**ORIGINAL ARTICLE**

**Size-dependent investment in tusk length, testis size and sperm length in a biparental geotrupid beetle**

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**Abstract**

Males of extreme small or large sizes often experience different levels of pre- and postcopulatory sexual selection that result in alternative ways to gain fertilization success. Here, we investigated such alternative mating tactics in *Lethrus apterus*, a biparental geotrupid beetle species with noticeable intermale size variation by comparing tusk (mandibular process) length, testis size and sperm length between large and small males. Firstly, we demonstrated evidence for male polymorphism as extremely large males had longer tusks relative to their body size than extremely small individuals. Secondly, large males had bigger testes than small males throughout the breeding season, however, a significant decrease was observed over time in large but not in small individuals. Thirdly, sperm length did not differ between the two size categories. Although these results do not explain how the observed large body size variation can be maintained in *L. apterus*, the non allometric relationship found between tusk length and body size and the lower decrease in testis size over the breeding season in case of small individuals might refer to the presence of alternative mating tactics in this species.

**Introduction**

Males are expected to maximize their reproductive success by optimal allocation of resources between pre- and postcopulatory reproductive traits (Simmons & Emlen, 2006; Simmons & Fitzpatrick, 2016). Precopulatory traits usually consist of high-cost ornaments, armaments or any behaviour with a purpose of gaining access to females (Simmons, Lüpold & Fitzpatrick, 2017), while postcopulatory traits, such as testis size and sperm quality, can influence fertilization success and sperm competitiveness (Simmons et al., 2017). Among these postcopulatory traits, testis size is most often associated with sperm production rate and sperm number (Møller, 1988; Stockley et al., 1997), that is well-known to influence paternity when more than one male’s sperm compete for the fertilization of the eggs, while sperm quality refers to traits such as sperm length, viability or mobility, that can further influence the fertilizing efficiency of the ejaculate even when controlling for sperm number (Snook, 2005). Longer sperm, for example, may be able to swim faster or can be more successful in displacing the sperm of the female’s previous mate, although empirical evidence is mixed (Snook, 2005; Simmons & Fitzpatrick, 2012) and relatively few studies found a positive relationship between sperm length and sperm competitiveness at the intraspecific level (LaMunyon & Ward, 1998, Vadlic, Afzelius & Bronnikov, 2002, Simmons et al., 2003, Simmons, Emlen & Tomkins, 2007).

When males compete to monopolize females, reproductive success depends highly on their precopulatory traits (Parker, 2016). However, when females mate with more than one male, resulting in sperm competition, high postcopulatory investment by males might be more beneficial (Simmons & Fitzpatrick, 2012; Parker, 2016). Indeed, a positive relationship was found between the level of postcopulatory investment and the risk of sperm competition at the interspecific level (Simmons & Fitzpatrick, 2012; Parker, 2016; Simmons et al., 2017), across populations (Brown & Brown, 2003; Dziminski et al., 2010) and in experimental selection lines (Hosken, Garner & Ward, 2001; Simmons & Garcia-González, 2008 but see also Crudgington et al., 2009; McNamara et al., 2016), but differences in relative expenditure on reproductive traits can also exist among males of the same population. These different responses to the reproductive competition are called alternative mating tactics (Taborsky et al., 2008; Buzatto et al., 2014a), which are particularly well studied in horned dung beetles (Scarabaeidae) with intrasexual dimorphism (Simmons & Ridsdill-Smith, 2011). In this group, large individuals often try to monopolize females by guarding the nest tunnel and fighting off rivals, while small males employ a parasitic strategy where they...
sneak into the tunnels to copulate with the female inside (Simmons et al., 2007; Emlen, 2008; Simmons & Ridsdill-Smith, 2011). As sneaks only have access to already-mated females, they usually experience a higher level of sperm competition than large ones, thus, they usually invest more in postcopulatory and less in precopulatory competitive traits (Parker, 1990; Simmons, Tomkins & Hunt, 1999; Neff, Fu & Gross, 2003), although the extent of this difference in expenditure is predicted to decrease as the number of sneaks, and hence the risk of sperm competition for guarding males, increases (Simmons et al., 1999). Another possible, but less discussed explanation of intrasexual polymorphism is that the two tactics are successful under different conditions. For example, small males may be able to avoid direct competition with large rivals and gain reproductive advantage at certain time periods by maturing earlier or by being active longer, as it happens in the Dawson’s burrowing bee (Alcock, 1997) and in some rhinoceros beetles (Eberhard, 1982; Kawano, 1995).

