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## Evolution and Development: *Onthophagus* Beetles and the Evolutionary Developmental Genetics of Innovation, Allometry and Plasticity

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### 7.1 Introduction

Over the past decade, horned beetles in the genus *Onthophagus* have emerged as a promising model system in evolutionary developmental biology (evo-devo) and ecological development (eco-devo). Specifically, *Onthophagus* beetles have attracted the attention of researchers due to:

1. the expression of horns, exaggerated and diverse secondary sexual traits lacking obvious homology to other insect traits;
2. rich phenotypic diversity including morphological, behavioural, and physiological traits;
3. the significance of environmental factors, especially nutrition, in guiding phenotype determination;
4. the fact that *Onthophagus* beetles stand out as the animal kingdom's most speciose genus.

*Onthophagus* beetles therefore offer a promising microcosm for integrating the developmental genetic underpinnings of phenotypic diversity with the physiological, behavioural and ecological mechanisms that shape this diversity in natural populations.

I begin this chapter by briefly summarizing recent methodological advances in *Onthophagus* research, including the development of gene expression assays, RNA interference-mediated gene function analysis and genomic/proteomic tools,

and by highlighting the power and limitations of each of these approaches. I then explore three frontiers in current evo-devo and eco-devo research and review how the use of genetic and developmental techniques for the study of *Onthophagus* beetles have helped advance these frontiers. Specifically, I explore the developmental genetic regulation of beetle horns as an evolutionary novelty at the heart of one of the most dramatic radiations of animal secondary sexual traits.

Next, I discuss what *Onthophagus* beetles have taught us so far about the developmental regulation of scaling, allometry and form, and some of the forces that shape these mechanisms in nature. And lastly, I review what is known to date about the developmental and genetic underpinnings of plasticity during *Onthophagus* development and its consequences for developmental evolution and diversification, including the origin of novel phenotypes.

*Onthophagus* beetles emerge as a powerful system with which to integrate micro- and macroevolutionary perspectives of development, and to explore the interplay between environmental, ecological and genetic factors in guiding morphological and behavioural evolution.

## 7.2 Evo-devo and eco-devo – a brief introduction

Evolutionary developmental biology – abbreviated commonly, for better or worse, as evo-devo – has a long, diverse and convoluted history (Raff, 1996). While the study of ontogeny as a means to gain insight into evolutionary history is as ancient as evolutionary biology itself, it was arguably only recently that evolutionary developmental biology has emerged as a coherent discipline with its own textbooks, journals, funding panels and philosophical debates (e.g. Carroll *et al.*, 2005; Samsom & Brandon, 2007).

In a nutshell, evolutionary developmental biology seeks to understand how developmental processes have originated and been shaped by evolution, and in turn how evolutionary outcomes have been influenced by the properties of development. Evo-devo clearly interfaces with both traditional evolutionary biology and genetics on one side, and with developmental biology on the other, but it cannot be subsumed within them. Specifically, evo-devo asks questions that neither contributing discipline is equipped to address by itself, such as:

- How do novel traits arise from the confines of homology and ancestral variation?
- Is macroevolution merely accumulated microevolution, or are both fundamentally different?
- How does development constrain or bias evolutionary trajectories?

Ecological developmental biology ('eco-devo'), on the other hand, is a discipline that arguably is just now being born, with one foot firmly in 'traditional' evo-devo but with another clearly stepping in directions where no discipline has gone before (Gilbert & Epel, 2009). Specifically, eco-devo seeks to identify the nature of interactions between ecological conditions and developmental processes and their consequences for the developmental biology, ecology and evolution of organisms.

With its focus on environmental factors and phenomena such as phenotypic plasticity, eco-devo builds on, but does not merely repeat, previous work on the evolutionary and quantitative genetics of genotype  $\times$  environment interactions (Schlichting & Pigliucci, 1998; DeWitt & Scheiner, 2003). Instead, while the latter treated development largely as a black box, eco-devo explicitly focuses on filling this box with biological reality.

Specifically, eco-devo addresses questions such as:

- How does the integration of ecological and genetic inputs during ontogeny shape development during the life time of an individual organism?
- How do interactions between ecological and developmental mechanisms affect the amount and type of phenotypic and genetic variation visible to selection?

Evo-devo and eco-devo therefore differ increasingly in their domains and foci. Both share, however, an immense relevance for our understanding of human health issues such as the evolution of diseases and the human body's ability and limits to mount effective defences, or the role of environmental factors in development and inheritance, such as the epigenetic effects of stress, endocrine disruptors or drugs (Gilbert & Epel, 2009).

Insect models have played a critical role in advancing basic evo-devo and eco-devo (Heming, 2003; Carroll *et al.*, 2005). Among them, and as introduced in the next section, dung beetles in the genus *Onthophagus* have emerged as particularly promising models for exploring the interplay between environmental, ecological and genetic factors in guiding morphological, developmental and behavioural evolution.

