RAPID ANTAGONISTIC COEVOLUTION BETWEEN PRIMARY AND SECONDARY SEXUAL CHARACTERS IN HORNED BEETLES

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Different structures may compete during development for a shared and limited pool of resources to sustain growth and differentiation. The resulting resource allocation trade-offs have the potential to alter both ontogenetic outcomes and evolutionary trajectories. However, little is known about the evolutionary causes and consequences of resource allocation trade-offs in natural populations. Here, we explore the significance of resource allocation trade-offs between primary and secondary sexual traits in shaping early morphological divergences between four recently separated populations of the horned beetle *Onthophagus taurus* as well as macroevolutionary divergence patterns across 10 *Onthophagus* species. We show that resource allocation trade-offs leave a strong signature in morphological divergence patterns both within and between species. Furthermore, our results suggest that genital divergence may, under certain circumstances, occur as a byproduct of evolutionary changes in secondary sexual traits. Given the importance of copulatory organ morphology for reproductive isolation our findings begin to raise the possibility that secondary sexual trait evolution may promote speciation as a byproduct. We discuss the implications of our results on the causes and consequences of resource allocation trade-offs in insects.

KEY WORDS: Aedeagus, horned beetle, *Onthophagus*, resource allocation trade-off, speciation.

The growth and differentiation of any biological structure requires resources. If two or more structures compete for a shared pool of resources, allocation trade-offs may arise, allowing the enlargement of one structure to be possible only at the expense of another. This may be particularly pronounced in organisms in which growth and differentiation occur during times when resource acquisition by the organism has ceased, as it is the case in holometabolous insects like beetles, butterflies, and flies that grow these traits right before and during pupation (Nijhout and Emlen 1998; Emlen 2001; Fry 2006). Even though the exact nature of resource allocation trade-offs remains obscure (but see Fry 2006), there is growing evidence that such trade-offs not only affect developmental outcomes but may also bias long-term evolutionary trajectories (Emlen 2001; Simmons and Emlen 2006). For example, surgical removal of hindwing discs in a butterfly, *Precis coenia*, led to an increase of the forewing (Nijhout and

Emlen 1998). Similarly, ablation of the genital disc in the dung beetle *Onthophagus taurus* resulted in a significant increase of relative horn length in males (Moczek and Nijhout 2004). However, artificial selection on relative horn length in male *O. acuminatus* led to an inverse response in relative eye size over just nine generations (Nijhout and Emlen 1998). Additionally, a recent comparison across *Onthophagus* species revealed a striking trade-off between the plasticity of head horn size and testes mass (Simmons and Emlen 2006). Here we hope to contribute to this body of research by combining both intra- and interspecific approaches on trade-off evolution in natural populations of *Onthophagus* beetles. Specifically, we focus on the relationship between the development of head horns, an exaggerated secondary sexual traits used as weapons in male combat, and the male copulatory organ or aedeagus, thought to play a major role in reproductive isolation (Eberhard 1985). We extend earlier experimental work

that has shown that inhibiting aedeagus development results in horn enlargement, and explore whether divergence in the relative investment into head horns in four natural populations of *O. taurus* and 10 *Onthophagus* species is paralleled by a corresponding inverse divergence in investment into copulatory organ growth. Lastly, we explore whether any compensatory responses in copulatory organ development are restricted to the copulatory organ itself or reflective of general changes in investment into nonhorn appendages.

