



# The evolution of relative trait size and shape: insights from the genitalia of dung beetles

Harald F. Parzer<sup>1,2</sup> · P. David Polly<sup>1,3</sup> · Armin P. Moczek<sup>1</sup>

Received: 25 April 2017 / Accepted: 9 January 2018  
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## Abstract

Insects show relatively little genital variation within species compared to extraordinary and often rapid diversification among species. It has been suggested that selection for reproductive isolation through differences in genital shape might explain this phenomenon. This hypothesis predicts that populations diverge faster in genital shape than in genital size. We tested this prediction in males from 10 dung beetle species with known phylogenetic relationships from the genus *Onthophagus* (Coleoptera: Scarabaeidae), including four species for which we were able to sample multiple populations. Specifically, we compared intra- and interspecific differentiation in shape and relative sizes of genitalia and calculated their respective evolutionary rates. We compared these rates to two similarly sized non-genital traits, the head and the fore-tibia. We found significant intraspecific variation in genital shape in all four species for which multiple populations were sampled, but for three of them we also identified significant relative size variation. We also found that genital shape evolved at higher rates than relative genital size. Genital shape evolved faster than head shape, but not fore-tibia shape. However, shapes of all measured structures evolved faster than their relative size. We discuss the functional constraints that may bias the developmental evolution of relative size and shape of genitalia and other morphological traits.

**Keywords** *Onthophagus* · Genitalia · Morphological evolution · Shape · Size · Evolutionary rates

## Introduction

Species diversity in animals is dominated by one taxon, the insects. According to an estimate by the International Union for the Conservation of Nature (IUCN), about 60% of all

described species are insects (IUCN 2008). In most insect groups, closely related species appear very much alike such that most traits cannot be used to reliably discriminate among species (e.g., Grimaldi and Engel 2005, for examples in specific taxa see Grimaldi and Nguyen (1999) for Diptera, Scoble (1995) for Lepidoptera, and Aspöck et al. (1991) for Raphidioptera). This implies that, despite high speciation rates, morphological disparity is often very low among closely related species. However, one class of traits represents a noteworthy exception to this rule: the male copulatory organ (aedeagus, sg.; aedeagi, pl.). Here, an astounding morphological diversity is found even among very closely related species (for an excellent overview, see Eberhard (1985)), which is at times so extreme that it is difficult to homologize aedeagal structures across species (e.g., Tuxen 1970; Scudder 1971).

Due to the close relationship between copulatory organs and reproduction, it has been hypothesized that genital divergence could play an important role in speciation (McPeck et al. 2008; Estrada and Prieto 2011; Bath et al. 2012; Kamimura and Mitumoto 2012; Richmond et al. 2012). The wide range of species-specific morphologies found among male copulatory organs of closely related species is consistent with this notion (Eberhard 1985; Grimaldi and Engel 2005). Analyses of

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This article is part of the Special Issue “Size and Shape: Integration of morphometrics, mathematical modelling, developmental and evolutionary biology”, Guest Editors: Nico Posnien—Nikola-Michael Ppirc.

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Communicated by Nikola-Michael Ppirc

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s00427-018-0602-2>) contains supplementary material, which is available to authorized users.

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✉ Harald F. Parzer  
[hparzer@fdu.edu](mailto:hparzer@fdu.edu)

<sup>1</sup> Present address: Department of Biology, Indiana University, Bloomington, IN, USA

<sup>2</sup> Department of Biology and Allied Health Science, Fairleigh Dickinson University, Madison, NJ, USA

<sup>3</sup> Department of Earth and Atmospheric Sciences, Indiana University, Bloomington, IN, USA

divergence patterns of male genitalia in damselflies have shown that, even though conspecific populations strongly overlap in genital shape, interspecific shape variation appears to arise during speciation events with little or no morphological change occurring after speciation (McPeck et al. 2008, 2011). Thus, regardless of time since common ancestry, male damselfly genitalia are similarly divergent. This finding led to the conclusion that either speciation itself drives aedeagal divergence or speciation is driven by the divergence of genitalia (McPeck et al. 2008). More generally, the rapid diversification of male copulatory organs observed in a wide variety of taxa has been suggested by Bertin and Fairbairn (2007) to pose an apparent “paradox,” because genital variation is limited *within* species studied so far (Eberhard et al. 1998; Hosken and Stockley 2004; McPeck et al. 2011), yet the phenotypic differences *among* closely related species are large and appear to arise quickly (e.g., Eberhard 1985; Grimaldi and Engel 2005; McPeck et al. 2008). In other words, the available data suggest that high rates of evolution are seen in a trait that has comparatively low phenotypic variance (and presumably low genetic variance). This raises the possibility that intraspecific variation may reside in genital traits that have so far escaped analysis, such as genital shape, relative genital size, or a combination of the two, which in natural populations may be able to fuel rapid interspecific divergence relative to non-genital traits.