### 7.3 *Onthophagus* beetles as an emerging model system in evo-devo and eco-devo

*Onthophagus* beetles have attracted the attention of evolutionary developmental biologists for a number of reasons (reviewed in Moczek, 2006a). First, a large number of *Onthophagus* species express horns – novel and highly diverse exaggerated secondary sexual traits used as weapons in male combat over breeding opportunities (Emlen, 2000; see Chapter 3 of this volume).

Second, onthophagine diversity is not merely restricted to horns. It extends to many other morphological, behavioural and physiological traits (e.g. resource specificity, nest construction, thermoregulation, sperm competition; see Chapter 1) in a variety of contexts, such as species-differences, sexual dimorphisms or the expression of alternative morphs within sexes (e.g. Simmons *et al.*, 1999; Moczek & Emlen, 2000; Shepherd *et al.*, 2008; see Chapter 6). The latter is particularly significant because, in many cases, the 'same' phenotypic differences, such as the presence or absence of horns, can be caused by different proximate factors, permitting researchers to juxtapose environmental regulation of phenotype determination with traditional genetic or allelic determination (Emlen, 1994).

Lastly, *Onthophagus* beetles stand out as the animal kingdom's most species-rich genus (Arrow, 1951; Balthasar, 1963b; Emlen *et al.*, 2007). At least one subset of

these species is now widely distributed and relatively easy to rear and maintain in the laboratory. *Onthophagus* beetles therefore offer a promising and accessible microcosm for integrating the developmental and genetic underpinnings of phenotypic diversity with the physiological, behavioural and ecological mechanisms that shape this diversity in natural populations.

More recently, this effort has been greatly advanced through the development of key genetic and genomic techniques and resources (Moczek *et al.*, 2007). Box 7.1 briefly summarizes the most relevant techniques, the information they can provide and some of their limitations. The sections that follow will discuss how the application of these techniques in *Onthophagus* beetles has begun to advance current frontiers in evo-devo and eco-devo research. Specifically, I will introduce three interrelated focal areas of current research in evo-devo and eco-devo and review the respective advances made possible through the study of dung beetles. Highlighted throughout are some of the most significant remaining gaps in our knowledge, alongside proposed avenues by which future work with dung beetles may be able to fill those gaps. I begin with a focal area as old as evolutionary biology itself: the origin of novelty.

### **Box 7.1** Developmental genetic tools available in *Onthophagus* beetles: utility and limitations

#### **Overview**

Genes and their messenger-RNA and protein products play a pivotal role in instructing developmental processes. Moreover, changes in when and where a given gene is expressed generate important avenues for changing aspects of phenotype expression. Visualizing, or otherwise documenting, if and to what degree a gene is expressed in a given context is therefore key to investigating the actions of developmental pathways and their potential contributions to developmental evolution. This can be achieved through a variety of techniques, several of which are now routinely utilized in *Onthophagus* beetles.

#### ***In situ* hybridization**

*In situ* hybridization relies on a labelled RNA strand complementary – and thus specific – to the mRNA of a given gene of interest to localize those tissue regions where this gene of interest is transcribed (Wilkinson, 1998). *In situ* hybridizations require that at least parts of the gene of interest have been cloned, sequenced, and are sufficiently unique to the gene of interest to exclude the possibility of false positives. Strictly speaking, *in situ* hybridizations can only document whether or not a gene of interest is transcribed. However, much gene regulation occurs post-transcriptionally, and it is commonplace for genes to be transcribed but for their protein

coding regions to not be translated (Gilbert, 2006). *In situ* expression data are therefore appropriate for surveying potential candidate genes given a certain phenotype, but extrapolations toward protein expression and gene function require caution.

### Immunohistochemistry

An immunohistochemical approach (antibody staining) can safeguard against some of the limitations of *in situ* hybridization because it relies on protein-specific antibodies to localize protein products, and thus it only detects those genes that have been successfully transcribed and whose protein coding regions have been translated. For researchers working with non-model organisms, the *de-novo* production of an antibody is still often too time-consuming and costly. However, a huge arsenal of antibodies have been developed for model organisms such as *Drosophila melanogaster*, and a subset of these (albeit a small one) can be used across insect orders or, in extreme cases, across phyla (e.g. Panganiban *et al.*, 1997).

While immunohistochemical approaches reduce false inferences that may arise due to post-transcriptional regulation, they are, of course, not without limitations. Post-translational modification of proteins and their functions are also ubiquitous during development (Gilbert, 2006), so a protein expressed at a certain time or place is no guarantee that the protein is actually carrying out the hypothesized function, or in fact any function at all. More generally, and similar to *in situ* hybridizations, immunohistochemistry permits investigations only one gene at a time, and only of genes whose sequence and function are at least in part known from other organisms. Both constraints are lessened in another expression method used now across a growing range of organisms—microarray hybridizations.

