col de Mantet (CDM), Font Romeu (FR), Merès-les-Vals (MER), Mireval (MIR), Peyriac de Mer (PEY), Pouzol (PZL), Puymorens (PUY), Vilamòs (VIL) (Greenfield et al., 2016; Party et al., 2015). We housed all individuals in their own plastic container, and males and females were reared in separate climate-controlled rooms maintained at 25 °C and 16:8 h light:dark. Males from the populations were reared through adulthood together and were thus exposed to the same daily mixed chorus that they created.

We followed the same mating protocol as before, mating a 10–14-day-old virgin male with a similarly aged virgin female in the presence of a chorus at a minimum distance of 5 m. We then mated males a second time with a different female from the same pool of females 8–12 days after the first mating. We measured spermatophore size as before, using an indirect approach.

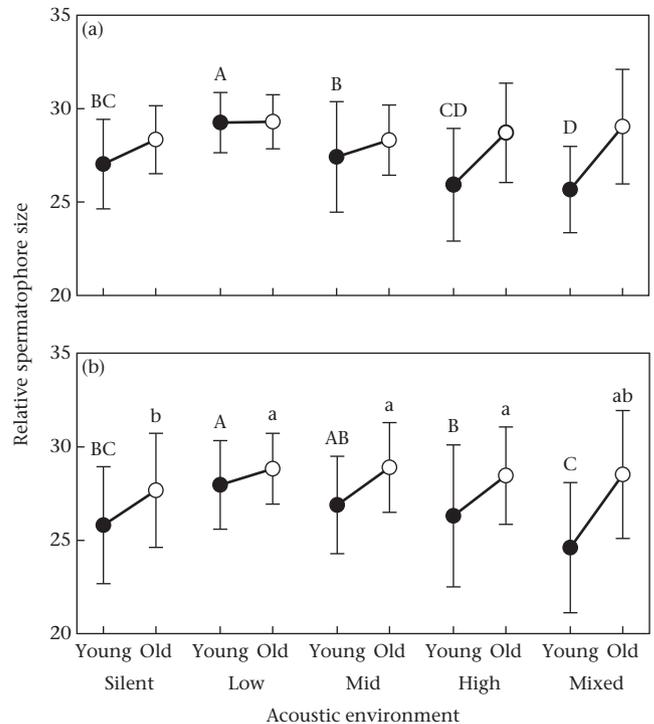
We used a linear mixed model to test for age-related effects on the relative size of spermatophores produced by males across populations. Our model included terms for population and age as fixed effects, along with a population\*age interaction term. We also included male identity nested within population as a random term. The population term tested for differences in the propensity to invest in spermatophores between populations. The age term tested for differences in spermatophores produced by young and old males. The population\*age interaction term tested for differences in the reaction norms across populations. We performed post hoc tests using the FDR method following a significant interaction term (see Results).

## RESULTS

### Acoustic Experience and Male Reproductive Investment

Acoustic experience influenced the size of spermatophores produced by males in both populations. There was a significant acoustic experience\*age interaction in both populations, indicating that the reaction norms differed significantly from one another (Fig. 1, Table 1). We thus disentangled the influence of acoustic experience by looking at the size of spermatophores produced by young and old males separately.

Whereas old males did not differ significantly or differed only minimally in spermatophore investment due to acoustic experience (Fig. 1), we found that young males significantly adjusted spermatophore size due to acoustic experience (Fig. 1). Post hoc pairwise comparisons revealed two commonalities in spermatophore adjustments. First, males responded to the presence versus absence of acoustic cues: young males increased spermatophore size significantly when exposed to the songs of unattractive males, and this effect was medium in magnitude (Silent versus Low: Vias:  $r = 0.46$ ; Font Romeu:  $r = 0.35$ ; Fig. 1). Second, the types of calls that males experienced influenced their spermatophore investment. Young males donated significantly smaller spermatophores to females when they (males) experienced attractive versus unattractive rivals (Low versus High; Fig. 1), and this effect was large to medium in magnitude (Vias:  $r = 0.55$ ; Font Romeu:  $r = 0.25$ ). Young males also decreased spermatophore size significantly as call variation increased when compared to unattractive rivals or rivals of average 'quality' (Low or Mid versus Mixed; Fig. 1). This effect was large to medium in magnitude (Vias:  $r = 0.66, 0.31$ ; Font Romeu:  $r = 0.48, 0.34$ ; Low or Mid versus Mixed, respectively).



