Trade-offs during the Development of Primary and Secondary Sexual Traits in a Horned Beetle

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ABSTRACT: Resource allocation trade-offs during development affect the final sizes of adult structures and have the potential to constrain the types and magnitude of evolutionary change that developmental processes can accommodate. Such trade-offs can arise when two or more body parts compete for a limited pool of resources to sustain their growth and differentiation. Recent studies on several holometabolous insects suggest that resource allocation trade-offs may be most pronounced in tissues that grow physically close to each other. Here we examine the nature and magnitude of developmental trade-offs between two very distant body parts: head horns and genitalia of males of the horned scarab beetle *Onthophagus taurus*. Both structures develop from imaginal disklike tissues that undergo explosive growth during late larval development but differ in exactly when they initiate their growth. We experimentally ablated the precursor cells that normally give rise to male genitalia at several time points during late larval development and examined the degree of horn development in these males compared to that of untreated and sham-operated control males. We found that experimental males developed disproportionately larger horns. Horn overexpression was weakest in response to early ablation and most pronounced in males whose genital disks were ablated just before larvae entered the prepupal stage. Our results suggest that even distant body parts may rely on a common resource pool to sustain their growth and that the relative timing of growth may play an important role in determining whether, and how severely, growing organs will affect each other during development. We use our findings to discuss the physiological causes and evolutionary consequences of resource allocation trade-offs.

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Allocation trade-offs during development may arise when two or more structures compete for a shared and limited pool of resources necessary to sustain their growth. A shared limiting resource could be a nutrient that is used up during the physical growth of a tissue or a hormone, growth factor, or morphogen that is sequestered by competing binding sites in different tissues. Such limiting resources may constrain the absolute sizes to which a structure can grow. If different tissues or organs are unequal in their ability to utilize or sequester those limiting resources, then this competition can play an important role in determining the relative sizes of those tissues (Nijhout and Wheeler 1996). In butterflies and beetles, various structures that develop from imaginal disks (wings, eyes, horns) experience resource allocation trade-offs during development, so that when the growth of one structure is experimentally inhibited, other structures develop to a disproportionally larger size (Nijhout and Emlen 1998).

A developmental mechanism that depends on the differential allocation of a limiting resource can pose a severe constraint on the magnitude and direction of evolutionary change. For instance, an evolutionary increase in overall size may be limited by the degree to which the limiting resource can increase. More important, perhaps, an evolutionary increase in one structure may cause a correlated decrease in another structure with which it shares a limiting resource (Nijhout and Wheeler 1996; Klingenberg and Nijhout 1998; Nijhout and Emlen 1998).