Based on preliminary data and visual inspection, Eberhard hypothesized that shape is the more important factor for morphological divergence than relative genital size (Eberhard et al. 1998; Eberhard 2009; Eberhard et al. 2009). Two predictions can be derived from this hypothesis: first, if male genital shape is the primary factor in divergence, then it is expected that divergence between populations should be greater in genital shape than genital size and thus shape and size should evolve at different rates relative to their within-species variation. And second, genital shape is expected to evolve at comparatively higher rates than the shape of non-genital traits.

To test these predictions, we compared patterns of genital variation of both shape and relative size to the corresponding patterns of variation in fore-tibiae and heads in the dung beetle genus *Onthophagus*, an increasingly recognized study system for the evolution and development of insect genitalia (e.g.,

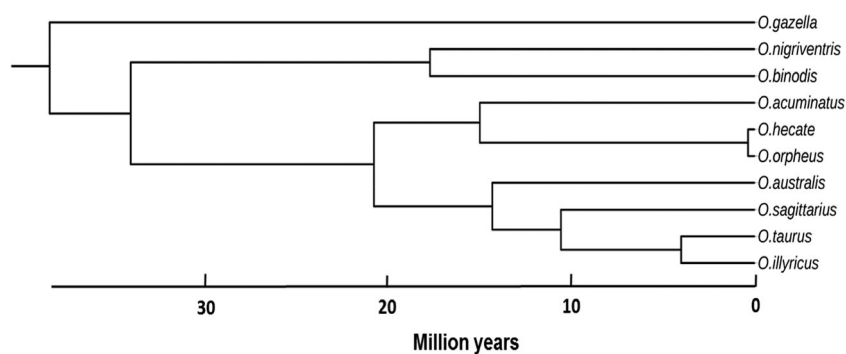
Palestrini et al. 2000; House and Simmons 2003, 2005, 2006, 2007, 2012; Moczek and Nijhout 2004; Pizzo et al. 2006a, 2006b, 2008, 2011, 2012; Simmons and Emlen 2006; Parzer and Moczek 2008; Werner and Simmons 2008; Simmons et al. 2009; Macagno et al. 2011a; Macagno et al. 2011b; Simmons and Garcia-Gonzales 2011; Snell-Rood and Moczek 2012). Even though *Onthophagus* species can often be distinguished based on external morphological traits, in particular male secondary sexual traits like horns, a large number of species can only be reliably distinguished based on male genitalia (well-studied examples from just the Mediterranean include five species within the *ovatus* group (Piera and Zunino 1986), the *fracticornis-similis-opacicollis* and *vacca* complex (Macagno et al. 2011b; Roessner et al. 2010); and the sister species *O. taurus* and *O. illyricus*, which were also used in this study (see below and Pizzo et al. 2006b)). Thus, new *Onthophagus* species description commonly rely on morphological differences of the aedeagus (e.g., Krikken and Huijbregts 2008; Tarasov and Kabakov 2010). For the purposes of this study, we focused on 10 *Onthophagus* species with known phylogenetic relationships (Pizzo et al. 2006b; Emlen et al. 2005; Fig. 1). Even though behavior, ecology, and natural history have been studied for three of these species (*Onthophagus acuminatus*, *O. binodis*, *O. taurus*, e.g., Hanski and Cambeftor 1991; Emlen 1997; Hunt and Simmons 2002; Price 2004; Moczek and Cochrane 2006; Simmons and Kotiaho 2007), relatively little is known about the remaining taxa. In addition, we measured intraspecific variation for all three traits in four of the 10 species and used phylogenetically corrected measurements across the full set of 10 species to compare and contrast evolutionary rates of size and shape.

## Materials and methods

### *Onthophagus* populations

To estimate intraspecific variation of the aedeagus (ae), head (h), and fore-tibia (t), individuals of four *Onthophagus* species, of which multiple populations were available, were dissected by HFP (Table 1). All specimens were collected in the

**Fig. 1** Phylogenetic relationships and divergence times for the 10 *Onthophagus* species used in this study (modified after Emlen et al. 2005; Pizzo et al. 2006)



**Table 1** Locations of *Onthophagus* populations and sample sizes for aedeagus ( $n_{\text{aedeagus}}$ ), head ( $n_{\text{head}}$ ), and fore-tibia ( $n_{\text{tibia}}$ )