**Figure 1.** Effect of acoustic experience and age on the relative size of spermatophores produced by males from (a) Vias and (b) Font Romeu. Relative spermatophore size was calculated as the percentage of body weight given to a female during mating. The responses of young males (filled circles) that share a capital letter are not significantly different. The responses of old males (unfilled circles) that share a lowercase letter are not significantly different (post hoc tests using the FDR method). Means  $\pm$  SDs are displayed.

**Table 1**

Linear mixed models testing for variation in the relative size of spermatophores produced by males due to acoustic experience, age and their interaction

Trait	Source	df	F	P
<b>Vias</b>	Spermatophore	Whole model	102, 85	<b>3.09</b> <0.001
		Acoustic experience	4, 5.06	<b>9.01</b> <b>0.016</b>
		Year [Acoustic experience]	5, 90.69	0.40 0.850
		Age	1, 85	<b>31.90</b> <0.001
		Acoustic experience * Age	4, 85	<b>4.06</b> <b>0.005</b>
		Male [Acoustic experience, Year]	88, 85	<b>2.49</b> <0.001
<b>Font Romeu</b>	Spermatophore	Whole model	96, 58	<b>4.97</b> <0.001
		Acoustic experience	4, 95.07	<b>2.54</b> <b>0.045</b>
		Age	1, 58	<b>65.23</b> <0.001
		Acoustic experience * Age	4, 58	<b>3.41</b> <b>0.014</b>
		Male [Acoustic experience]	87, 58	<b>4.31</b> <0.001

Males from each population were mated as young and old adults, with a minimum of 8 days between matings. The models included acoustic experience and age as fixed effects, along with their interaction. We included a year term nested within environment as a random effect for the Vias population because individuals were collected over 2 years. Male identity was nested within environment (and year for Vias) as a random effect. Spermatophore size was calculated with respect to the size of the male at mating. Significant tests are in bold.

### Age-related Effects on Male Reproductive Investment

Males from Vias and Font Romeu increased their relative investment in spermatophores as they aged (age term, Table 1, Fig. 1). This response resulted in no difference across acoustic environments, evidenced by the disappearance of the inverted U-shaped curve in favour of an equivalent investment pattern in old males across environments (Table 1, Fig. 1). However, some of this effect

**Table 2**  
Linear mixed model testing for adjustments in the relative size of spermatophores produced by males as they aged across eight populations of *E. diurnus*

Trait	Source	df	F	P
Spermatophore	Whole model	175, 160	<b>3.38</b>	<b>&lt;0.001</b>
	Population	7, 160	<b>19.19</b>	<b>&lt;0.001</b>
	Age	1, 160	<b>26.94</b>	<b>&lt;0.001</b>
	Population*Age	7, 160	<b>8.64</b>	<b>&lt;0.001</b>
	Male [Population]	160, 160	<b>1.69</b>	<b>&lt;0.001</b>

The model included population and age as fixed effects, along with their interaction. We also included male identity nested within population as a random effect. Significant tests are in bold.

may have been confounded with adjustments to acoustic experience (acoustic experience\*age interaction terms; Table 1, Fig. 1).

We then compared the spermatophores that males produced as young and old adults across eight populations, including replication of Font Romeu. We controlled for acoustic effects by rearing males in a common acoustic environment. We found significant differences in spermatophore size between populations, across ages and in their interaction (Table 2). Post hoc tests revealed that males when old produced significantly larger spermatophores in five of the eight populations, including Font Romeu, and only one population produced smaller spermatophores as they aged (Fig. 2). These results confirm our findings in the two main study populations: males did indeed invest more by producing larger spermatophores when older.

#### Repeatability of Spermatophore Investment

Male identity accounted for a significant amount of variation in spermatophores for both populations (Table 1), indicating that males consistently varied in spermatophore investment. We quantified repeatability as the male identity percentage variance component of the models in Table 1 using the REML method (Lessells & Boag, 1987), finding relative spermatophore size to be repeatable (Vias:  $r = 0.43$ ; Font Romeu:  $r = 0.66$ ). We then used Pearson product-moment correlations to look at the relationship within each acoustic environment. We found a positive relationship within each acoustic environment (Pearson  $r$ ; Vias:  $r = 0.15$ – $0.67$ ; Font Romeu:  $r = 0.19$ – $0.79$ ), but small sample sizes restricted further interpretation. We thus pooled across environments, and verified the variance component estimates by looking at the overall correlation (Pearson  $r$ ; Vias:  $r = 0.40$ ,  $N = 90$ ,  $P < 0.001$ ; Font Romeu:  $r = 0.63$ ,  $N = 63$ ,  $P < 0.001$ ).