In spite of the great number of studies on alternative mating tactics in the Scarabaeoidea superfamily (e.g. Simmons et al., 2007), so far there is no evidence, to our knowledge, of alternative mating tactics in the Geotrupidae family. Here, we examined expenditure on precopulatory and postcopulatory morphological reproductive traits of males in the biparental beetle, Leathrus apterus (Laxmann, 1770). In this, species pairs build a 50–90 cm deep tunnel that has six to eight brood chambers in which the larva develop (Emich, 1884; Kosztolányi et al., 2015; Rosa et al., 2017). While females collect leaves for the offspring (Kosztolányi et al., 2015), males guard the tunnel against intruders (Frantsevich et al., 1977; Rosa, Bartá & Kosztolányi, 2018) presumably to monopolize access to the female. Sexes are dimorphic, and males have two mandibular processes (tusks) that are most likely under precopulatory sexual selection by serving as a weapon or an ornament. There is a big size variation among males, and small ones are known to be less willing to initiate contests against residents and, on average, are assumed to be less successful in precopulatory competition than large ones (Rosa et al., 2018). Thus, for small males it might be advantageous to adopt alternative mating tactics such as mating opportunistically by sneaking in the tunnels in the absence of the resident male. This way they do not have to deal with the costs of fights, parental care and mate guarding, but in turn they must cope with a high level of sperm competition (Parker, 1990; Taborsky et al., 2008).

In this study we investigated whether male polymorphism is present in L. apterus, and compared tusk length, testis size and sperm length of large and small males to estimate their expenditure on pre- and postcopulatory reproductive traits. If alternative mating tactics exist in this species, we predict that small males invest more in postcopulatory traits (i.e. have larger testes or longer sperm) and less in precopulatory traits (i.e. have shorter tusk) than large individuals. We also examined temporal changes in pre- and postcopulatory traits to test whether the relative advantage of the two tactics differ early and late in the breeding season.

Materials and methods

Field work was carried out during the active period of L. apterus (4 March–25 May) in 2017 on 12 hectares of grassland in Debrecen, Hungary (47°31' 28.6"N 21°34' 30.4"E), where a rather dense population of L. apterus was found. Right tusk length and the maximum width of the pronotum (that we used as a proxy of body size) was measured with a calliper to the nearest 0.01 mm in 231 males that were randomly picked up from the ground and then were released right after the measurements. Similarly, pronotum width of 279 females was also measured. Over 1000 active individuals were present in the population at the time of sampling, thus the probability to include the same individual twice into the sample is negligible.

In order to investigate precopulatory (tusk length) and postcopulatory (testis size and sperm length) traits, male beetles of two extreme size categories (small: pronotum width $\leq$11.00 mm, 14.29% of the population; large: pronotum width $\geq$13.00 mm, 11.69% of the population) were collected early (29 March–06 April; 15 small and 15 large) and late (04 May–12 May; 15 large and 15 small) in the breeding season. After collection and measurement of pronotum width and right tusk length, alive beetles were kept individually in 50 mL tubes at 4°C for 1–4 days until dissection. The number of days elapsed between the day of collection and dissection did not affect testis size (linear regressions, big males: $P = 0.896$; small males: $P = 0.908$) or sperm length (big males: $P = 0.462$; small males: $P = 0.669$).

Individuals were dissected in Ringer solution for insects (Ephrussi & Beadle, 1936) under a Euromex StereoBlue SB.1903 microscope. The reproductive system of L. apterus is similar to that of other Geotrupidae species with well-developed accessory glands and two testes that each consists of six round testicular follicles (Martínez & Trotta-Moreu, 2010). After removing both testes, we separated the 12 follicles and photographed them with a Euromex CMEX-5 USB camera (Euromex Microscopan bv, Arnhem, The Netherlands) using the ImageFocus4 analysing software (version 2.6, Euromex Microscopan bv, Arnhem, The Netherlands) at a magnification of seven. The area of the 12 follicles was measured in pixel units with the elliptical selections tool of the ImageJ software (version 1.51k, Rasband. W., Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA. https://imagej.nih.gov/ij/docs/index.html; Schneider, Rasband & Eliceiri, 2012). Pixel units were converted to $\text{mm}^2$ by determining pixel size using images of millimetre paper that were taken with the same settings. To test the reliability of our measurements we measured 20 follicles, each from different individuals, five times. Measurements were highly repeatable ($R > 0.99$, $P < 0.001$; maximum difference between measurements of the same individual was 1.49%±0.16 mean ± sd; rptR package, version 0.9.21; Stoffel, Nakagawa & Schielzeth, 2017).