### Microarray hybridizations

Many different types of microarray currently exist (reviewed in Gibson & Muse, 2009). What they all share is that they consist of thousands of microscopic spots, each of which in turn is made up of relatively short DNA molecules. If the sequence of these short DNA molecules is specific for different genes, each spot can act as a probe for a different gene. As such, microarrays permit the simultaneous examination of expression levels of thousands of transcripts. A common route to microarray development is through the development of a normalized cDNA library, i.e. a collection of the mRNA population (converted to cDNA) present in a particular tissue at a particular stage of development. Clones from such a library can then be used to manufacture a corresponding array.

In most cases, arrays are used to detect relative differences in transcript abundance by competitively hybridizing RNA samples obtained from two

different tissues or treatments. Samples are labelled with different fluorescent markers and reflectance can be used as a proxy to determine relative expression differences across tissues or treatments. Similar to the methods discussed above, microarray hybridizations typically do not allow strong inferences toward gene function, but they represent a powerful tool for quickly surveying gene expression differences across large numbers of genes, or even on a genome-wide scale.

Two types of arrays currently exist for *Onthophagus* beetles: a spotted cDNA array consisting of 4,000 spots representing approximately 2,700 genes (Kijimoto *et al.*, 2010); and a NimbleGen High Density Array consisting of 138,000 spots representing approximately 14,000 genes (Kijimoto, Moczek & Andrews, unpublished data).

### Next-generation sequencing

Next-generation sequencing refers to several innovative sequencing techniques that are capable of economically generating large amounts of sequence data. Their main limitation is that individual sequence reads are relatively short (presently 70–500 base pairs, depending on the specific approach used). However, given the extreme volume of reads generated in a single sequencing run, much overlap exists between reads which can therefore be assembled bioinformatically to larger contiguous sequences (contigs).

Next-generation sequencing is a highly effective and sensitive technique to characterize transcript diversity and to measure transcript abundance, using the number of overlapping reads of the same transcript as a proxy for expression intensity. It is most effective if the genome of the organism to be investigated has already been fully sequenced and can serve as a reference to place reads. If this is not the case, next-generation sequencing itself can be used to generate and assemble a reference transcriptome or genome.

As of the writing of this chapter, the first next-generation sequencing effort for dung beetles has been completed using Roche/454 GS-FLX Titanium platform sequencing. This effort generated >1.3 million sequence reads with >580 megabases of sequence information, which assembled into approximately 50,000 contigs and singletons. Sequences matched >14,000 genes from other organisms already described in public databases, and they also included a large number of protein coding sequences that lack obvious homology to known genes (Choi *et al.*, in press).

### 2D Differential Gel Electrophoresis (DIGE)

A similar approach to microarrays, albeit on the protein level, involves 2D Differential Gel Electrophoresis (DIGE). Here, multiple protein samples are

labelled fluorescently, combined and then separated according to their isoelectric focusing point and size on a two-dimensional gel (Unlu *et al.*, 1997). Proteins shared by samples migrate together, whereas differentially expressed or post-transcriptionally modified proteins occupy unique spots on the gel. Proteins of interest can then be extracted, and their mass and their amino acid sequence can be determined via mass spectrophotometry and blasted against available databases.

### RNAinterference

All methods listed above have the power to implicate genes and their products in processes determining phenotype expression. However, all of them ultimately rely on correlations between gene expression and phenotype expression. To examine gene function, gene expression must be perturbed experimentally. Experimental over-expression of candidate genes is commonplace in genetic and developmental model systems, but it is currently beyond the reach of most non-traditional model systems. Experimental down-regulation of gene expression, on the other hand, is feasible via RNA interference (RNAi), a relatively straightforward technique with incredible power (Novina & Sharp, 2004).

Specifically, as an experimental approach, RNAi involves injections of double-stranded RNA fragments, thereby activating a cellular response mechanism that digests exogenous RNA. Digestion fragments are then used to detect other matching RNA molecules, including those made by the organism itself, which are subsequently targeted for digestion as well. Experimental injection of dsRNA that matches a selected target gene made by the organism itself allows researchers to use the organism's own RNAi machinery against itself to deplete transcript abundance of its own genes, including those of interest to researchers.

RNAi has revolutionized experimental evolutionary developmental genetics due to its applicability across a wide range of organisms (Novina & Sharp, 2004) including *Onthophagus* beetles (Moczek & Rose, 2009). It does require pre-existing knowledge of candidate genes (such as sequence and expression data) and is relatively work-intensive, but it is extremely powerful when it comes to identifying and comparing gene function.

## 7.4 The origin and diversification of novel traits

The origin of novelty in evolution has captivated evolutionary biologists ever since the inception of the discipline (Raff, 1996; Wilkins, 2002; West-Eberhard, 2003). What has to come together – genetically, developmentally or ecologically – for complex novel traits to arise and diversify in nature? Is the origin of novel traits underlain by processes fundamentally different from those that facilitate quantitative changes in pre-existing traits, or is innovation merely an extrapolation of

diversification over time (Erwin, 2000; Davidson & Erwin, 2006; Moczek, 2008)? Furthermore, how is innovation initiated within the confines of homology and descent with modification? Where exactly does ancestral variation end and novelty begin?