In spite of their important developmental and evolutionary implications, the nature and physical basis of resource allocation trade-offs remain poorly understood (Klingenberg and Nijhout 1998). Until now, allocation trade-offs have been studied exclusively among tissues that develop in close physical proximity to each other (Nijhout and Emlen 1998; Emlen 2001). In fact, at least one study suggested that resource allocation trade-offs may be restricted to only neighboring body parts (Emlen 2001). This could be the case if tissues primarily utilize local pools of
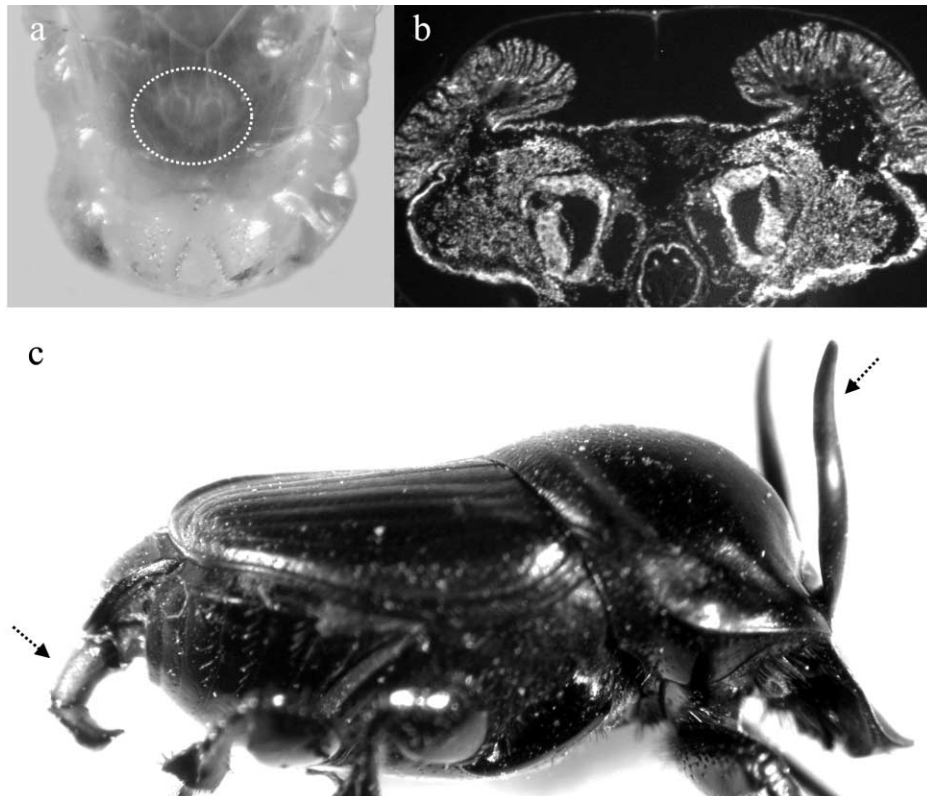
Figure 1: *a*, Genital imaginal disks (indicated in dashed circle) in a third-instar *Onthophagus taurus* larva. Genital disks give rise to the male copulatory organ and testicular tissue. *b*, Developing horn primordia in the head of an early prepupal male *O. taurus*. Future horns are visible as two mushroom-like outgrowths of the dorsal epidermis with a high degree of tissue folding. Once the animal sheds the larval cuticle at pupation, horn primordia expand to form the fully developed pupal and adult horn. *c*, Male *O. taurus* with fully developed head horns (*right, arrow*) and everted copulatory organ (*left, arrow*).

resources for growth or if tissues communicate through some kind of short-range signal, such as a paracrine morphogen (Gibson and Schubiger 2000), to regulate each other's growth. Recent studies, however, have implicated hormones and insulin-like growth factors in the regulation of growth and size of insect appendages (Kawamura et al. 1999; Oldham et al. 2000; Brogiolo et al. 2001; Nijhout and Grunert 2002). These factors are known to circulate freely in the hemolymph (see, e.g., Nijhout 1994), and it is unclear why any allocation trade-off mediated by these factors should be restricted to only neighboring body parts. Alternatively, resource allocation trade-offs may actually occur over long distances but may have escaped the attention of earlier studies. If this were the case, then the constraints on evolution imposed by such trade-offs would have the potential of being far more widespread and profound.

Here we test the notion that only neighboring body parts can exhibit significant trade-offs during development by examining two very distant body parts: head horns and genitalia of males of the horned scarab beetle *Onthophagus taurus*. Male *O. taurus* develop a pair of long horns on their heads and a large copulatory organ in their abdomen (fig. 1). Both structures develop from imaginal disklike tissues (henceforth referred to as disks) but differ in exactly when they initiate their growth: genital disks begin to grow early in the third (=last) larval instar and continue to grow throughout the remainder of the larval stage, whereas horn disks undergo brief but explosive growth approximately 8–10 d later, when larvae enter the prepupal stage. At this stage larvae are no longer able to acquire resources from the outside, and subsequent growth and differentiation are sustained by resources stored up during larval life. Here we report the results of a microcautery study in which we surgically removed genital imaginal disks at different points during larval development and tested whether this ablation influences the relative sizes of head horns that develop at the other end of the animal and whether the relative timing of growth periods influences the magnitude of this interaction.