Material and Methods
ONTHOPHAGUS POPULATIONS AND SPECIES

To explore resource allocation trade-offs within species we investigated three exotic (North Carolina, Western Australia, and Eastern Australia) and one native (Italy) population of *O. taurus* (Fig. 1A). We selected these populations because earlier studies have documented rapid, differential divergence in male horn investment in less than 50 years since the introduction from a common European ancestor (Moczek et al. 2002; Moczek 2003; Moczek and Nijhout 2003). To explore resource allocation trade-offs between species we sampled 10 additional *Onthophagus* species. All species included in this study possess paired head horns (with the exception of *O. fracticornis*). Beetles of each population and species were collected in the field and males were chosen for measurements at random. Sample sizes and collection sites were as follows: *O. acuminatus*, Guatemala, Santa Rosa Guana, *n* = 5; *O. dama*, Nepal, Chitwan, *n* = 5; *O. fracticornis*, *n* = 15, Austria, Niederoesterreich; *O. gazella*, Hawaii, Waimea, *n* = 18; *O. hirculus*, Argentina, La Vina Salte, *n* = 7; *O. illyricus*, Italy, Piemonte, *n* = 45; *O. sagittarius*, Waimea, Hawaii, *n* = 43; *O. taurus*: USA, North Carolina, Orange County, *n* = 50; Italy, Piemonte, *n* = 30; Western Australia, Narrikup, *n* = 48, Eastern Australia, Tumut, *n* = 47; *O. watanabei*, Malaysia, Sabah *n* = 31; as well as an unknown *Onthophagus* species (*O. spec*, Costa Rica, Mt. Arenal, *n* = 20) whose identity is currently being determined. All specimens within a species were collected from a single population with the exception of *O. acuminatus*, *O. hirculus*, and *O. dama*, where the exact population-level origin could not be determined. All specimens were stored in ethanol and male copulatory organs were dissected by hand from each specimen.

MEASUREMENTS

Head horns, copulatory organ size, and body size were measured using a standard two-dimensional morphometric setup. Head horns were measured as described in Moczek 2006 (see also for justification of horn measurements). Male copulatory organ size was measured as the sum of the lengths of both paramere and phallobase combined (for further details see online supplementary Fig. S1). Body size was measured as pronotum width as
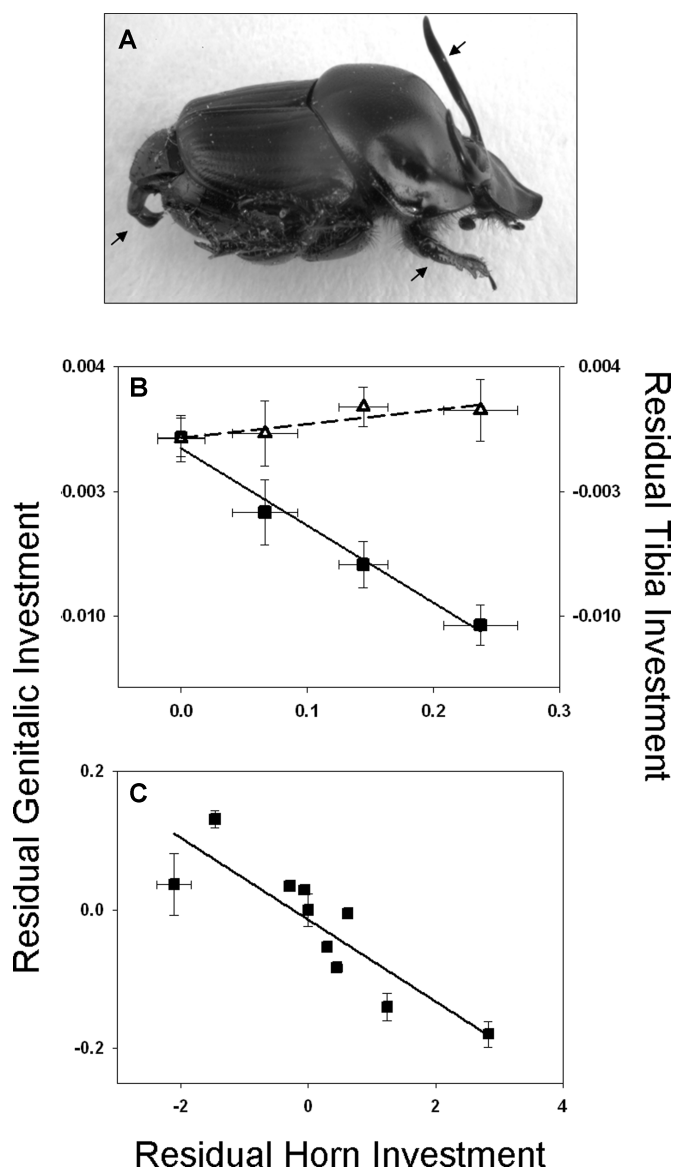