For instance, one of the strictest definitions of novelty, advocated by Müller and Wagner (1991), is the absence of homology or homonymy (serial homology). Here, novelty begins where homology ends (Moczek, 2008), but exactly where homology ends has become more and more difficult to define, primarily for two reasons (Brigandt, 2002): On the one hand, we have come to understand that a tremendous amount of morphological diversity, including clearly non-homologous traits, is made possible through the use of a relatively small toolkit of ancient, conserved and homologous developmental pathways (Carroll *et al.*, 2005; Shubin *et al.*, 2009). Clearly, homology of development need not imply homology of form. On the other hand, the opposite is also true: unambiguously homologous traits often diverge dramatically with respect to the developmental genetic mechanisms that regulate their expression during development (True & Haag, 2001; Palmer, 2004), a phenomenon also known as developmental systems drift (True & Haag, 2001) or phenogenetic drift (Weiss & Fullerton, 2000).

Homology of form, apparently, need not imply homology of development either. Therefore, where homology ends and novelty begins is murkier than ever. It is precisely at this intersection that research on dung beetles has made several important contributions, focusing on the developmental genetic regulation of horns and horn diversity in the dung beetle genus *Onthophagus*.

#### 7.4.1 Dung beetle horns as novel traits

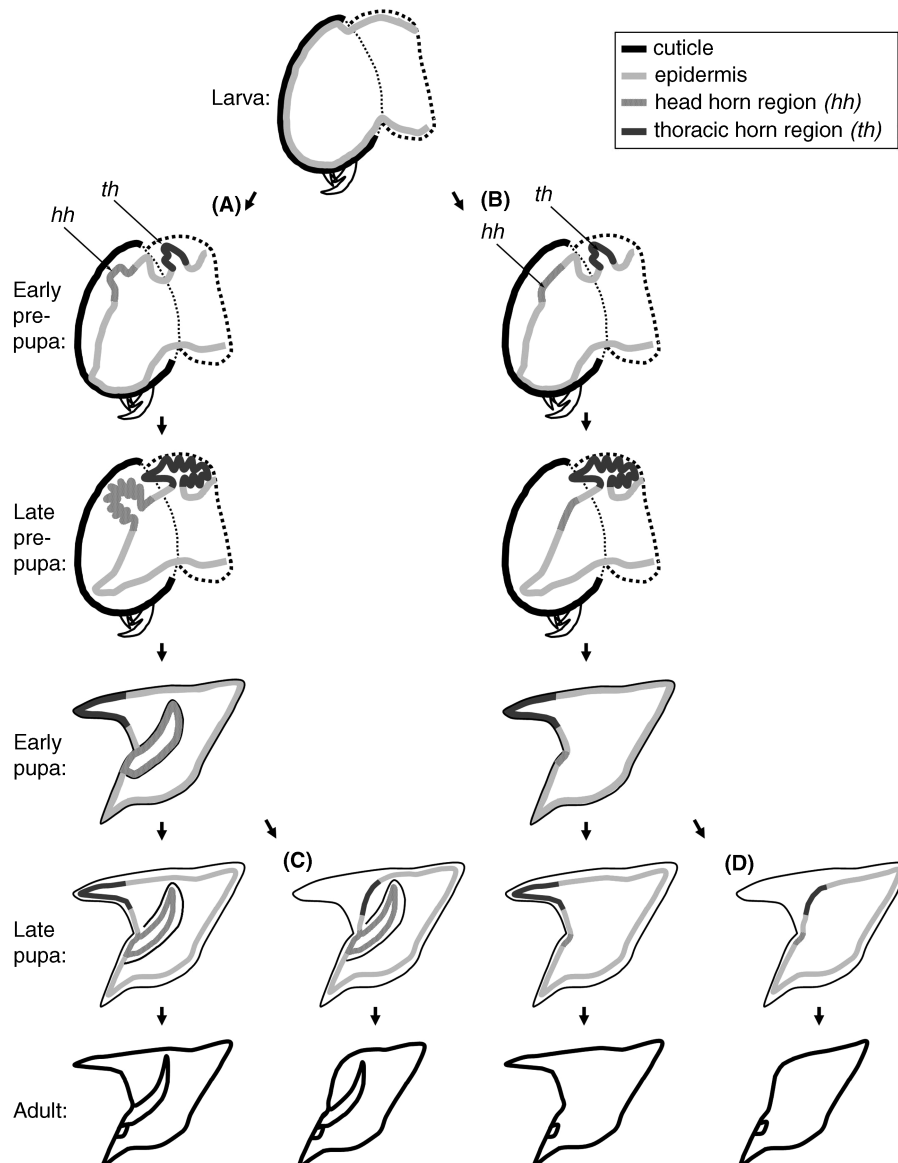
Beetle horns lend themselves well to the study of innovation in evolution, primarily for the following reasons. Beetle horns are often large, solid, three-dimensional outgrowths that function as weapons in male competition over breeding opportunities (Emlen, 1997a; Moczek & Emlen, 2000; see Chapter 3 of this volume). Beetle horns thus shape in many ways both the morphology and the behavioural ecology of their bearers. At the same time, beetle horns differ dramatically in size, shape, number and location of expression, and much of this variation can be found not only between species but also between sexes, and frequently within sexes, creating much opportunity for comparative approaches.