Material and Methods

Rearing

All individuals used in this study were the offspring of a laboratory colony of *Onthophagus taurus* kept at a constant-temperature insectary at 26°C and 60% relative humidity under a 16L : 8D cycle. Beetles were bred in plastic containers (25 cm tall, 20 cm diameter) filled three-quarters of the way with a moist sand/soil mixture. Five pairs of beetles were added to each container and provided with ~0.5 L of homogenized dung. Six days later beetles were recaptured and brood balls were collected and placed in individual containers until emergence (see also Moczek et al. 2002). Brood balls were opened occasionally to determine the age of larvae but were otherwise left undisturbed until larvae had molted into the third instar.

Microcautery

Larvae were allowed to develop to approximately day 4 of the third instar and sexed based on the presence or absence of visible genital imaginal disks (for details, see Moczek and Nijhout 2002*b*). Male larvae were then cauterized either at this stage (early), approximately 5 d later (mid), or just before entering the prepupal stage an additional 5 d later. At this stage, larvae undergo a series of physiological changes such as the gut purge, which can be used as markers to indicate the transition to the prepupal stage. After disk ablation, larvae were returned to their original brood ball, placed in an individual container filled with moist sand, and reared to adulthood. Sham-operated males were treated in the same fashion except that instead of ablating the genital disks we ablated an area of similar size adjacent to the disks. Untreated control males were allowed to complete larval development inside their brood ball without disturbance. All ablations were done under a dissecting scope (Wild) using a Hyfrecator (Birtcher) to electrosurgically cauterize genital disks or control regions. After emergence, adult males of all treatment groups were frozen and stored in Ethanol for morphometric measurements. All experimental and sham-operated animals were dissected after measurements to confirm the absence of male genitalia (experimental) or other internal damage (sham operated).

Morphometric Measurements

All individuals were measured using a standard two-dimensional image analysis setup at the Duke University Morphometrics Laboratory (for details, see Moczek et al. 2002). We used thorax width as an estimate for body size (for justification, see Moczek and Emlen 1999).

Statistical Analyses

Allometric differences between samples were first analyzed globally using a residual analysis (for earlier application of this approach see, e.g., Emlen 1996, 1997, 2001; Moczek 2002, 2003; Moczek and Nijhout 2003). A four-parameter nonlinear regression model of the form

$$\text{horn length} = y_0 + \frac{a(\text{body size})^b}{c^b + (\text{body size})^b}$$

was used to describe the average scaling relationship between horn length and body size of all untreated control males combined (y_0 specifies minimum horn length, a describes the range of horn lengths in the sample, b specifies a slope coefficient, and c represents the body size at the point of inflection of the sigmoid; parameter values were obtained via Sigma Plot curve-fitting procedures). We obtained the following relationship

$$\text{horn length} = 0.4787$$
$$+ \frac{3.7104291 \times (\text{body size}) \exp 51.975}{5.0729 \exp 51.975 + (\text{body size}) \exp 51.975}$$

as the equation with the best fit ($r^2 = 0.93$). This equation was then used to predict horn lengths for all possible body sizes. Individual males were characterized by the degree to which their real horn length differed from the horn length predicted by this reference function, that is, by their residual horn length. Treatment groups that differed in their scaling relationship between horn length and body size could then be recognized by consistent differences in residual horn lengths (see also Emlen 1996, 1997, 2001; Moczek 2002). Horn length residuals were usually not normally distributed, and we therefore used nonparametric Kruskal-Wallis *H*-tests for statistical analyses (Sachs 1992; Sokal and Rohlf 1995). When significant differences in residual horn lengths were indicated, we repeated the curve-fitting procedure separately for each treatment group to obtain group-specific mean and standard error estimates for each regression parameter. We then used repeated unpaired *T*-tests to examine the degree to which differences in particular regression parameters, such as the inflection point or slope, explained allometric differences between samples (Sachs 1992; Sokal and Rohlf 1995). All significance levels reported below are adjusted for multiple comparisons using sequential Bonferroni corrections where this was necessary (Sachs 1992; Sokal and Rohlf 1995). Unless otherwise noted, all data are presented as means ± SE.