Figure 1. Trade-offs between primary and secondary sexual characters in populations and species of *Onthophagus* beetles. (A) Horned male *Onthophagus taurus*. Arrows highlight horns (secondary sexual character), everted aedeagus and the fore tibia. Note that in live animals the aedeagus is normally only everted during copulation and otherwise held inside the body. (B) Relative investment into copulatory organ size (left, squares) and fore tibia size (right, triangles) as a function of relative investment into horn size in four allopatric populations of *O. taurus*. Error bars represent one standard error. (C) Relative investment into copulatory organ size as a function of relative investment into horn size in ten different *Onthophagus* species. Data are corrected for differences in body size. Relative investment into head horns exhibits a strong negative correlation with the relative investment into copulatory organ size across populations and species.

in Emlen (1994). All measurements were taken by HFP. Samples of *O. taurus* populations were anonymized prior to measurement

and the origin of each sample was identified only after all morphometric data had been collected.

To determine whether changes in copulatory organ investment are restricted to the copulatory organ, or instead may be a reflection of general changes in investment into nonhorn appendages, we also measured the size of the front tibia, a heavily sclerotized portion of the first pair of legs used in walking and digging, across all four populations. Such a comparison is only meaningful provided that the focal trait has not yet had the opportunity to be subject to independent diversifying selection, for example due to habitat differences. This is unlikely to be case as all four *O. taurus* populations studied here occur in Mediterranean climate zones and exhibit no obvious differences in habitat use (A. Moczek, pers. obs.).

To determine whether a trade-off signature is also detectable in long-term evolutionary trajectories we compared the relative investment into primary and secondary sexual characters in 10 *Onthophagus* species. Males of all species express very similar paired head horns, yet differ substantially in relative investment into horn size. Because all 10 species differ, at times substantially, in their native habitat types including soil conditions and are therefore likely to have been subject to long periods of diversifying selection that may have differentially affected the size of the tibia, we did not include traits other than horns and copulatory organs in this analysis.

ANALYSIS

Population comparison

The relative investment of horns, genitalia, and tibia was calculated by standardizing their respective sizes relative to pronotum width. Differences between populations in the investment into any of these three traits were examined via a residual analysis. To do so, we used the population with the largest sample size (US) as a reference population to quantify the relationship between their relative investment into horn size, copulatory organ size, and tibia size, as a function of male body size and we calculated the residuals of each population from this curve.

The horn investment relationship was estimated by

$$\text{horn length} = \text{minimal horn length} + \frac{a\,(\text{body size}^b)}{c^b + (\text{body size}^b)},$$

where a represent the range of observed horn lengths, b corresponds to maximum slope of horn increase, and c signifies the inflection point of the sigmoid curve.

The genitalic and tibial investment relationships were described with a linear regression

$$\text{aedeagus (or tibia) length} = \text{minimal aedeagus (tibia) length} + a\,\text{body size},$$

where a specifies the slope of the linear regression line. We then calculated the deviation of each measurement from the expected value (= residuals) as given by the above regressions, for each individual in each population. To test for trade-offs between traits we determined the mean of each residual for each population. We then assessed the relationship of tibial, genitalic residuals, and horn residuals. An inverse, or negative, correlation is indicative of a trade-off. Although this analysis corrects for differences between populations or species brought about by differences in mean body size, it risks the possibility of altering correlation coefficients through the introduction of autocorrelations (LaBarbera 1989). We therefore replicated the residual analysis using nonstandardized traits. However, population and species clearly differ in average body size and thus in their respective absolute trait sizes. Because this study is primarily interested in relative trait investment, we focus on the analysis of standardized traits and report the results of nonstandardized regressions (absolute trait size) in parentheses.

