Larger testes are associated with a higher level of polyandry, but a smaller ejaculate volume, across bushcricket species (Tettigoniidae)

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While early models of ejaculate allocation predicted that both relative testes and ejaculate size should increase with sperm competition intensity across species, recent models predict that ejaculate size may actually *decrease* as testes size and sperm competition intensity increase, owing to the confounding effect of potential male mating rate. A recent study demonstrated that ejaculate volume decreased in relation to increased polyandry across bushcricket species, but testes mass was not measured. Here, we recorded testis mass for 21 bushcricket species, while ejaculate (ampulla) mass, nuptial gift mass, sperm number and polyandry data were largely obtained from the literature. Using phylogenetic-comparative analyses, we found that testis mass increased with the degree of polyandry, but decreased with increasing ejaculate mass. We found no significant relationship between testis mass and either sperm number or nuptial gift mass. While these results are consistent with recent models of ejaculate allocation, they could alternatively be driven by substances in the ejaculate that affect the degree of polyandry and/ or by a trade-off between resources spent on testes mass versus non-sperm components of the ejaculate.

1. INTRODUCTION

A positive relationship across species between relative testes size and the extent to which females engage in polyandrous mating has been demonstrated in a wide range of taxa, including fish, amphibians, birds, mammals and insects [1,2]. Experimental evolution experiments have provided further evidence for the positive effect of increased polyandry on relative testes size (reviewed in [3]). At least two potentially

interrelated hypotheses could account for this pattern: numerical sperm competition on the one hand and male mating rate on the other [2,4], although relationships between testes mass and polyandry could be more complex than simple cause and effect [5].

Traditionally, it has been assumed that larger testes produce more sperm per ejaculate, giving the male an advantage in numerical sperm competition [6]. Early models of ejaculate allocation predicted that as the level of sperm competition increases, ejaculate size and sperm number per ejaculate should increase alongside testes size [6]. These predictions have been upheld by many studies (reviewed in [1,2]). An increase in the extent of polyandrous mating, however, will not only increase the level of sperm competition, but, all else being equal, will also entail an increase in the number of mating opportunities available for the male. Recent models of ejaculate allocation that take male mating rate into account predict that relative ejaculate size may in some circumstances actually *decrease* as the size of testes and sperm competition intensity (the number of competing ejaculates, above two ejaculates) increase ([2,4]; for another recent model, see [7]): a higher number of mating opportunities could select for males to partition their limited sperm supplies into smaller, but more numerous, ejaculates [2,4]. There is currently little empirical support for these models, other than Katvala *et al.* [8], who found that across bruchid beetles, smaller ejaculates correlated with faster female re-mating rates and larger testes. Vahed [9] similarly showed that, across bushcricket species, there was a negative correlation between relative ejaculate volume and the degree of polyandry in field-mated females, although testis mass was not measured. While these studies appear to support predictions of recent models of ejaculate allocation [2,4], it should be noted that such models have not taken into account the possibility that substances in the ejaculate might directly affect the receptivity of the female to further matings (as occurs in many insect taxa), and thus the degree of polyandry [9].

The present study follows from Vahed [9], with the addition of data on testis mass for largely the same set of bushcricket species. The aims of this study are: (i) to test the prediction that across bushcricket species, testis mass should increase as the level of polyandry increases and (ii) to examine the relationship between testis mass and ejaculate volume and sperm number per mating across species. We also examined the relationship between testis mass and spermatophylax mass (a gelatinous nuptial gift that is attached to the ejaculate-containing ampulla of the spermatophore). Because the spermatophylax appears to function to allow ejaculate transfer, spermatophylax mass may be used as an indirect measure of ejaculate volume (although other hypotheses for spermatophylax function exist, such as paternal investment and the induction of refractory periods in females, reviewed in [10]).

2. MATERIAL AND METHODS

Males of 21 bushcricket species (electronic supplementary material, S1) were collected as adults in mid-August/early September from the field (by K.V. and G. Carron) from a variety of European locations (for further details, see [9]) and were preserved in 75 per cent alcohol

at 4°C until dissection. The species sampled included all but one of those in Vahed [9], plus four further species/sub-species (*Gampsocleis glabra*, *Metrioptera saussuriana*, *Anonconotus alpinus alpinus* and *Anonconotus pusillus*).

Testes were removed, blotted using tissue paper for 5 s and then weighed using an electronic balance to an accuracy of 0.1 mg. The mean mass of the right and left testis for each specimen was calculated before data analysis. Data on ampulla mass (the portion of the spermatophore that contains the ejaculate), total number of sperm per ejaculate, spermatophylax mass and male body mass were taken from the literature [9,11–13]. Additional novel data on sperm number were added for *Tettigonia viridissima*, while novel data on ampulla and spermatophylax mass were added for *Eupholidoptera chabrieri* (using methods given in [11]).