Lastly, and most importantly, beetle horns are unique structures in a sense that they lack clear homology to other traits in insects. Horns are not modified versions of traditional appendages such as mouth parts, antennae or legs, but instead exist alongside these structures in body regions in which insects normally do not produce outgrowths (Moczek, 2005; 2006a). Horns can therefore be considered an example of an evolutionary novelty that horned beetles invented at some point during their history, giving rise to one of the most impressive radiations of secondary sexual traits in the animal kingdom (Arrow, 1951; Balthasar, 1963a; Emlen *et al.*, 2007; Emlen, 2008).



### 7.4.2 How horns develop

The horns of beetles become first detectable during the last larval stage as the animal nears the larval-to-pupal moult (Figure 7.1; reviewed in Moczek, 2006a; Moczek and Rose, 2009). At this stage, certain epidermal regions detach from the larval cuticle and proliferate. The resulting tissue folds as it is trapped underneath the larval cuticle, then expands once the animal moults to the pupal stage. It is at this stage that horns become externally visible for the first time.



This period of prepupal horn growth is then followed by a period of pupal remodelling of horn primordia. During the pupal stage, horns undergo at times substantial remodelling in both size and shape – and, in some cases, complete resorption prior to the adult moult. After the pupal-to-adult moult is complete, horns have then attained their final adult size and shape.

The horns of adult beetles therefore develop, at least in part, in a similar manner to traditional appendages in other holometabolous insects (Svácha, 1992) and may thus be thought of, at least in developmental terms, as highly simplified appendages. Recent studies now show that many important components of the developmental machinery employed in the making of traditional appendages have, indeed, been recruited into the development and evolution of horns.

### 7.4.3 The developmental genetics of horn growth

Expression studies using immunohistochemical approaches and *in situ* hybridization (Box 7.1) were the first to implicate several important appendage-patterning genes in the making of horns. Specifically, several transcription factors known to play important roles in establishing the proximo-distal axis of insect appendages (*Distal-less (Dll)*, *aristaless (al)*, *dachshund (dac)*, *homothorax (hth)* and *extradenticle (exd)*) were also found to be expressed during the formation of horns in the prepupal stage (Moczek & Nagy, 2005; Moczek *et al.*, 2006a). Moreover, all of them but *dac* were expressed in regions of the future horn that were at least consistent with a conservation of gene function.

**Fig. 7.1** Schematic development of (A) horns and (B–D) horn dimorphisms in *Onthophagus* beetles. (A) During the last larval instar, the larval epidermis (light gray) fully lines the larval cuticle (black). At the onset of the prepupal stage, the larval epidermis detaches from the cuticle (apolysis) and selected regions (shown here for a head horn (*hh*) and thoracic horn (*th*)) undergo rapid cell proliferation. The resulting extra tissue folds up underneath the larval cuticle. The epidermis subsequently secretes the future pupal cuticle, which, upon the moult to the pupal stage, forms the outermost layer of the pupa, lined once again by a layer of epidermal cells. During this pupal moult, horn primordia are able to expand and unfold and are now visible externally. During the second half of the pupal stage, epidermal cells detach once more. This time, however, no significant growth of horn tissue follows detachment. Instead, epidermal cells secrete one last cuticle and the pupa undergoes one last moult to the final adult stage. (B) Development of horn dimorphisms through differential proliferation of prepupal horn tissue (illustrated here for head horns (*hh*) only). During the prepupal stage, presumptive horn tissue proliferates little or not at all, resulting in the absence of external horns in pupae and the resulting adults. This mechanism is used to generate sexual dimorphisms as well as alternative male morphologies for head horns in many species. (C) and (D) Development of horn dimorphisms through differential loss of pupal horn tissue (illustrated here for thoracic horns (*th*) only). Pupal horn epidermis is resorbed prior to the secretion of the final adult cuticle, most likely via programmed cell death. In many cases, resorption of pupal horn tissue can completely erase the former presence of a thoracic horn. This mechanism contributes to sexual dimorphisms for thoracic horns in many species, and can occur in the presence or absence of (differential) head horn development. Modified after Moczek, 2006a.

Recent gene function analyses (Box 7.1) using RNA interference (RNAi) mediated transcript depletion clarified these inferences substantially, and in the process they underscored the limitations arising from inferring gene function purely from comparative gene expression data. Specifically, Moczek & Rose (2009) examined the function of three of these genes – *dac*, *hth*, and *Dll* – during horn development in two species of *Onthophagus* beetles. Irrespective of any involvement in horn development, larval RNAi-mediated transcript depletion of all three genes generated phenotypic effects identical or similar to those documented by previous studies in other taxa (e.g. Prpic *et al.*, 2001; Angelini & Kaufman, 2004; Kojima, 2004). This observation was important because it documented that all three patterning genes exhibited conservation of function with respect to the patterning of traditional appendages, as well as the general feasibility of larval RNAi in *Onthophagus* beetles.