For determining sperm length, one follicle from each testis was ruptured and transferred to a slide then pressed with a coverslip. Sperm was photographed under a Euromex iScope 1153.PL-PH microscope (Euromex Microscopan bv, Arnhem, The Netherlands) at 200× magnification. We determined the length of 30 randomly chosen sperm per testis (i.e. 60 per individual) using the segmented line tool in ImageJ. Pixel units were calibrated to mm using the same methods as above. Repeatability was tested by performing five repeated measurements on 20 randomly chosen sperm ($R = 0.99$, $P < 0.001$). The maximum difference between any two measurements on the same sperm was 3.16% ± 0.28 (mean ± sd).

In case of seven males (six small and one large male) collected late in the breeding season, the structure of the testes...
differed from normal ones and we did not find any sperm in their follicles. As the cause of this structural difference was unknown, these males were excluded from all analyses (i.e. final sample size at the end of the season was nine small and 14 large males). All measurements were done without knowledge of the size category of the individuals.

Statistical analysis

All analyses were carried out in R (version 3.5.3; R Core Team, 2018). To analyse the relationship between tusk length and pronotum width, the steps outlined in Knell (2009) were followed. Neither the scatterplot nor the histogram of tusk length (Fig. 1) indicated a discontinuous relationship, so we compared the fit of different models describing continuous relationship similarly to the procedure of Painting & Holwell (2013). In particular, we examined eight models: (1) linear regression, (2) second order orthogonal polynomial regression (using poly function in R), (3) two-parameter power (using nls function and equation \( Tusk = a \times Pronotum^b \)), (4) three-parameter power (using nls function and equation \( Tusk = int + a \times Pronotum^b \)), (5) Weibull growth curve (using nls and SSweibull functions), (6) four-parameter logistic (using nls and SSfpl functions), (7) segmented with one breakpoint and (8) segmented with two breakpoints (segmented function in segmented R package version 0.5.3.0). Segmented models with three breakpoints were unstable between runs and the confidence intervals of the breakpoints overlapped (results not shown), therefore, these models were not investigated further. The fit of the eight models was compared using AIC values.

Historically allometries were investigated on the logarithmic scale (because by taking the logarithm of the variables, a power function simplifies to a linear model), however, there may be issues with this method (Packard, 2009, but see also Kerkhoff & Enquist, 2009), and the use of non-linear fitting methods do not require log transformation. Nevertheless, we also analysed the natural log transformed data (results not shown), and that analysis gave the same best model.

Absolute testis size was calculated as the summed area of the 12 follicles per individual and absolute sperm length was estimated as the mean length of 60 sperms per male. To estimate the relative investment into postcopulatory traits by the two male size categories, we also calculated relative tusk length and relative testis size by dividing the measurements with pronotum width. Data were analysed by two-way ANOVAs where the size category of the individuals (large or small), the time of the breeding season (early or late) and their interaction were the explanatory variables. In case of significant interaction, post hoc comparisons (lsmeans package, version 2.6.3) were used to compare the effect of the breeding season and size categories. Non-significant interactions were removed before testing main effects.

Results

Male polymorphism

We found a notable body size variability in males that was significantly greater than in females (pronotum width of males: 12.11 ± 0.92, \( n = 231 \); females: 11.70 ± 0.74, mean ± se, \( n = 279 \); Levene test for equality of variances, \( F_{1,508} = 7.314, P = 0.007 \)). The scatterplot of tusk length against pronotum width indicated a non-linear, continuous S-shaped relationship in males (Fig. 1). From the eight fitted models the three sigmoid shaped models had the lowest AIC values (Table 1). In particular, the two-breakpoint segmented and the Weibull growth curve performed equally well (\( \Delta AIC = 1.034 \)), whereas the four-parameter sigmoid model had a little worse fit (\( \Delta AIC = 3.021 \)). These models suggest that there are groups of males with either small or large tusks, and a group of intermediate males where tusk length increases sharply with body size. The breakpoints predicted by the two-breakpoint segmented model (10.76 ± 0.129 and 12.39 ± 0.066, estimate ± se) are close to the values that were used in the field to discriminate extreme small and large males (≤11.00 and ≥13.00, respectively).

Tusk length

Similarly, to the population data, males from the large size category had longer absolute \( (F_{1,50} = 926.200, P < 0.001; \) Fig. 2a) and relative \( (F_{1,50} = 699.340, P < 0.001; \) Fig. 2b) tusks than small males independently of the time of the breeding season (absolute tusk length: \( F_{1,50} = 0.403, P = 0.529; \) relative tusk length: \( F_{1,50} = 1.397, P = 0.243 \)). The interaction was not significant in either analysis (absolute tusk length: \( F_{1,49} = 0.635, P = 0.429; \) relative tusk length: \( F_{1,49} = 0.900, P = 0.347 \)).