Species	Location	$n_{\text{aedeagus}}$	$n_{\text{head}}$	$n_{\text{tibia}}$
<i>O. australis</i>	Narrikup, Australia	8	8	8
	Adelaide, Australia	1	1	1
	Tallangata, Australia	1	n/a	1
	Canberra, Australia	23	24	22
	Cootamundra, Australia	1	1	1
<i>O. binodis</i>	Braidwood, Australia	8	9	9
	Canberra, Australia	6	8	8
	Sutton, Australia	4	5	5
	Waimea, Hawaii	21	24	23
<i>O. gazella</i>	Serengeti National Park, Tanzania	4	3	22
	Masai Mara, Kenya	2	3	3
	King William's Town	2	2	2
	Garsen, Kenya	4	4	4
	Galana Game Ranch, Kenya	2	3	2
	Caconda, Angola	1	1	n/a
	Gweru, Zimbabwe	1	1	1
	Maun, Botswana	1	1	1
	Nyanga, Zimbabwe	1	1	1
	Marble Hall, South Africa	3	3	3
	Ka'a'awa, Oahu, Hawaii	22	28	26
<i>O. taurus</i>	Sylvania, USA	7	5	5
	Canberra, Australia	26	24	24
	Bussoleno, Italy	18	16	13
	Durham, NC, USA	33	18	19
	Tumut, Australia	27	22	20

field by APM or collaborators and stored in ethanol (80%). The male copulatory organs were dissected by hand from each specimen and were stored in ethanol (80%). Export of specimens from Australia was authorized under Commonwealth of Australia Wildlife Protection Permits 04/04684 and 04/08351 to APM. No import permit was required to import preserved Australian dung beetles into the USA. Specimens are stored and available at Indiana University, Bloomington, USA.

### *Onthophagus* species

For the analysis of interspecific evolutionary rates of size and shape of the aedeagus, head, and fore-tibia, individuals of 10 *Onthophagus* species were dissected by HFP (Table 2). All species were collected in the field by APM or collaborators, dissected, and stored in ethanol as described above.

### Divergence times

Divergence times were estimated for the phylogenetic tree of the *Onthophagus* species using the nuclear and mitochondrial

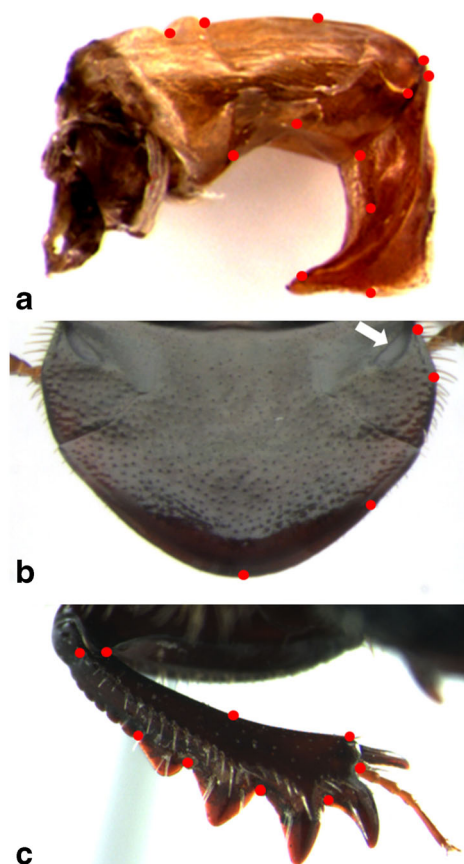
**Table 2** Locations of 10 *Onthophagus* species and sample sizes for aedeagus ( $n_{\text{aedeagus}}$ ), head ( $n_{\text{head}}$ ), and fore-tibia ( $n_{\text{tibia}}$ )

Species	Location	$n_{\text{aedeagus}}$	$n_{\text{head}}$	$n_{\text{tibia}}$
<i>O. acuminatus</i>	Santa Rosa, Guatemala	4	4	4
<i>O. australis</i>	Combined locations (Table 1)	39	36	35
<i>O. binodis</i>	Combined locations (Table 1)	43	47	47
<i>O. gazella</i>	Combined locations (Table 1)	43	50	50
<i>O. hecate</i>	Durham, NC, USA	4	4	3
<i>O. illyricus</i>	Bussoleno, Italy	19	25	25
<i>O. nigriventris</i>	Waimea, Hawaii, USA	18	20	25
<i>O. orpheus</i>	Hutcheson Memorial Forest, NJ, USA	5	9	9
<i>O. sagittarius</i>	Ka'a'awa, Oahu, Hawaii, USA	29	20	20
<i>O. taurus</i>	Combined locations (Table 1)	37	29	29

sequence data published by Emlen et al. (2005) with the “r8s” algorithm (Sanderson 2006), assuming a mutation rate of 1.2% sequence divergence per million years (Brower 1994), as has been done previously for *Onthophagus* (Pizzo et al. 2006b). Two methods were used, Langley-Fitch and Penalized Likelihood methods (for details see Langley and Fitch 1974; Sanderson 2002), which yielded similar divergence times and which were averaged to reduce error. Note that time calibration does not affect the tests for differences in rate between traits because a different calibration would affect all rates equally.