Results

Experimental male larvae developed into normal adults but lacked the male copulatory organ as well as testicular tissue in all cases ($n = 130$). This suggests that ablation of genital imaginal disks during the larval stage effectively prevents the development of the male genital apparatus. Sham-operated males developed into normal adults without obvious external or internal damage. Larval mortality was similar in all three treatment groups ($P > .1$ for each comparison; multiple χ^2 tests).

Experimental males whose genital disks were ablated early in the third larval instar developed horn sizes similar to control and sham-operated males of the same body size. However, experimental males whose genital disks were ablated in the middle of and late in the final larval stage expressed on average longer horns than control or sham-operated males of the same body size (fig. 2). Late genital disk ablation showed the most dramatic effect and caused males to overexpress their horns by more than 26% (± 7.2; $n = 34$) compared to control males of the same body size (fig. 2). Genital disk ablation therefore resulted in greater horn overexpression the later genital disks were ablated during development. In contrast, sham-operated males expressed horn lengths similar to those of control males of the same body size independent of when genital disks were ablated (fig. 2).

To identify the degree to which differences in particular components of the scaling relationship between horn length and body size, such as the inflection point or slope, could account for the observed differences in horn length residuals, we contrasted individual parameter estimates between treatment groups. We found no significant differences between treatment groups in either the range of horn lengths (a) or the minimum horn length (y_0). Instead, we found that differences in horn length residuals between treatment groups could be attributed largely to differences in parameter c, or the average threshold body size at which males switched from the hornless to the horned phenotype (fig. 3; table 1). Experimental males whose genital disks were ablated early in the third larval instar exhibited an average switch point similar to those of control and sham-operated males. Experimental males whose genital disks were ablated in the middle of the third instar exhibited a tendency to switch at smaller body sizes ($T = 1.69$, df $= 193$, $P = .09$). Experimental males whose genital disks were ablated just before the prepupal stage showed the strongest effect and expressed a switch point highly significantly smaller than that of control males ($T = 6.96$, df $= 169$, $P = .0001$; fig. 3). In addition, late-disk ablated males also exhibited a slightly but significantly higher slope coefficient than their untreated counterparts ($T = 2.14$, df $= 169$, $P = .03$; fig. 3). When combined,
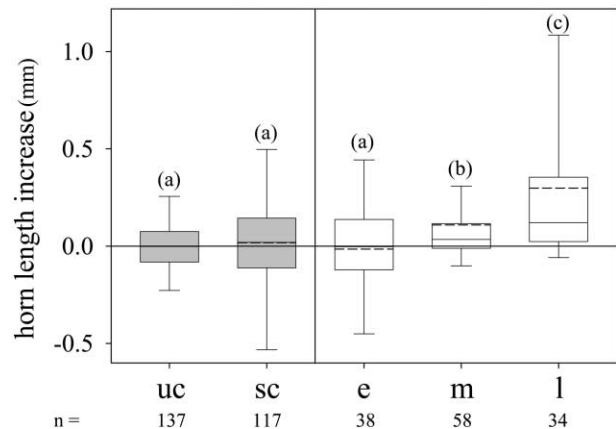

Figure 2: Box plot (with 90%/10% confidence intervals) of horn length increase in male *Onthophagus taurus* whose genital imaginal disks were ablated early in (*e*), midway through (*m*), and at the end of (*l*) the third instar (*open bars*) relative to untreated control (*uc*) and sham-operated males (*sc*; *gray bars*). Sham-operated males showed no significant difference from untreated control males independent of whether sham surgery occurred early, midway, or late in the instar and are therefore illustrated by a single combined bar only. Dashed lines in boxes indicate means. Different letters above the bar indicate significant differences (Kruskal-Wallis *H*-test, $P < .01$).

these results suggest that the increases in relative horn lengths observed in the mid and late treatment groups were not distributed equally across the entire range of body sizes but instead were largely due to increased horn development in small and medium-sized males, that is, males that otherwise would have developed only small horns or no horns at all.