In addition, however, this study yielded many surprising insights into the functional regulation of horn development. For example, *dac* did not appear to play any obvious role in the regulation of size, shape or identity of horns, even though it is expressed widely throughout prepupal horn primordia (Moczek *et al.*, 2006a). Thus, even though *dac*RNAi individuals expressed severe *dac* knockdown phenotypes in their legs and antennae, thoracic and head horn expression was completely unaffected. In contrast, *hth*RNAi had a dramatic effect on horn expression, but only affected thoracic, not head, horns in the same individuals, even though *hth* is expressed during the development of both horn types.

The results of *Dll*RNAi made matters even more complicated. While *Dll*RNAi affected the expression of both head and thoracic horns, it did not do so in the same individuals or even the same species. In *Onthophagus taurus*, head horn expression was only affected in large males, whereas horn expression in small and medium-sized males was unaffected. Similarly unaffected was the expression of pupal thoracic horns in both males and females regardless of body size. In *O. binodis*, on the other hand, *Dll*RNAi did affect the expression of thoracic horns in both males and females, though the effect was strongest in larger individuals.

Combined, these results illustrate that horn development evolved via differential recruitment of at least some proximo-distal-axis patterning genes normally involved in the formation of traditional appendages. On the one hand, these results contribute to a by now-common theme in the evolution of novel traits: new morphologies arise through the recruitment of existing developmental mechanisms into new contexts, rather than the evolution of novel genes or pathways (Shubin *et al.*, 2009). On the other hand, they highlighted an unexpected degree of evolutionary lability in the developmental regulation of horns, including the absence of patterning function (*dac*), patterning function in selected horn types (*hth*, *Dll*) and function in one size class, sex or species but not another (*Dll*).

Different horn types, and even the same horn type in different species, may therefore be regulated at least in part by different pathways. If this is correct, different horn types may thus have experienced distinct, and possibly independent, evolutionary histories. These conclusions receive further confirmation when we take a closer look at the regulation of the second developmental period relevant to adult horn expression: pupal remodelling.

#### 7.4.4 The developmental genetics of pupal remodelling

During the pupal stage, horns are sculpted into their final adult shape. As such, pupal remodelling of horns is not unusual; indeed, all pupal appendages and body regions of holometabolous insects undergo at least some degree of sculpting during the pupal stage (e.g. Nijhout, 1991). What is unusual, however, is the often extreme nature of pupal horn remodelling, especially as it occurs in thoracic horns (Moczek, 2006b). Here, pupal remodelling can result in the complete resorption of pupal horn primordia, causing fully horned pupae to moult into thorax-horn-less adults. Pupal horn resorption occurs in at least one (female or male) or both sexes in all 21 *Onthophagus* species examined to date, suggesting that it is widespread, yet evolutionarily labile, with respect to the sex in which it occurs (Moczek *et al.*, 2006b).

Recent work now implicates programmed cell death (PCD) in the resorption of horn primordial tissue (Kijimoto *et al.*, 2010). PCD is an ancient, highly conserved physiological process employed by all metazoan organisms to remove superfluous cells and their contents during development (Potten & Wilson, 2004). For example, PCD is responsible for removing inter-digit tissue during embryonic development of the human hand; the removal of the tadpole's tail during metamorphosis; and the sculpting of the hind wing projections of swallowtail butterflies (Nijhout, 1991; Gilbert, 2006).

Recent work has now shown that PCD plays an important and dynamic role in the resorption of pupal horn primordia during *Onthophagus* pupal development. Using two bioassays, one for detecting PCD-characteristic DNA breakage, the other for detecting the expression of activated caspases (a class of enzymes used for protein digestion during cell death), Kijimoto *et al.* (2010) showed that most PCD occurred between 24 and 48 hours of pupal life. Most importantly, the same study showed a tight correlation between occurrence of PCD and subsequent resorption of pupal horn primordia.

In *O. taurus*, pupal thoracic horns of both sexes revealed high levels of PCD, matching the sex-uniform, complete resorption of thoracic horn primordia seen in this species. However, very little PCD was detected in the head horns of large male *O. taurus*, which undergo little resorption. In contrast, in *O. binodis*, high levels of PCD were only observed in female, but not male, thoracic horn primordia, this time matching the female-specific thoracic horn resorption characteristic for this species.

Thus, the amount of cell death-mediated horn resorption depended strongly on species, sex, and body region, suggesting the existence of regulatory mechanisms that can diversify quickly. Combined, the regulation of pupal remodelling therefore reinforces many of the conclusions reached above for the regulation of prepupal horn growth. As before, a pre-existing developmental machinery, this time PCD, has become recruited into a new developmental context, the sculpting of horns. At the same time, this appears to have permitted the rapid evolution of modifier mechanisms, allowing an ancient developmental process to contribute to species-, sex-, and body region-specific expression of horns.