### Geometric morphometric analysis

Shape of the aedeagus, head, and fore-tibia was calculated with geometric morphometric analysis using a landmark-based approach (Bookstein 1991). To do so, we placed each dissected body part in a horizontal plane using a plasticine supporter and photographed it with a digital camera (Scion, Frederick, MD, USA) under a stereoscope (Leica MZ-16, Bannockburn, IL, USA). Thin plate spline format (tps) files were created with tpsUtil (Rohlf 2010a) and subsequently, landmarks (lm) were digitized with tpsDig2.16 (Rohlf 2010b) on each image of the aedeagus (lm = 12) and the fore-tibia (lm = 9) (Fig. 2). Due to object symmetry in the landmark configuration of the head, we used only the right side (lm = 4). To minimize error, photographing and placing the landmarks were done by the same person (HFP) for all species, populations, and traits. All landmarks were homologous and represented by clear, easily recognized features. For each trait, a least-squares Procrustes superimposition (Rohlf and Slice 1990; Zelditch et al. 2004) was conducted to remove differences in rotation, translation, and scaling. Shape variables were obtained from a principal components analysis (Dryden and Mardia 1998) using MorphoJ (Klingenberg 2011) and the Morphometrics for Mathematica packages (Polly 2016a). Shape differences were



**Fig. 2** Position of landmarks (lm) placed on the aedeagus (lm = 12), head (lm = 4) and fore-tibia (lm = 9). Images were taken from *Onthophagus gazella*. Landmarks of the aedeagus were placed onto the endophallus (7 lm) and right paramere (5 lm). Landmarks of the head were placed on each of the following: anterior margin of the eye (white arrow indicates location of the left eye), widest point of head, and anterior margin of the head, and the midpoint between the latter two. Landmarks of the fore-tibia were placed onto the most proximal and distal point of the tibia, the midpoint between the latter, and between each tibial tooth. Tibial teeth were not used due to the variance induced by wear

measured in Procrustes distances, which is the square-root of the sum of the squared distances between the corresponding landmarks of two Procrustes superimposed objects (Bookstein 1991). These distances can be calculated from the Procrustes superimposed landmarks or shape variables.

Centroid size (the square root of the sum of squared distances of all landmarks from their centroid) was obtained through MorphoJ (Klingenberg 2011) and used to estimate trait size (e.g., Bookstein 1991; Workman et al. 2002). Since we were interested in relative size evolution across taxa, we divided centroid size by pronotum length, a common estimate of body size in *Onthophagus* beetles (Emlen 1994). Pronotum length was measured by using digital images obtained with the same setup as described for each dissected body part and further analyzed with the software program ImageJ (Schneider et al. 2012).

## Variation among populations and species

To test for shape differences among populations and species, we used a MANOVA on the principal components scores. To test for relative size difference, we used a one-way ANOVA on relative centroid size for each of the traits. Significance of ANOVA was tested with F-test and MANOVA with Wilk's lambda ( $\lambda$ ), a multivariate generalization of the F-test. All statistical analyses were conducted in Minitab 17 Statistical Software (Minitab 17 Statistical Software 2010). We used sequential Bonferroni corrections to test for significant differences in shape and size for each trait in all species pairs (Supp. Table 1).

## Estimation of evolutionary rates

Evolutionary rates of size and shape were estimated in standard deviation units using phylogenetically independent contrasts (PIC; Martins and Garland 1991; Garland 1992) method on Mahalanobis distances, which are pairwise measures of univariate or multivariate trait differences scaled in variance units (Mahalanobis 1936), adjusted for the number of variables in each trait. Specifically, we used the PIC algorithm to estimate evolutionary rate from standardized independent contrasts, which are phylogenetically independent pairwise phenotypic distances scaled to unit branch length (Felsenstein 1985). Each standardized contrast is an independent estimate of the amount of divergence per unit time, so the overall least squares estimate of the rate is the mean of the contrasts (Martins and Garland 1991; Garland 1992). With the caveats discussed below, this approach produces rate estimates comparable to the phylogenetic generalized least squares (PGLS) method (Martins and Hansen 1997), its multivariate likelihood-based extensions (O'Meara et al. 2006; Revell and Harmon 2008), and newer multivariate distance-based algorithms (Adams 2014). Each PIC was measured using the square root of the Mahalanobis distance ( $D$ ) normalized by the number of degrees of freedom (dimensions) of each trait. For each trait ( $x$ ), we estimated the mean relative size and shape and associated pooled within-species covariance matrices for each species. For size traits, this simply meant calculating the mean for each species and the average within-species trait variance. For shape, each trait was Procrustes superimposed and transformed to shape variables. The mean and covariance matrix for each species were calculated using the first 10 shape variables (which accounted for more than 97% of shape variance for all three traits). For each PIC, the normalized Mahalanobis  $D$  was calculated as,

$$D = \sqrt{\frac{(\bar{x}_i - \bar{x}_j)^T S^{-1} (\bar{x}_i - \bar{x}_j)}{n}} \quad (1)$$



where  $(\bar{x}_i - \bar{x}_j)$  are the distances between the endpoints of each contrast,  $S^{-1}$  is the inverse of the pooled covariance matrix (or the mean within-species variance in the case of size), and  $n$  is the number of degrees of freedom (1 for size and  $2k - 4$  for shape, where  $k$  is the number of landmarks and 4 degrees of freedom are removed by Procrustes analysis with rescaling, rotating, and translating in two dimensions). Mahalanobis distances are scaled by the multivariate covariance structure of the data, so the square roots of the rates are equal to rates scaled in standard deviation (SD) units. This scaling allows size and shape traits to be compared even though they are measured in different units and are composed of different numbers of variables (Turelli et al. 1988; Lynch 1990; Amegard et al. 2010; Carlson et al. 2011). Our rates are thus normalized for differences in trait dimensionality and are expressed in SD units, thus allowing them to be compared.