In bushcrickets of the subfamily Tettigoniinae, the lifetime degree of polyandry of a female can be measured directly by counting spermatodoses within the spermatheca (reviewed in [9]). Mean spermatodose counts for field-caught females at the end of the season were taken from Vahed [9], while additional data were obtained for the four *Anonconotus* taxa and for *M. saussuriana* and *G. glabra* using methods outlined in Vahed [9]. For the phaneropterines and bradyporines, the degree of polyandry was taken from the literature. Data were analysed by fitting phylogenetic generalized least-squares models (PGLS) [14,15]. Further details of the analysis are provided in the electronic supplementary material, S2.

3. RESULTS

The absolute mean mass of a single testis ranged from 2.7 mg in *Metrioptera roeselii* to 70.1 mg in *Platycleis affinis* (electronic supplementary material, S1). In proportion to total male body mass, the smallest testis was found in *G. glabra* and *Ephippiger ephippiger* (combined testes = 1% of male body mass in both species), while the largest was found in *P. affinis* (combined testes = 13.8% of male body mass).

In models of testis mass, the two best models incorporated either (i) body mass and ampulla mass only or (ii) body mass and polyandry only (change in Akaike's Information Criterion, corrected for sample size (ΔAICc) < 2; table 1). Accounting for body mass and phylogeny, testis mass was associated positively with polyandry (figure 1) and, in a separate model with similar AICc, was associated negatively with ampulla mass (figure 2), but models including both variables performed poorly (table 1). This may be explained by the fact that, accounting for body mass, ampulla mass and polyandry themselves covaried negatively (PGLS: likelihood ratio test (LRT) statistic = 11.20, d.f. = 1, $p < 0.01$; see also [9]), so models incorporating both variables may be penalized for over-parametrization. Pagel's λ was moderately high (0.56–0.65) for the best models, indicating a degree of phylogenetic structure in testis mass even after accounting for variation in body size and either ampulla mass or polyandry. Models including spermatophylax mass or sperm number performed poorly and we concluded that these were not important predictors of testis mass.

4. DISCUSSION

Testis mass varied considerably across the bushcricket species sampled. It is noteworthy that the species with the largest testes in this study, *P. affinis*, appears to have the largest testes, as a percentage of male body mass, so far recorded for any animal [16]. Testis mass was found to increase with the degree of polyandry across the species sampled—a pattern that has been found in numerous other taxa (reviewed in

Table 1. AICc tables and model coefficients for the top 10 phylogenetic models of testis size, ranked by AICc. Standard errors are given in italics. Key: bm, body mass; amp, ampulla mass; pa, polyandry; sphlx, spermatophylax mass; λ, Pagel's lambda; k, number of parameters; Δ, AICc difference from top model. 'A : B' denotes an interaction term between A and B. The best models (ΔAICc < 2) are given in bold.