Discussion

We have shown that male *Onthophagus taurus* beetles develop disproportionally larger horns on their heads when genital imaginal disks located at the other end of the animal are removed from the pool of tissues that undergo rapid growth during late larval development. Sham-operated males, who received an ablation of similar intensity of an area of similar size adjacent to the genital disks, did not show any overexpression of male horns, suggesting that our results cannot be attributed to trauma or wound healing processes. In fact, if wound healing would have been the predominant consequence of ablation, we would have expected sham-operated and experimental males to express relatively smaller rather than larger horns, since wound healing is more likely to use up resources than generate new ones. Instead, sham-operated males showed no effect, and experimental males dramatically overexpressed their horns.
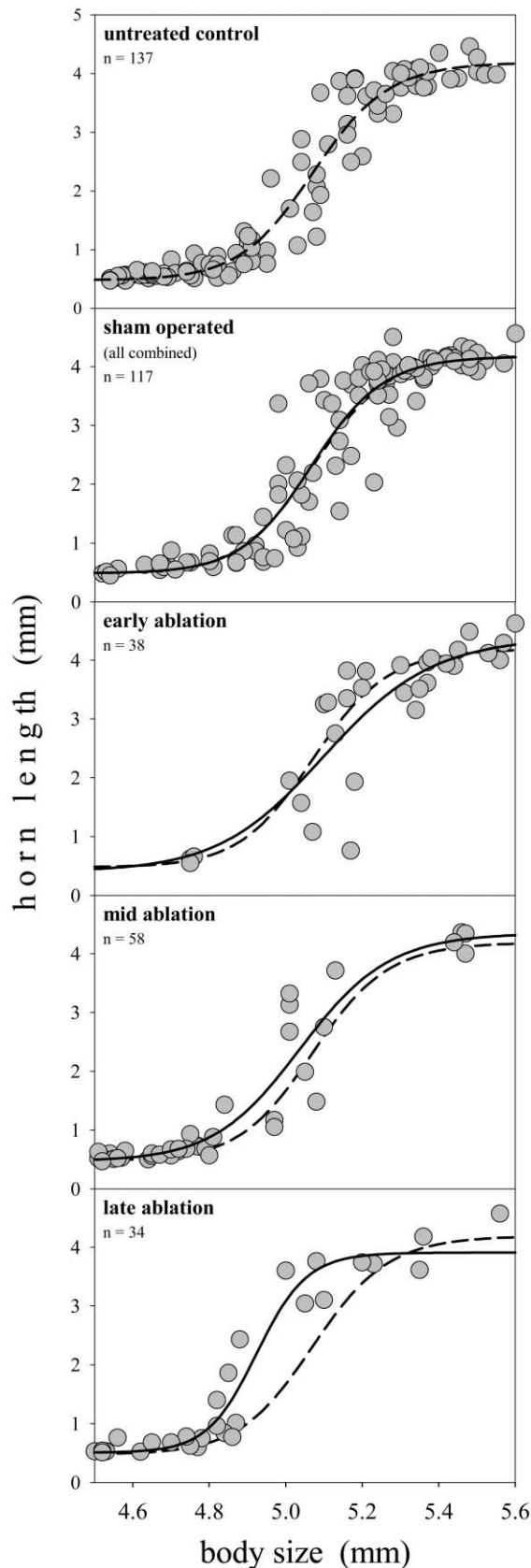
Our results therefore suggest the existence of a substantial resource allocation trade-off between head horns and genitalia in male *O. taurus* and that ablation of genital disks frees up resources that at least in part become allocated to the development of horns at the other end of the animal. These findings run contrary to recent studies that suggested that mainly adjacent, neighboring structures are likely to exhibit significant trade-offs during development (Nijhout and Emlen 1998; Emlen 2001). Instead, our results suggest that even distant body parts can affect each other's development through competition for a shared and limited pool of resources.