SPECIES COMPARISON

We used the same approach for the species comparison as described in the population study. Unlike allometries obtained from different *O. taurus* populations, species-specific allometries differed significantly in shape (horns) and slope (aedeagus) and we therefore logarithmized the measurements. Furthermore, some species were only represented by relatively few individuals. We therefore repeated the entire analysis without species with fewer than six individuals, which, however, did not change the general outcome of the results.

Results

POPULATION COMPARISON

The relative investment into head horns exhibited a strong negative correlation with the relative investment into copulatory organ size (SS = 0.0001, $F = 107.84$, $r^2 = 0.92$, $P = 0.009$; Fig 1B; nonstandardized regression: SS = 0.0046, $F = 4.6620$, $r^2 = 0.55$, $P = 0.1635$), revealing a strong signature consistent with a resource allocation trade-off between both structures. At the same time, relative investment into fore tibia size was unaffected (SS < 0.00, $F = 7.370$, $r^2 = 0.68$, $P = 0.11$; nonstandardized regression: SS < 0.00, $F = 4.0893$, $r^2 = 0.51$, $P = 0.1805$), consistent with the hypothesis that this trade-off may not involve other appendages. Thus, an increase in the investment into head horns coincided with a significant decrease in the investment into aedeagus but not legs. Scaling relationships are shown in the online Supplementary material (Fig. S2A–C). Using the native (Italy) *O. taurus* sample as a reference population did not change the general outcome of the analysis.

SPECIES COMPARISON

The relative investment into head horns exhibited a strong negative correlation with the relative investment into copulatory organ size across species (SS $= 0.0581$, $F = 24.788$, $r^2 = 0.73$, $P = 0.0011$; Fig. 1C; nonstandardized regression: SS $= 0.0597$, $F = 38.48$, $r^2 = 0.81$, $P = 0.0003$). As in the population comparison, an increase in the investment into head horns between species coincided with a decrease in aedeagus investment.

Discussion

Our study identified a significant negative correlation between relative investment into horn growth and development of the male copulatory organ both within and between species. Although phenotypic correlations cannot distinguish between cause and effect, our results match predictions derived from an earlier experimental study that documented increased investment into horns following experimental ablation of copulatory organs during late larval development (Moczek and Nijhout 2004). We therefore interpret the negative correlations between relative investment in horns versus copulatory organ detected here as being consistent with a resource allocation trade-off, likely arising during late larval development, and capable of leaving a clear signature across a range of phylogenetic distances. Below we briefly discuss the most significant results and their implications.

VARIATION IN COPULATORY ORGAN SIZE IS LOW WITHIN POPULATIONS BUT UNEXPECTEDLY HIGH BETWEEN POPULATIONS

Earlier studies generally emphasized minimal variation in copulatory organ size relative to body size within species of arthropods (e.g., Eberhard 1998). In other words, even though conspecific males may differ substantially in body size there is typically little corresponding difference in the sizes of their respective copulatory organs. In the present study we detected similarly low levels of variation within each population examined. However, comparing across populations revealed unexpected and significant differences. Specifically, although each of the four populations examined here still exhibited a shallow allometric scaling relationship between copulatory organ size and body size, y-intercepts differed significantly, causing all four populations to exhibit consistent differences in copulatory organ size over the same body size range (analysis of covariance [ANCOVA], SS $= 0.18$, df $= 3$, $F = 14.65$, $P < 0.001$). Importantly, the most extreme differences between populations (W. Australia vs. Eastern US) were similar in magnitude to at least some of the differences detected between species. (e.g., *O. taurus* vs. *O. sagittarius*). Our results suggest that earlier studies may have been underestimating the amount of standing phenotypic variation in copulatory organ size within species, possibly by limiting analysis to specimens derived from single populations (but see Kawano 2004). However, the presence of substantial between-population differences in copulatory organ morphology appears to be restricted to size but not shape, which appeared remarkably invariant across populations (H. Parzer, preliminary data). If correct, this would support the hypothesis by Eberhard et al. (1998), which suggests greater evolutionary liability of genitalia size over shape in arthropods. Studies are now under way to quantify the evolution of genitalia shape and size within and between *Onthophagus* species.