Testis size

In case of absolute testis size, there was a significant interaction between size and the time of the breeding season \( (F_{1,49} = 5.958, P = 0.018; \) Fig. 3a). Large males had significantly bigger testes than small ones over the breeding season (post hoc comparisons, early: \( t_{1,49} = 8.457, P < 0.001; \) late: \( t_{1,49} = 4.017, P < 0.001 \)). However, absolute testis size decreased significantly over the season in large males \( (t_{1,49} = 5.509, P < 0.001) \), whereas in small males there was no significant change \( (t_{1,49} = 1.601, P = 0.116) \). In case of relative testis size, the interaction between male size and the time of the breeding season was not significant \( (F_{1,49} = 3.459, P = 0.069; \) Fig. 3b). Large males had bigger testes relative to their body size than small ones \( (F_{1,50} = 16.369, P < 0.001) \) and relative testis size decreased significantly over the breeding season in both size category groups \( (F_{1,50} = 30.201, P < 0.001) \).

Sperm length

For sperm length, the interaction between the size categories and the time of the season was not significant \( (F_{1,49} = 1.708, P = 0.197) \). Sperm length did not differ between small and large males \( (F_{1,50} = 0.015, P = 0.903; \) Fig. 4) and was not affected by the time of the breeding season \( (F_{1,50} = 1.256, P = 0.268) \).

Discussion

We showed that small individuals invested less in the measured precopulatory trait (tusk length) and while large
Figure 1 Allometric relationship between tusk length and pronotum width for male Lethrus apterus (n = 231). The solid line shows the fitted values of the segmented model with two breakpoints, the dashed line shows the fitted values of the Weibull growth curve, whereas the dotted line shows the fitted values of the four-parameter logistic model. The vertical solid lines show the estimated breakpoints of the segmented model and the vertical broken lines show the 95% confidence limits of the breakpoints. The histogram on the top shows the distribution of male pronotum width with kernel density estimation, whereas the histogram on the right shows the distribution of male tusk length with kernel density estimation. The rugs along the bottom axis of the plot show the distribution of female pronotum width (n = 279). The line drawings illustrate the head and pronotum of a large and a small male.
Table 1 Comparison of model fits describing the relationship of tusk length and pronotum width of male *Lethrus apterus* (*n* = 231). ΔAIC is calculated between the best and the actual models.

| Model                          | AIC   | ΔAIC  | Model parameters                        |
|-------------------------------|-------|-------|-----------------------------------------|
| Segmented with two breakpoints| 114.795 | 0.000 | Intercept = –3.17                      |
|                               |       |       | Slope 1 = 0.40                         |
|                               |       |       | Slope 2 = 1.47                         |
|                               |       |       | Slope 3 = 0.41                         |
|                               |       |       | Breakpoint 1 = 10.76                   |
|                               |       |       | Breakpoint 2 = 12.39                   |
| Weibull growth curve          | 115.829 | 1.034 | Asymptote = 3.92                       |
|                               |       |       | Drop = 3.36                            |
|                               |       |       | In rate constant = –37.51              |
|                               |       |       | Power = 15.21                          |
| Four-parameter logistic       | 117.816 | 3.021 | Lower asymptote = 0.66                 |
|                               |       |       | Upper asymptote = 4.10                 |
|                               |       |       | Scale = 0.52                           |
|                               |       |       | Inflection point = 11.57               |
| Segmented with one breakpoint | 179.748 | 64.953 | Intercept = –10.49                     |
|                               |       |       | Slope 1 = 1.12                         |
|                               |       |       | Slope 2 = 0.44                         |
|                               |       |       | Breakpoint = 12.59                     |
| Linear regression             | 201.915 | 87.120 | Intercept = –9.42                      |
|                               |       |       | Slope = 1.03                           |
| Three-parameter power         | 203.621 | 88.826 | Intercept = –7.28                      |
|                               |       |       | a = 0.49                               |
|                               |       |       | b (power) = 1.22                       |
| Second order orthogonal polygon | 203.819 | 89.024 | Intercept = 3.04                       |
|                               |       |       | Slope 1 = 14.35                        |
|                               |       |       | Slope 2 = 0.11                         |
| Two-parameter power           | 252.999 | 138.204 | a = 4.93 × 10^-6                     |
|                               |       |       | b (power) = 4.41                       |

Figure 2 Absolute (a) and relative (b) tusk length in large (black circles) and small (white circles) *Lethrus apterus* males early (large males: *n* = 15, small males: *n* = 15) and late (large males: *n* = 14, small males: *n* = 9) in the breeding season.

individuals showed a significant decrease in testis size over time, it was not present in small males. However, contrary to our predictions, large males had significantly bigger testes throughout the breeding season even after controlling for body size, and sperm length did not differ significantly between the two groups.