Note that if there are strong outliers among the contrasts, such as when one branch is evolving notably faster than others, then the rate estimate will be unduly affected by that outlying point. In our data, the contrast between *Onthophagus orpheus* and *Onthophagus hecate*, which are recently diverged sister species, was unusually large given their very recent common ancestry (Supp. Fig. 1). This contrast was therefore excluded to avoid inflating the overall rate estimates (see “Results” and “Discussion” below).

Standard errors on the rates were estimated by bootstrap (Manly 2007). To do this, random subsamples were drawn 10,000 times with replacement from the contrasts and the rate was re-estimated from each subsample. Rate calculations were performed using the *Phylogenetics for Mathematica* and *Morphometrics for Mathematica* packages (Polly 2016a, b).

Note that our approach produces a single evolutionary rate for each trait, regardless of whether it is univariate (size) or multivariate (shape). Our rates are therefore unlike Revell and Harmon’s (2008) multivariate rate matrices, which contain a

separate rate for each individual variable and covariances between rates, and like Adam’s (2014)  $\sigma^2$ -multi, which provides a single overall rate for a multivariate shape trait (indeed our approach yields identical results if  $\sigma^2$ -multi is scaled in standard deviate units). We chose to use a single rate for our shape traits because the individual variables are landmark coordinates, which are not biologically interesting in their own right, and to compare the overall rate of shape evolution to size. Rates which were more than two standard errors apart were considered as significantly different.

## Results

### Within-species variation

Populations within a single species were significantly different in some of the traits analyzed (Table 3). This was particularly pronounced in *Onthophagus australis* and *O. taurus*, in which all traits exhibited significant differences among their respective populations in mean shape and mean size (with the exception of fore-tibia size in *O. australis*) (Table 3). *O. binodis* populations exhibited significant differences in shape, but not in size, in all traits measured (Table 3). In *Onthophagus gazella*, aedeagus and head shape, as well as aedeagus size exhibited significant differences among populations (Table 3). These results are further illustrated in supplemental Figs. 2–5. Due to small sample sizes of some of the populations, we reanalyzed our data yet included only populations with three or more individuals. This re-analysis confirmed all earlier results with the exception of aedeagus and head sizes between *O. australis* populations which were no longer significantly different (Supp. Table 2).

**Table 3** Shape and size divergence of aedeagus, head, and fore-tibia between populations

Species	Trait	Wilks’ $\lambda$	Shape divergence	F-value	Size divergence
<i>O. australis</i>	Aedeagus	0.000491	$p < 0.0001^{***}$	11.45	$p < 0.0001^{***}$
	Head	0.02669	$p < 0.0001^{***}$	20.55	$p < 0.001^{**}$
	Fore-tibia	0.05154	$p < 0.0001^{***}$	0.7	$p > 0.05$
<i>O. binodis</i>	Aedeagus	0.04502	$p < 0.0001^{***}$	0.6	$p > 0.05$
	Head	0.42276	$p < 0.01^{**}$	0.23	$p > 0.05$
	Fore-tibia	0.21493	$p < 0.0001^{***}$	2.2	$p > 0.05$
<i>O. gazella</i>	Aedeagus	0.01302	$p < 0.05^*$	2.26	$p < 0.05^*$
	Head	0.12165	$p < 0.05^*$	1.87	$p > 0.05$
	Fore-tibia	0.2352	$p > 0.05$	0.51	$p > 0.05$
<i>O. taurus</i>	Aedeagus	0.21202	$p < 0.0001^{***}$	59.03	$p < 0.05^*$
	Head	0.42180	$p < 0.0001^{***}$	4.63	$p < 0.01^{**}$
	Fore-tibia	0.36242	$p < 0.0001^{***}$	4.35	$p < 0.01^{**}$

## Variation among species

We detected significant differences in shape and size variation for the aedeagus, head, and fore-tibia at the species level (Table 4, Fig. 3, and Supp. Table 1).

## Evolutionary rates

*Onthophagus orpheus* and *O. hecate* exhibited far greater phenotypic differences in all traits except fore-tibia size compared to their very recent phylogenetic divergence when compared with any other pair of species (Supp. Fig. 1). The large change over a short divergence time results in an uncharacteristically high rate of evolutionary change for this pair, which created an extreme outlier in the standardized independent contrasts used to estimate rates of evolution. We therefore excluded this pair from rate calculations to prevent upward bias in the rate estimations, although for reference we report rates calculated with (Supp. Fig. 6) and without this pair (Fig. 4).