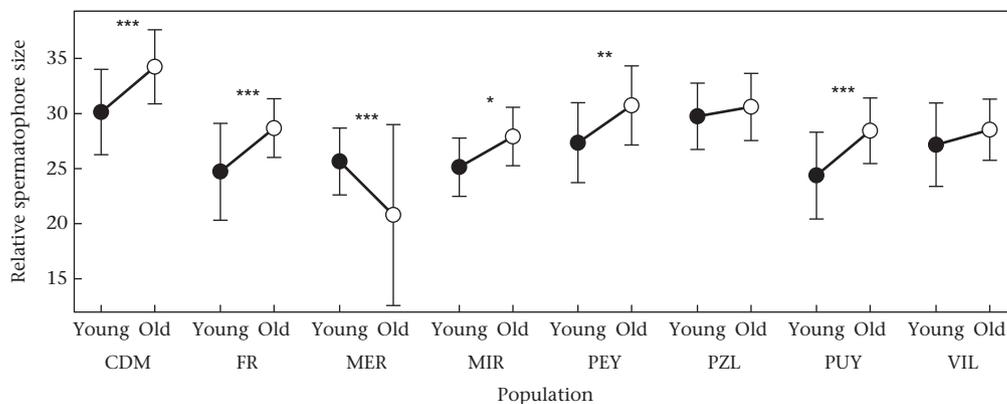
## DISCUSSION

By exploring the interplay between acoustic experience and age, we found support for a robust pattern of reproductive plasticity in the bushcricket *E. diurnus*. In several ways our results expand on a small but growing number of studies documenting experience-mediated plasticity in male ejaculates (e.g. Bretman, Westmancoat, et al., 2011; Gray & Simmons, 2013; Rebar et al., 2016). First, we found that rival phenotypes influenced a male's investment strategy. By varying call syllable number, an important trait under selection via female preference (Barbosa et al., 2016a; Rebar et al., 2016), males made fine-scale adjustments by decreasing the size of spermatophores given to females as rival attractiveness increased. Second, we found a shift in reproductive investment strategy, where adjustments to acoustic experience were overshadowed by age-related adjustments. We documented this age-related effect in several populations of *E. diurnus*, a pattern consistent with life history theory. Finally, we noted consistent variation in male investment strategies in spite of experience or age, suggesting that underlying genetic components may regulate the relative amount of energy that males can expend in producing spermatophores.

Why did young males adjust their investment in spermatophores in response to acoustic experience? Males expend much energy in ejaculate production (e.g. Dewsbury, 1982; Wedell et al., 2002), and the adjustments in ejaculate expenditure to acoustic cues are consistent with a response to perceived sperm competition risk and intensity. Here, males increased spermatophore size with increased risk due to the presence versus absence of acoustic cues, but only when experiencing unattractive rivals. That males in the other environments did not produce larger spermatophores may reflect other influences on both males reared in silence and those reared in variable environments, which we outline below.

In the absence of acoustic experience, males may have produced larger spermatophores than expected because mating was facilitated by the presence of a chorus. Feedback from the chorus may have resulted in real-time adjustments, as *E. diurnus* males produce spermatophores during mating. Alternatively, a lack of rivals in the environment may have indicated a lack of suitable mates as well, suggesting that males should invest more in their current mating event, as future events would be unlikely.

In the presence of acoustic experience, males may have decreased spermatophore investment as rival attractiveness increased due to a perceived increase in sperm competition intensity. Directional female preferences for calls with more syllables



**Figure 2.** Reaction norms of the relative size of spermatophores produced by males when young and old for eight different populations of *Ehippiger diurnus*. Filled circles display the mean spermatophore size of young males, unfilled circles those of old males. Means  $\pm$  SDs are displayed. Asterisks denote significant differences following post hoc tests (FDR method). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

(Barbosa et al., 2016a; Rebar et al., 2016) suggests that female remating rates would increase in environments with increasing rival attractiveness, resulting in increased sperm competition intensity. Our results are consistent with this prediction: males exposed to attractive calls invested less in spermatophores than males exposed to unattractive calls. A recent meta-analysis on sperm competition risk and intensity found strong evidence supporting a response to risk, but mixed evidence for a response to intensity (Kelly & Jennions, 2011). Our results indicate that the quality of rivals above and beyond their mere presence may be important in regulating male investment, as males produced similarly small spermatophores when only attractive calls were presented or mixed with other less attractive calls. This could help explain the mismatch between theory and empirical evidence.