| model rank	intercept	± s.e.	amp	± s.e.	bm	± s.e.	pa	± s.e.	sphlx	± s.e.	amp:bm	± s.e.	bm:pa	± s.e.	λ	k	AIC	AICc	Δ	wt
1	**−8.26**	**2.02**	**−0.68**	**0.23**	**1.91**	**0.38**									**0.65**	**5**	**46.35**	**50.35**	**0**	**0.39**
2	**−5.70**	**1.56**			**1.10**	**0.21**	**0.47**	**0.17**							**0.56**	**5**	**47.55**	**51.55**	**1.20**	**0.21**
3	−7.11	2.01			1.68	0.38									0.56	5	48.98	52.98	2.63	0.10
4	−3.97	1.64			0.95	0.23			−0.42	0.17					0.67	4	50.68	53.18	2.83	0.09
5	−7.72	2.06	−0.43	0.31	1.65	0.44	0.26	0.23							0.63	6	48.20	54.20	3.85	0.06
6	−8.74	2.10	−0.51	0.29	2.03	0.40			−0.20	0.21					0.65	6	48.74	54.74	4.40	0.04
7	−11.22	3.43	0.31	0.86	2.44	0.62					−0.17	0.14			0.47	6	49.30	55.30	4.95	0.03
8	−6.31	2.08			1.29	0.49	0.37	0.29	−0.12	0.29					0.58	6	50.02	56.02	5.67	0.02
9	2.41	0.01													−0.13	3	54.80	56.21	5.86	0.02
10	−3.67	2.97			0.79	0.44	−0.57	1.34					0.16	0.21	0.53	6	50.23	56.23	5.89	0.02
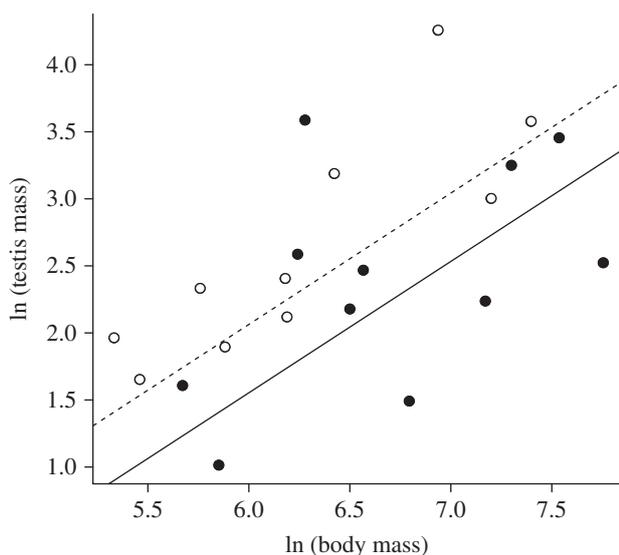
Figure 1. Relationship between testis mass and body mass in bushcricket species with low (filled circles with less than 5.3) and high (open circles with greater than 5.3) degrees of polyandry.
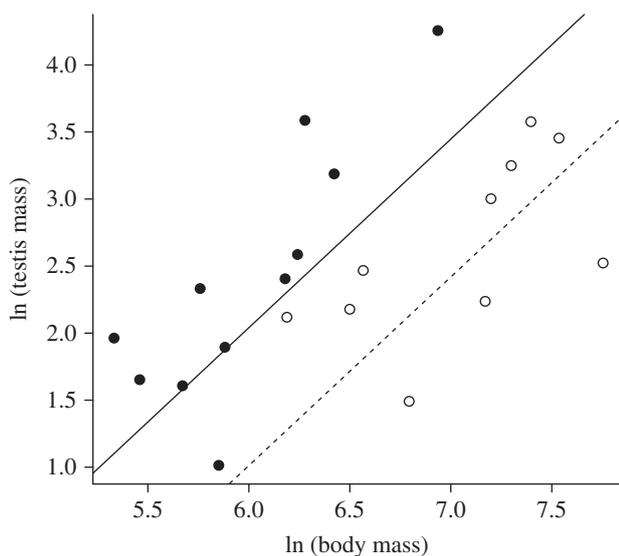

Figure 2. Relationship between testis mass and body mass in bushcricket species with small (filled circles with less than 16 mg) and large (open circles with greater than 16 mg) ampulla mass.

[1,2]). Unlike previous studies, however, we found a negative relationship across species between testis mass and ejaculate volume (ampulla mass) and no significant relationship between testis mass and sperm number (or spermatophylax mass). We also found a negative relationship between ampulla mass and the degree of polyandry across species (as reported previously [9]). Taken together, it could be argued that these data are consistent with the predictions of recent models of ejaculate allocation that take into account the effect of male mating rate [2,4], although insects may not conform to all of the assumptions of these models [9].

The lack of a significant relationship between sperm number and either the degree of polyandry [9] or testis mass (the present study) is consistent with the predictions of Fromhage *et al.*'s [2] model for cases in which

paternity is determined largely by mating order. The pattern of sperm precedence is known for only four of the species in the present study, and this ranges from an apparent fair raffle to pronounced last-male sperm precedence (reviewed in [17]), so it is unclear whether paternity is determined by mating order for the majority of species sampled. Unlike sperm number, ejaculate volume (ampulla mass) was negatively associated with testes mass across species. Variation in ampulla mass may be better explained by variation in non-sperm portions of the ejaculate that might function to affect female reproductive behaviour rather than variation in sperm numbers [18].

The negative relationship between testes mass and ampulla mass found in the present study could be driven by the effects of such non-sperm substances in the ejaculate: it could be that large ejaculates are associated with a much lower number of potential matings for males owing to their negative effects on female receptivity. The costs of producing large ejaculates might further enhance this negative effect on male mating rate [12]. This in turn could relax selection for large testes (as males would not be selected to produce ejaculates in rapid succession), leading to the evolution of smaller testes. Note that the non-sperm substances in the ejaculate that affect female receptivity are likely to be produced by reproductive accessory glands, rather than by the testes, as appears to be the case in *Drosophila* [19]. The negative relationship between testes mass and ampulla mass across species found in the present study could, alternatively, result from a macro-evolutionary trade-off between investment in testes mass and investment in non-sperm portions of the ejaculate. Apparent trade-offs between testes mass and a range of other traits have been documented in previous studies (reviewed in [20]).

Our results highlight the importance of considering male mating rate as a selective pressure influencing testes size, in combination with sperm competition (see also [21]). The lack of a positive relationship between testis size and sperm number and the significant negative relationship between testis size and ejaculate volume also demonstrates that it is unsafe to assume that larger testes necessarily equate with more voluminous ejaculates and/or higher sperm counts.

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