COPULATORY ORGAN SIZE MAY DIVERGE AS A BYPRODUCT OF EVOLUTIONARY CHANGES IN HORN LENGTH

Diverging genitalia are thought to be an exquisite primer for evolving reproductive isolation in arthropods (Eberhard 1985). Here we show that several populations of *O. taurus* have diverged significantly in male copulatory organ size since the establishment from a common ancestor less than 50 years ago. Earlier studies demonstrated that these populations differ, at times dramatically, in the ecological context in which male horns function (Moczek et al. 2002; Moczek 2003). Specifically, local densities of competing males differ by as much as three orders of magnitude across these populations. The frequency of horned males as well as the body size at which horn expression is initiated covaries correspondingly, such that horned males are rarer, and horn expression is restricted to larger sizes, in populations in which male competition is most intense. Under the latter set of circumstances, male–male encounter rates are extremely high and only the very largest males appear to benefit from engaging in fights, and hence the possession of horns, whereas smaller males have to resort to nonaggressive sneaking tactics, which do not require horns and may even select against them. This suggests that the likelihood of male–male encounters is driving the evolution of horn investment. (Moczek 2003; Tomkins and Brown 2004).

The tight correlation between local densities and population-wide differences in morph ratios and body size thresholds is consistent with the hypothesis that differences in local densities have selected for differential optimal investments into horn expression in disparate populations since these populations were established in their new locations (Moczek 2003). If correct, combining these earlier findings with those presented here would suggest that this rapid evolutionary change in horn expression may have caused diversification of male genitalia as a byproduct, possibly priming these diverging populations for the subsequent evolution of reproductive isolation. However, although density-dependent selection on weapon expression is not only predicted by game theory (e.g., Gross 1996) and documented in at least one other insect taxon, earwigs, (Tomkins and Brown 2004) we presently cannot rule out the opposite scenario, namely that selection acted on aedeagus size and brought about correlated changes in

horn investment across populations. Further research is therefore needed to elucidate the evolutionary causes and consequences of this developmental relationship. If trade-offs between horns and male genitalia are indeed driving speciation in *Onthophagus* this might help explain how *Onthophagus* beetles were able to radiate into over 2400 extant species, making it the most specious genus in the animal kingdom (Arrow 1951). Furthermore, this would call into question the common notion that the evolution of male copulatory organs is driven solely by sexual selection (reviewed in Hosken and Stockley 2004) and might suggest that under specific conditions changes of the male copulatory organ could be caused by pleiotropic effects (Mayr 1963).