Our results show that males in *L. apterus* are polymorphic with respect to tusk length, similarly to many species with alternative mating tactics (Moczek & Emlen, 2000; Simmons *et al.*, 2007; Buzatto *et al.*, 2014a). Among the three groups that were predicted by the segmented model, the large size category contained the largest number of individuals (46.8%), while the small category contained only 9.5% of the males. The model also distinguished an intermediary group, that contained 43.7% of the individuals. According to Parker’s (1990) game theory model, differences in gametic expenditure
between small and large males should decline as the frequency of minor (or sneak) males in the population increases. Supporting this prediction, Simmons et al. (1999) showed that in the dung beetle *Onthophagus binobis*, where 30% of the males were minors, small individuals possessed larger testes and longer sperm than major males, while in *Onthophagus taurus*, where the ratio of small individuals was more than 60%, no difference in gametic traits was observed. Even though, the low ratio of small males found in *L. apterus* is even less than what was observed in *O. binobis*, we did not find signs of higher postcopulatory investment in small individuals. The fact that large males had relatively bigger testes than small individuals is not an unexpected result as positive correlation between reproductive traits and body size has been observed in many species (Evans & Garcia-Gonzalez, 2016). Additionally, the relatively bigger testes of large males can be associated with more frequent copulations (probably with the social partner) that requires higher sperm production rate (Vahed & Parker, 2012). Similar results were found for example in sunfish (Neff et al., 2003) and in yellow dung flies (Blanckenhorn et al., 2004). The decrease of testis size in both groups during the breeding season is an interesting result, that could have been caused by sperm depletion or by the preparation for the inactive period until the arrival of the next spring (*L. apterus* lives for more than 1 year, and spends the inactive period buried underground), which might also explain why no sperm was found in seven individuals late in the breeding season (which were excluded from the analyses). The less intense decrease in absolute testis size in case of small individuals suggests that they may be able to gain fertilization advantage during the later periods. It is also possible that the observed decrease in testis size is caused by differences in the average age of the active beetles early and late in the breeding season as testis shrinkage over age was shown in Lepidoptera (Hiroyoshi & Reddy, 2018) and older individuals may be more eager to exploit every last mating opportunity and hence start preparing for the inactive period later than 1-year old ones. It is important to note, however, that more small individuals were excluded due to abnormal testis structure and a lack of sperm late in the season than large one (six and one, respectively), which might have contributed to these results.

While testis size and tusk length seem to depend more on the individual’s condition, the lack of difference in sperm length between large and small individuals suggests that sperm has an optimal length regardless of body size which is expected under strong stabilizing postcopulatory sexual selection towards superior sperm quality (Calhoun, Ihmler & Birkhead, 2007; Fitzpatrick & Baer, 2011). Thus, the observed canalization of sperm length can be a sign of high level of sperm competition where sperm quality is a more important determinant of competitiveness than testis size. In the light of this finding, it seems possible that both size categories, and not just small individuals, are under high-risk sperm competition.
Nevertheless, even though large males had larger testes and longer tusks than small individuals throughout the breeding season, this does not necessarily mean that they have higher reproductive advantage as there may be other, unmeasured variables affecting the fertilization success of males, for example, sperm viability (Hunter & Birkhead, 2002), accessory gland size (Crudgington et al., 2009; Simmons & Fitzpatrick, 2012), strategy-specific behaviour (e.g. copulation duration, Taborsky et al., 2008) or the relative length of the different sperm components (head, mid-piece, flagellum) that has been shown in other taxa to influence the competitive ability of sperm (Simpson et al., 2013). However, a difference in the reproductive success between small and large males is also possible, providing that the adopted reproductive strategies depend on developmental constrains (e.g. the amount of food resources available during the larval stage) and not on genetics.

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In species with significant intermale size variation, small individuals are often expected to adopt alternative mating tactics to ensure reproduction, for example by investing less in their pre- and more in their postcopulatory reproductive traits. We investigated this pattern in a biparental beetle, Lethrus apterus by comparing tusk (mandibular process) length, testis size and sperm length of extremely large and small males. We showed that males in this species are polymorphic in respect to tusk length as large individuals possess longer tusks relative to their body size. However, contrary to our predictions, large individuals also had relatively bigger testes than small ones and sperm length did not differ between the two size categories. These results do not explain the observed body size variation in this species, but indicate that tusk length and testis size depend mostly on individual condition, while sperm may have an optimal length regardless of body size.