The rate of evolution of aedeagus shape ( $0.54 \pm 0.09$ ;  $0.64 \pm 0.12$  SD/myr with *O. orpheus* and *O. hecate*) was significantly higher than size ( $0.29 \pm 0.07$ ;  $0.60 \pm 0.29$  SD/myr with *O. orpheus* and *O. hecate*) (Fig. 4). Aedeagus shape evolved faster than both fore-tibia shape ( $0.42 \pm 0.05$ ;  $0.59 \pm 0.17$  SD/myr with *O. orpheus* and *O. hecate*) and head shape ( $0.39 \pm 0.04$ ;  $0.80 \pm 0.39$  SD/myr with *O. orpheus* and *O. hecate*), but relative aedeagus size evolved at about the same rate as relative fore-tibia size ( $0.27 \pm 0.07$ ;  $0.26 \pm 0.06$  SD/myr with *O. orpheus* and *O. hecate*), both of which evolved much faster than relative head size ( $0.08 \pm 0.02$ ;  $0.21 \pm 0.13$  SD/myr with *O. orpheus* and *O. hecate*). While evolutionary shape rates of all traits are not significantly different from each other when the outliers *O. orpheus* and *O. hecate* are included, the evolutionary rates for head and fore-tibia size remain significantly lower than all evolutionary shape rates.

## Discussion

In this study, we compared and contrasted phenotypic variation of the relative size and shape of male copulatory organs, as well as their evolutionary rates, to that of heads and fore-tibiae across multiple dung beetles species. Several important

results emerge. First, male copulatory organs exhibited significant shape differences among conspecific populations of all four species examined, whereas among-population differences in relative size were significant in three of the four species. At the same time, *interspecific* comparisons across 10 species revealed significant differences in both shape and size of the aedeagus. Second, evolutionary rates for copulatory organ shape were almost twice as high as the evolutionary rate for copulatory organ size. This supports the hypothesis that genital divergence may be fueled by changes in shape and, to a lesser degree, by the evolution of relative size (e.g., Eberhard et al. 1998). Surprisingly, while the rate of genital shape evolution was higher than that of the head, it was not significantly different to that of the fore-tibia. We discuss the most important implications of each of these findings below.

## Intra- and interspecific variation

As predicted, populations in all four species analyzed in this study exhibited significant among-population variation in aedeagus shape. However, three of the species also showed significant among-population variation in aedeagus size, albeit to a lesser degree. Due to the small sample size of some of the populations, we reanalyzed our data including only populations with samples of three or more individuals. These results still show significant among-population variation in copulatory organ size for populations in two species (Supp. Table 2). Thus, our results provide only modest support for the hypothesis that the ratio of among-population shape variation to within-population shape variation exceeds the corresponding ratio for size variation. Similarly, we found significant among-population variation in head shape in all four species measured, and in fore-tibial shape in three of the four species. Lastly, we found significant among-population variation in relative head and fore-tibia size in a subset of species.