These insights into the developmental regulation of prepupal growth and pupal remodelling of horns illustrate that regulatory genes whose functions are otherwise highly conserved nevertheless remain able to acquire new functions, and that little phylogenetic distance is necessary for the evolution of sex- and species-specific

differences in these functions. Moreover, tracing the diversity of developmental regulation of beetle horns through the phylogeny of horned beetles is beginning to provide surprising insights into the very origins of the first adult horns, as discussed in the next section.

#### 7.4.5 The origin of adult thoracic horns through exaptation

If evolutionary change is, indeed, dominated by descent with modification, everything new has to come from something old (Wake, 1999; 2003). However, tracing this ancestry is often difficult, as ancestral character states may be obscured by long periods of independent evolution of diverging lineages. Alternatively, or in addition, signatures of ancestral character states are often hidden in developmental stages other than the adult. Evolutionary scenarios inferred solely on the basis of adult trait expression are thus bound to overlook these signatures.

While this problem is widely recognized and appreciated, opportunities to integrate developmental perspectives into ancestral character state reconstructions are often limited by the degree to which the ontogenies in question are experimentally accessible. PCD-mediated resorption of pupal thoracic horn tissue, as discussed in the previous section, has provided a good example of how incorporating developmental data can be used to refine evolutionary hypotheses.

PCD-mediated resorption of pupal thoracic horn tissue is ubiquitous among *Onthophagus* species, and all species examined so far express pupal prothoracic horns in both males and females, followed by horn resorption in either one or both sexes in each species (Moczek *et al.*, 2006b). This raises the question as to the adaptive significance, if any, of such transient horn expression. Experimental approaches have now revealed that pupal horns play a crucial role during the larval-to-pupal moult, and especially the removal of the larval head capsule, and do so regardless of whether they are resorbed or converted into an adult structure (Moczek *et al.*, 2006b).

Unlike in larval-to-larval and pupal-to-adult moults, larvae that moult into pupae have absorbed most of their muscle tissue. Instead, they shed old cuticle by means of peristaltic contractions to increase local haemolymph pressure and the swallowing of air to inflate selected body regions. This suffices for the removal of the highly membranous thoracic and abdominal cuticle of larval scarab beetles, but shedding the larval head capsule poses greater challenges, since it is composed of extremely thick and inflexible cuticle. During larval life, this robust cuticle and its inward projections provide important attachment points for the powerful jaw muscles of fibre-feeding scarab larvae, such as *Onthophagus*. Histological studies have now shown that, during *Onthophagus*' prepupal stage, thoracic horn primordia enter into the space between the larval head capsule and corresponding epidermis, fill with haemolymph and then expand. This expansion appears to cause the larval head capsule to fracture along pre-existing lines of weakness. As the larval head moults into a pupal head, the thoracic horn primordium is the first structure to emerge from the head capsule (Moczek *et al.*, 2006b).

Experimental manipulations support a moulting function of pupal thoracic horns. When the precursor cells that would normally give rise to thoracic horn primordia are ablated early in larval development, the resulting pupae do not

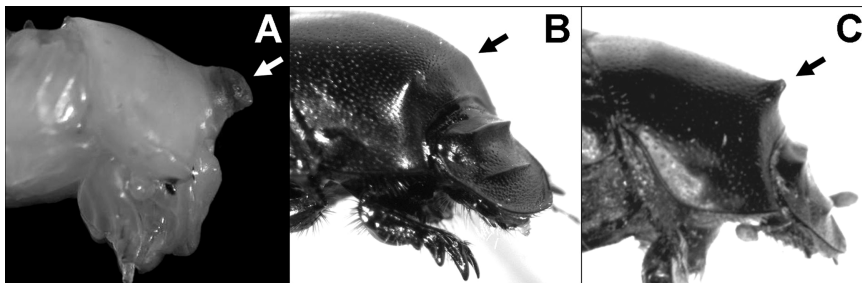
express a thoracic horn and fail to shed their larval head capsule (Moczek *et al.*, 2006b). Replicating this approach for two *Onthophagus* species yielded similar results, but it failed to elicit any effect in the sister genus *Oniticellus*, i.e. the same surgical manipulation did not impede shedding of the larval head capsule. This suggests that this putative moulting function of thoracic horn primordia may be unique to onthophagine beetles.

Phylogenetic analyses further suggest that the function of horns as a moulting device may have preceded the horns-as-a-weapon function of the adult counterparts and that, ancestrally, all pupal horns were resorbed prior to the adult moult (Moczek *et al.*, 2006b). If correct, this would explain why prepupal thoracic horns appear ubiquitous among *Onthophagus* species, even though only a relatively small subset of species uses them to build a functional structure in the adult.