We also found that the magnitude of horn overexpression depended on the exact timing of genital ablation. We originally expected horn overexpression to be more pronounced in males whose genital disks were ablated early in larval development because this would maximize the amount of resources available to the development of horns that would otherwise have gone to the genitalia. Instead, we found that horn overexpression increased the later genital imaginal disks were ablated. One possible interpretation of this unexpected increase in horn overexpression may be that resources set free by early genital disk ablation are no longer available at the time horn disks begin to proliferate, possibly because those resources have been consumed by other tissues in the meantime. As the time between genital ablation and onset of horn proliferation decreases, horn disks become increasingly likely to share temporarily varying resources with the growing genitalia.

An important implication of this finding is that it may be the relative timing of their respective growth periods, rather than the relative location of growing organs, that determines whether, and how severely, two growing structures will interact with each other during development. This may be most pronounced in organisms such as holometabolous insects, whose adult structures develop in short episodes of very rapid growth. In such organisms, changes in the relative timing of growth periods may therefore provide an important avenue for morphological evolution to escape potentially constraining developmental trade-offs.

Late genital disk ablation did not affect the overall range

Figure 3: Scaling relationship between horn length and body size for untreated, sham-operated, and experimental male *Onthophagus taurus*. Dashed curve (same in all panels) indicates a best-fit nonlinear regression fitted to all untreated control males combined. Solid curves indicate best-fit nonlinear regressions calculated separately for each treatment group. Sham-operated males showed no significant difference from untreated control males independent of whether sham surgery occurred early, midway, or late in the instar and are therefore illustrated by a single panel only. See table 1 for regression parameters.

Table 1: Parameter values (mean ± SE) for each treatment group

	Regression parameters				
	a	b	c	y_0	n
Control:					
Untreated control	3.710 ± .104	51.97 ± 4.213[a],*	5.073 ± .010[b],***	.479 ± .040	137
Sham ablation	3.688 ± .155	54.36 ± 6.557[a],*	5.069 ± .013[b],***	.486 ± .092	117
Experimental:					
Early ablation	3.989 ± .481	36.20 ± 12.12	5.105 ± .039	.408 ± .219	38
Mid ablation	3.879 ± .212	43.95 ± 7.401	5.041 ± .020	.472 ± .071	58
Late ablation	3.400 ± .195	75.49 ± 14.19[c],*	4.925 ± .018[d],***	.508 ± .096	34

Note: Regression parameters are as follows: a specifies the range of horn lengths within a sample (amplitude), b specifies a slope coefficient, c represents the body size at the point of inflection of the sigmoid, and y_0 specifies minimum horn length. Different superscript letters indicate significant differences between samples (multiple t-tests [two-tailed] including sequential Bonferroni correction for multiple comparisons).

* $P < .05$.

*** $P < .001$.

of horn lengths (a) or the minimum horn length expressed by small males (y_0). Instead, the increase in relative horn lengths observed among late genital disk–ablated males was mainly the result of a shift of the body size threshold above which males begin to express horns to smaller body sizes. In other words, genital disk ablation caused intermediate-sized males, which normally would have expressed short or no horns at all, to develop relatively longer horns, whereas smaller and larger males remained largely unaffected. This is interesting because it suggests that whatever resources are made available through the removal of genital disks does not affect all males equally. This could be because small males do not possess horn primordia competent to take up surplus resources. However, it is unclear why providing additional resources would then manifest itself in a shift in the threshold to smaller body sizes unless the resources made available through genital ablation could themselves somehow induce horn expression in presumptive hornless males. Hormones such as juvenile hormone (JH), ecdysteroids, as well as various growth factors play important roles in regulating the timing and degree of imaginal disk proliferation in virtually all insects studied so far (Nijhout 1994; Miner et al. 2000; Brogiolo et al. 2001; Nijhout and Grunert 2002). Recent studies have shown that elevating JH levels via application of JH analogues can induce horn expression in male *O. taurus* larvae that normally would have developed into hornless males (Emlen and Nijhout 1999; Moczek and Nijhout 2002*a*). If imaginal disks could somehow act as sinks for a hormone or growth factor, then the removal of one disk, such as the one for genitalia, would leave a certain fraction of these growth regulators unused and free to induce the proliferation of other disks or disklike tissues, such as horn primordia, which otherwise would proliferate to a lesser degree or not at all. This would provide an important and so far overlooked mechanism that would allow body parts to influence each other's development independent of exactly where they are located.