DEVELOPMENTAL BASIS OF RESOURCE ALLOCATION TRADE-OFFS BETWEEN PRIMARY AND SECONDARY SEXUAL TRAITS

Despite their important developmental and evolutionary implications, the nature and physical basis of resource allocation trade-offs remain poorly understood (Klingenberg and Nijhout 1998). Earlier studies emphasized the importance of physical proximity between structures in determining the degree of resource allocation trade-offs that may be manifest between them. The closer two structures grow to each other, the more likely they should be to compete with each other for a shared pool of resources (Nijhout and Emlen 1998; Emlen 2001). This may be expected if tissues use local resource pools for growth, or if tissues communicate with each other through short-range signals such as paracrine morphogens (Gibson and Schubiger 2000). Recent studies, however, have implicated hormones and insulin-like growth factors in the regulation of growth and size of insect appendages (reviewed in Shingelton et al. 2007). In insects, these factors circulate freely in the haemolymph, and it is unclear why any allocation trade-off mediated by these factors should be restricted to only neighboring body parts (Nijhout 1994). Alternatively, resource allocation trade-offs may actually occur over long distances, but may have escaped the attention of earlier studies. There are several studies supporting this notion. For example, Moczek and Nijhout (2004) showed that ablation of the male genital disc resulting in increased horn growth in male *O. taurus* is dependent on the exact timing of ablation. This implicates the relative timing of growth, rather than physical proximity, as the primary determining factor for the strength of resource allocation trade-offs. Similarly, Simmons et al. (2006, 2007) found a consistent negative correlation between investment into horn growth and testes mass between populations and species of *Onthophagus* beetles. In all three studies, the organs implicated in the trade-off developed on opposite sides of the animal, demonstrating that resource allocation trade-offs need not be restricted to neighboring structures. The present study further supports this notion, implicating copulatory organ size and

horn length in a resource allocation trade-off shaping both short-term and long-term antagonistic coevolution. Our data suggest that at least in the context of short-term divergences between populations, this trade-off appears to be restricted to horns and copulatory organs, and does not extend to other appendages such as legs. As explained above, past studies support the hypothesis that between-population divergences in *O. taurus* were driven by selection acting on relative horn length due to population-wide changes in the social context within which horned males compete for females (Moczek 2003). We are not aware, however, of any obvious developmental mechanisms such as competition for specific nutrients or growth factors, or regulation through joint developmental pathways that would somehow link the development of horns and copulatory organs to the exclusion of other appendages. For example, the same basic patterning genes govern expression of legs and genitalia in flies (Chen and Baker 1997; Chen et al. 2005) and horns, legs, and genitalia in beetles (Parzer and Moczek, preliminary results). Furthermore, primordia of all three appendage types grow late in larval development, and even though there may be subtle differences in the exact onset and duration of growth, these do not appear to be relevant because leg primordia proliferate after genitalia, but before horns. We therefore speculate that the observed trade-offs may not arise due to some kind of limiting resource shared between horns and genitalia. Instead, we suggest that although all appendage types share and compete for the same types of resources to sustain their growth, pairwise trade-offs may arise simply due to differences in the functional consequences that would result from changes in scaling relationships between body size and different appendage types. For example, appendages such as legs scale nearly isometrically with body size. This is likely reflective of the severe functional constraints imposed by any deviation from the optimal scaling relationship between legs and body size, causing slightly longer or shorter legs to carry a strong functional penalty. In contrast, copulatory organs in arthropods generally exhibit very shallow allometries, causing large males to express copulatory organs very similar in size to that of small males. Therefore, expression of copulatory organ size within populations is largely body-size independent. As a consequence, population-wide changes in copulatory organ size may not result in great functional repercussions. If correct, this would make population-wide changes in copulatory organ size a possible evolutionary line of least resistance (Schluter 1996) toward freeing up resources during late larval development to fuel the exaggeration of other traits such as horns during both development and evolution.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Lateral view of copulatory organ (aedeagus) of *Onthophagus taurus* consisting of two basic regions, the phallobase (left) and parameres (right). Distal is to the right. Red lines indicate how size measurements of both the phallobase (dashed) and paramere (solid) were obtained. The sum of both lengths was used as a size measurement of an individual male's copulatory organ.

Figure S2. Scaling relationships between body size and (A) horns, (B) aedeagus and (C) tibia (all in mm) in four populations of *Onthophagus taurus* (USA: $n = 50$; W-Australia: $n = 48$, E-Australia: $n = 47$; Europe: $n = 30$).

Supporting information may be found in the online version of this article.

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