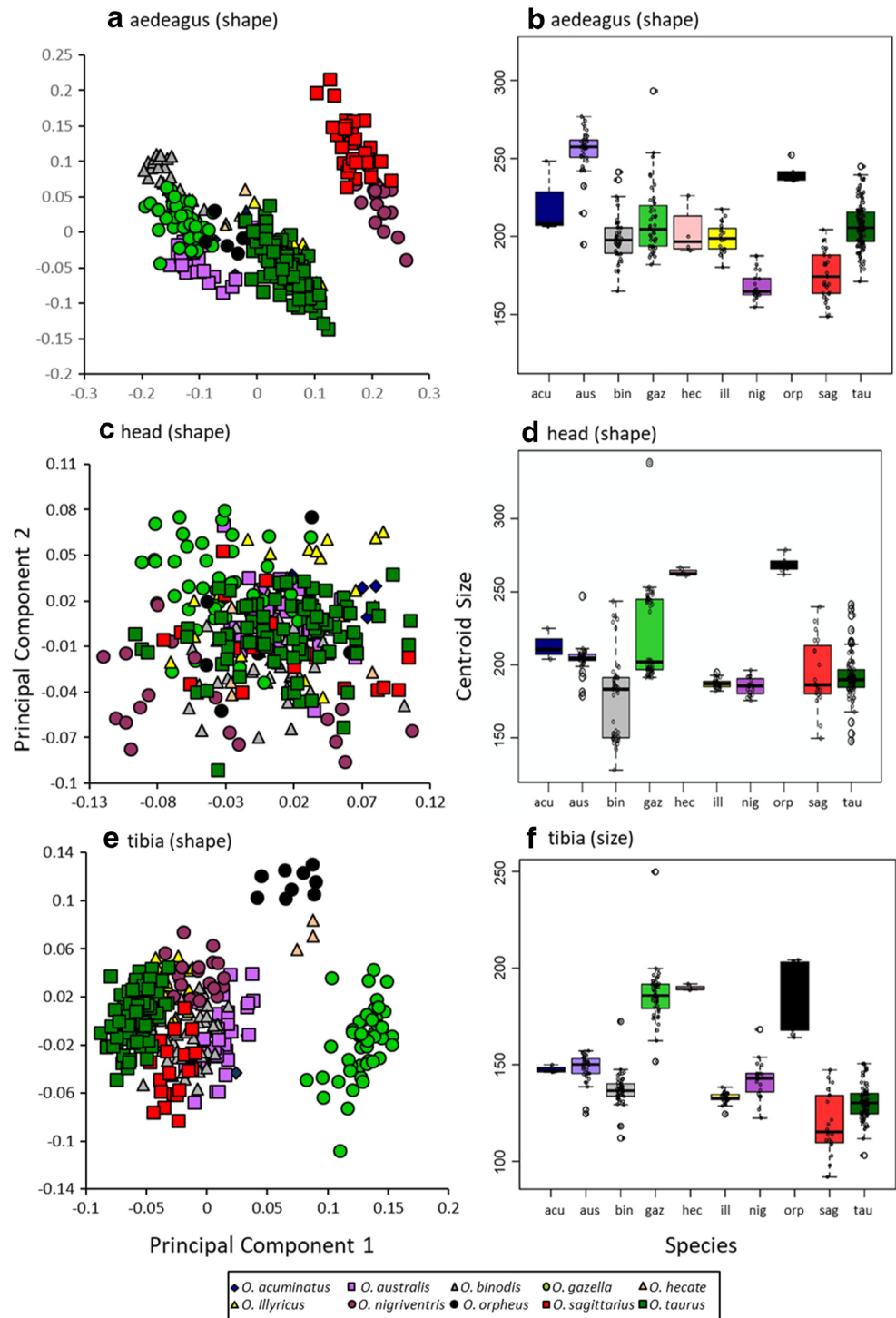
Interspecific comparisons across 10 species revealed significant differences for both genital shape and size. However, the estimated evolutionary rates indicate that this variation is significantly greater for aedeagal shape compared to size. Thus, if confirmed in other taxa (but see McPeck et al. 2011), this pattern is consistent with the hypothesis that within-species variation in shape may indeed contribute significant morphological substrate to facilitate rapid phenotypic divergences in genitalia among recently formed species, as hypothesized originally by Eberhard et al. (1998). Stabilizing selection on relative genital size in combination with diversifying selection on genital shape may account for the observed pattern.

Interestingly, clusters are formed by different species depending on the trait measured. For example, in our genital shape analysis, two distinct clusters emerge. One of these clusters is formed by *Onthophagus sagittarius* and *Onthophagus nigriventris*, indicating a distinct genital shape for these two species when compared with all other species (Fig. 3a).

**Table 4** Shape and size divergence of aedeagus, head, and fore-tibia between species

Trait	Wilks' $\lambda$	Shape divergence	F-value	Size divergence
Aedeagus	0.00028	$p < 0.0001^{**}$	65.56	$p < 0.0001^{**}$
Head	0.051104	$p < 0.0001^{**}$	32.49	$p < 0.0001^{**}$
Fore-tibia	0.00301	$p < 0.0001^{**}$	147.78	$p < 0.0001^{**}$

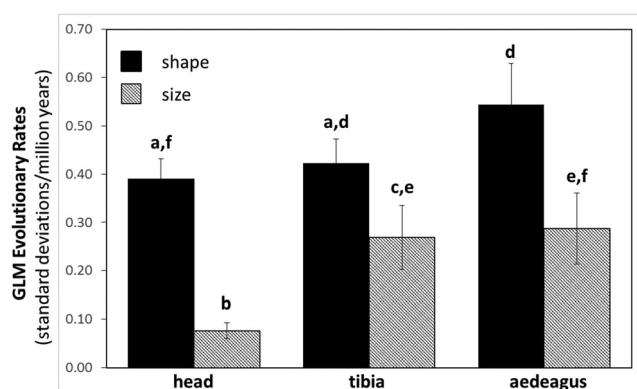
**Fig. 3** Shape and size variation (phylogenetically uncorrected) of the male copulatory organ (**a, b**), the head (**c, d**) and the fore-tibia (**e, f**) in 10 dung beetle species. Left panels show shape variables (principal components 1 and 2) for each trait. Each symbol represents an individual. Right panels show the relative size (centroid size) for each trait for each species. Each dot represents an individual



However, these two species strongly overlap with other species in our tibial shape analysis. Here, *O. orpheus*, *O. gazella*, and *O. hecate* each form their own cluster, while all other species form one large cluster with overlap for some, but not all species (Fig. 3e). Interestingly, none of such clusters appear in our head shape analysis (Fig. 3c). Thus, morphological shape divergence in the genus *Onthophagus* varies in respect of the trait measured, as well as the species investigated.