Recent studies support the notion that hormones and growth factors indeed regulate the sizes of insect body parts and therefore have the potential to mediate allocation trade-offs. For instance, in the buckeye butterfly *Precis coenia*, wing disk growth requires an optimal concentration of ecdysone plus the insulin-like growth factor, bombyxin, with the concentration of the latter fluctuating with the nutritional state of the larva (Nijhout and Grunert 2002). Insulin-like peptides and other growth factors have also recently been implicated in the control of somatic and imaginal disk growth in *Drosophila melanogaster* (Kawamura et al. 1999; Oldham et al. 2000; Brogiolo et al. 2001). However, although these growth factors appear to be modulated in such a way that imaginal disk growth remains proportional to somatic growth, it remains unknown whether these growth regulators occur in sufficiently low concentrations to function as mediators of allocation trade-offs.

Several studies provide support for a link between horn and genitalia development and evolution in horned beetles. Recent work on two horn-dimorphic *Chalcosoma* species showed that sympatric, but not allopatric, populations of both species differed dramatically in horn length–body size allometries and genitalia length (Kawano 2002). *Chalcosoma caucasus* males that occurred in sympatry with a second species expressed the minor, largely hornless morph over a much wider range of body sizes and switched to the major, fully horned morph at a larger body size threshold than when they occurred in allopatry. Sympatric populations of *C. caucasus* therefore showed, on average, a reduction in the relative expression of horns compared to their allopatric counterparts. Interestingly, relative male genitalia length was significantly larger in sympatric than in allopatric *C. caucasus* populations (Kawano 2002).

Males of the second species, *Chalcosoma atlas*, exhibited the opposite pattern for both traits. In sympatric *C. atlas* populations the switch from minor to major morphs occurred at much smaller body sizes compared to allopatric populations. Sympatric *C. atlas* males therefore expressed horns already at very small body sizes compared to their allopatric counterparts, and relative male genitalia length was significantly smaller in sympatric than in allopatric populations (Kawano 2002). While these results were primarily discussed in the context of reproductive character displacement, they are clearly also consistent with the existence of trade-offs in the development and evolution of beetle horns and genitalia.

Recent studies on the behavioral ecology of *Onthophagus* beetles provide additional interesting insights into a connection between beetle horns and genitalia. Horned and hornless *Onthophagus* beetles typically rely on alternative reproductive tactics to gain access to females. Horned males use their horns as weapons in intermale combat, whereas hornless males rely on sneaking behaviors to acquire mating opportunities (Moczek and Emlen 2000). Furthermore, small, hornless males of several *Onthophagus* species, including *O. taurus*, rely on sperm competition mechanisms to increase their fitness (see, e.g., Simmons et al. 1999; Tomkins and Simmons 2002). Small, hornless males typically copulate more frequently with females, develop significantly greater amounts of testicular tissue, and produce larger ejaculate volumes than their large, horned counterparts (Cook 1990; Simmons et al. 1999; Tomkins and Simmons 2002). Interestingly, small male *O. taurus* also develop relatively larger copulatory organs than large, horned males (Palestrini et al. 2000). This latter finding has generally been interpreted as an adaptation for males that allows them to copulate successfully with females of a wide range of body sizes (Eberhard et al. 1998; Palestrini et al. 2000). While we are in no position to evaluate any of these hypotheses, our data suggest an interesting developmental mechanism that may have provided the conditions that facilitated the evolution of the observed relationship between the relative sizes of horns, genitalia, and ejaculates in horn-dimorphic beetles.

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