The interaction between context and condition can mask our ability to detect important and relevant patterns of reproductive plasticity. The shift towards uniformly large spermatophores in old males suggests that an individual's life history state strongly influences when environmental cues are important. That is, the adjustments to perceived competition when young and condition when old were probably driven by a trade-off between current and future mating events. Although this effect could reflect male age, mating status of the male or female or any combination thereof, we suggest it was age for the following reasons. First, previous work on related populations of *E. diurnus* has shown that older males donate larger spermatophores to females (Jarrige et al., 2013; Wedell & Ritchie, 2004). This adjustment occurred regardless of male mating history (Wedell & Ritchie, 2004) or female mating history or age (Jarrige et al., 2013). Second, males incur a significant cost from increased spermatophore investment by having reduced immune functions (Barbosa et al., 2016b). As old males are unlikely to mate again, they may be more willing to accept this cost. Third, one might predict that males mating with older, previously mated females would provide smaller, rather than larger, spermatophores: such females are less likely to mate again, and these females are less likely to elicit a larger gift. We found the opposite pattern, implying that the problem of using females in a different state for testing older males was not an issue. Therefore, these factors suggest that the increased spermatophore investment largely reflects male age.

Nevertheless, several alternatives may explain the observed interaction between context and condition. First, males may assess their own attractiveness versus that of their competitors to adjust their investment as they age. For example, young males surrounded by attractive competitors should invest less in current reproduction and save their resources for future reproduction because, on average, they are less attractive than their competition. The response to acoustic environments suggests that males can assess call syllable number to a certain degree, but whether they are aware of their own acoustic prowess is unknown. We also note that male elytra were waxed together throughout adulthood, rendering the male mute and thus restricting such relative assessment. Second, multiply mated females may be more successful at reproduction than singly mated females of similar age, such that males invest more in previously mated females. This would require males to be able to detect whether a female has previously mated, which is unknown for this species. Further, we have found that females deposit fewer eggs after their second mating (Rebar & Greenfield, n.d.). Third, nonrandom sperm utilization by females favouring second male (P2) precedence could contribute to selection favouring greater reproductive investment by older males. Although P2 precedence has been documented in *E. diurnus* in the laboratory, field studies are less conclusive (Hockham et al., 2004), suggesting that such effects may not be as strong as believed. Thus, male age remains the most parsimonious explanation for the shift to uniformly large spermatophores in old males.

Patterns of age-related investment are widely predicted (Clutton-Brock, 1984; Williams, 1966). Although many studies have found support for terminal investment in other reproductive behaviours (e.g. Bowers et al., 2012; Creighton, Heflin, & Belk, 2009; Lafaille, Bimbard, & Greenfield, 2010), the fewer studies on male spermatophores have found various adjustments, including size and composition (e.g. Duffield, Hunt, Rapkin, Sadd, & Sakaluk, 2015; Jarrige et al., 2013; Thanda Win, Kojima, & Ishikawa, 2013). We documented a broad pattern of plasticity consistent with theory, finding a significant increase in spermatophore size in five of eight populations, positive reaction norms in two more populations and only one reaction norm opposite to this prediction. Whether spermatophore and ejaculate investment in *E. diurnus* reflects parental or mating investment remains unknown, as is the case for many bushcricket species (Lehmann, 2012), but both hypotheses predict that larger spermatophores would result in delayed female remating. Males will have increased fitness returns under either hypothesis for the following reasons. First, the two components of the spermatophore, the sperm-filled ampulla and spermatophylax, strongly and positively covary with one another (Jarrige, Body, Giron, Greenfield, & Goubault, 2015; Wedell, 1994b), meaning larger spermatophores transfer more sperm. Second, larger spermatophores have larger spermatophylaxes (Jarrige et al., 2013), which could influence a male's fitness by nutritional investment in offspring or by delaying the female's subsequent mating. Third, females average a 5-day postmating refractory period during which time they deposit eggs (Vahed, 2007; Wedell, 1993), such that any lengthening of that period would increase the number of eggs deposited before remating. In concert with female nonrandom sperm use and male trade-offs with immune function, such reproductive adjustments may be adaptive.

In summary, we have documented reproductive plasticity in male bushcrickets in response to acoustic experience and age, and showed that differences in the reaction norms all converge on increased investment as they aged in a manner consistent with life history theory. Our results highlight that males can attend to variation in competition mediated by acoustic cues and respond in ways that may optimize their reproductive fitness. Future work on reproductive plasticity should consider the interaction between context and condition, as robust patterns of plasticity in strategic investment may exist across taxa.

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