### Evolutionary shape rates differ between copulatory organ and head, but not between copulatory organ and fore-tibia

The evolutionary rates of shape we found in the aedeagus, head, and fore-tibia are only partially consistent with predictions about genital evolution made by earlier studies (e.g., Eberhard 1985; McPeck et al. 2008; Arnqvist 1998; Rowe and Arnqvist



**Fig. 4** Evolutionary rates (without outlier) of shape (black shaded columns) and size (gray shaded columns) for aedeagus, head, and fore-tibia of 10 dung beetle species. Error bars represent one standard error. Bars which share the same exponent are not significantly different from each other

2012), which identified the male copulatory organ as evolving at higher rates than other structures. Although the exact mechanism underlying accelerated aedeagal diversification is unclear (Hosken and Stockley 2004), sexual selection has generally been accepted as the driving force (Eberhard 1985). A growing number of studies, including on dung beetles, support this notion (e.g., Arnqvist 1998; House and Simmons 2003; Bertin and Fairbairn 2007; Hotzy and Arnqvist 2009; Polak and Rashed 2010; Rowe and Arnqvist 2012).

While the elevated evolutionary rates for aedeagal shape justify the common entomological practice of using male copulatory organs to identify and describe new species (e.g., Diptera: Grimaldi and Nguyen (1999); Lepidoptera: Scoble (1995); Raphidioptera: Aspöck (1991)), we could not find any significant differences between the evolutionary rates of aedeagal and tibial shape. Thus, our results also suggest that fore-tibia shape might provide important additional characters for species description, at least in the genus *Onthophagus*. Several mechanisms might explain why especially fore-tibia may be evolving more quickly in *Onthophagus* beetles compared to other taxa. First, the fore-tibia constitutes the principal digging tool of tunneling scarabs. Since dung beetles can be found on every continent (except Antarctica) (Emlen et al. 2005), it is likely that different species experience differences in soil conditions and consequently evolved different morphological solutions to adapt to their soil environment. In fact, a recent study on two *Onthophagus* species, as well as native and exotic populations of one species, found evidence that fore-tibial size and shape are remarkably evolutionarily labile, and co-evolve at least in part in conjunction with nesting depth (Macagno et al. 2016). Furthermore, Tomkins et al. (2005) suggested that forelegs might act as secondary sexual trait compensators (SSTC) in *Onthophagus taurus*, a pattern which has been found in other sexually dimorphic beetles (e.g., Okada et al. 2012; Ito et al. 2017). In *O. taurus*, males with large head horns also develop relatively larger fore-tibia,

which they might use as a stabilizer during aggressive encounters in tunnels. Since males of all species measured in this study develop diverse head horns used for male-male competition (Emlen 1997; Emlen et al. 2005), SSTC might further elevate the evolutionary rate of fore-tibial shape. In addition, tibiae in other Scarabaeine taxa have undergone extensive morphologic changes, probably due to sexual selection (e.g., Huxley 1932; Eberhard 1977; Emlen and Nijhout 2000). At least one of the species measured in this study exhibits a strong sexual dimorphism in tibia length (*O. gazella*, Parzer and Moczek, unpublished data), suggesting that sexual selection on the fore-tibia could additionally contribute to the high rates of tibia shape evolution.

A similar hypothesis may explain the apparently rapid evolution of head shape, albeit lower than the evolution of aedeagal or fore-tibia shape. Most male *Onthophagus* beetles develop head horns on the dorsal anterior head surface. Horn shape and position vary greatly among species, which in turn has the potential to affect head shape (Tomkins et al. 2005; Macagno et al. 2009). Therefore, the relatively high rates of head shape may be explainable as a secondary consequence of divergence in head horns.

### Discrepancies in the evolution of relative size and shape within and among traits

We showed that shape evolution proceeded at different rates among our three focal traits and that all of them evolved faster than their respective relative sizes. The multivariate nature of shape space might enable an increased accumulation of variation than what is possible for the univariate relative trait size. Thus, the relatively higher amount of variation might permit more rapid evolution of shape than size. However, we standardized our rates by the number of trait variables and by their covariances, which should, in principle, eliminate this possibility. Our results document evolutionary size rates for aedeagus and fore-tibia about half that of their respective shape rates, whereas relative head size evolved to a much lesser degree when compared to head shape. We suggest that functional constraints might underlie differences in relative size rates. For example, Frankino et al. (2005) showed that traits, such as wings in butterflies, function optimally only when scaled tightly with body size. Thus, these traits are more severely constrained in the amount of relative size evolution, similar to what we found for head size in *Onthophagus* beetles. Relative aedeagus and fore-tibia sizes, while still evolving at only half their shape rate, may be experiencing fewer functional constraints that are tied to body size.

**Acknowledgements** We would like to thank Tami Cruickshank who graciously calculated divergence times, Anna Macagno for statistical questions, Teiya Kijimoto for providing images for each trait, Elise Morton for helping us with the creation of the size figures, and two anonymous



reviewers for constructive comments that improved the manuscript.

**Funding information** This study was supported in part by National Science Foundation grants IOS 0445661 and IOS 0718522 to APM.

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