How could the first adult horns have originated from pupal ancestral structures? The results presented above raise the possibility that the first adult horns could have been mediated by a simple failure to remove a pupal-specific projection via failure to activate PCD at the right developmental time and location. Several anecdotal studies suggest that such events do occur in natural populations, at least occasionally (e.g. Paulian, 1945; Ballerio, 1999; Ziani, 1994; see also Figure 7.2). Clearly, such a failure would have resulted in an adult outgrowth that at first would probably have been small. However, behavioural studies have shown that even very small increases in horn length are sufficient to bring about significant increases in fighting success and fitness (Emlen, 1997a; Moczek & Emlen, 2000; Hunt & Simmons, 2001).

Importantly, the possession of horns is not a prerequisite for fighting, since fighting behaviour is generally widespread among beetles and occurs well outside horned taxa (reviewed in Snell-Rood & Moczek, in press). The first pupal horn that failed to be removed before the pupal-to-adult moult could thus have provided an immediate fitness advantage. Thoracic beetle horns may, therefore, be a good example of a novelty that arose via exaptation (*sensu* Gould and Vrba, 1982) from traits originally selected for providing a very different function at an earlier development stage.

How do these observations help revise earlier hypotheses regarding the evolutionary origin of onthophagine horns? Mapping adult morphologies onto a



**Fig. 7.2** Example of a failure to remove pupal thoracic horns through programmed cell death in *Onthophagus taurus*. Female *O. taurus* express a thoracic horn as (A) pupae but remove it via programmed cell death prior to moulting to an adult (B). (C) A female *O. taurus* obtained from L.W. Simmons' laboratory culture failed to fully remove the pupal thoracic horn primordium and moulted into a horned female adult.

molecular phylogeny, Emlen *et al.* (2005b) concluded that thoracic horns must have originated a minimum of nine independent times in males and seven in females among just 48 *Onthophagus* species to explain present-day patterns of horn expression. This is a staggering number of independent gains over a remarkably short phylogenetic distance, but it is the inescapable conclusion if adult morphological data are the only source for inferring ancestral character states.

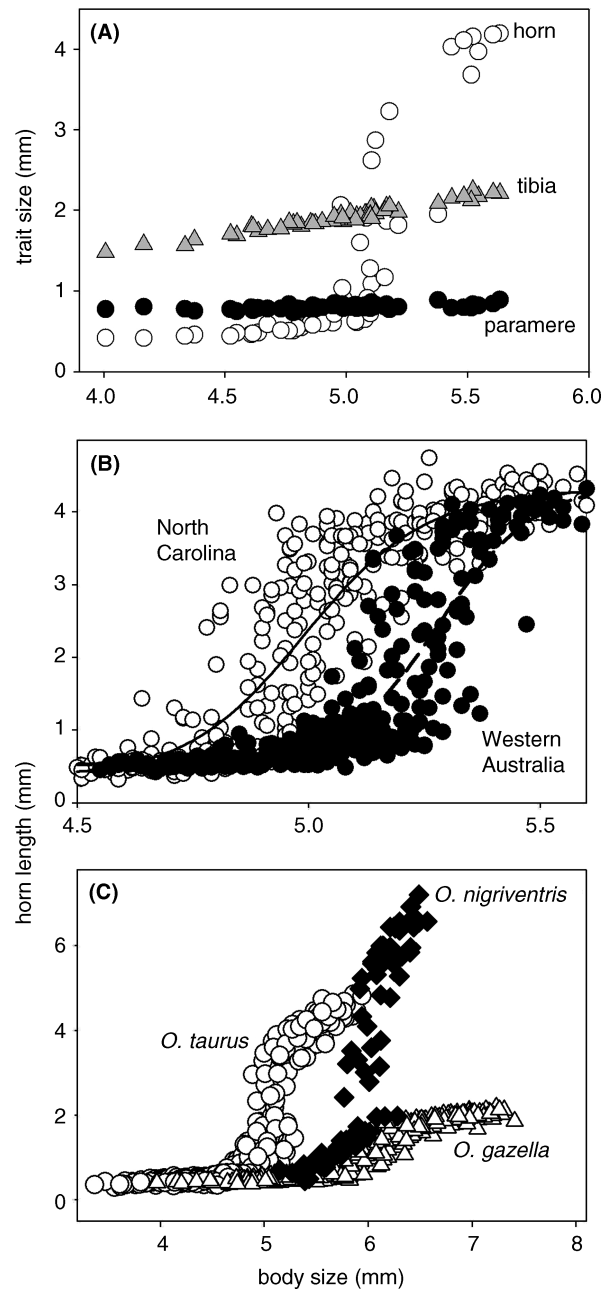
Unfortunately, only a subset of the 48 species in this phylogeny have known pupal morphologies. Incorporating the pupal morphologies of those nine species for which pupae have been described into a re-analysis (and treating the remaining 39 species as treated by the original analysis) is sufficient, however, to make the ancestral character state at all nodes either horned or undeterminable (Moczek *et al.*, 2006b). This suggests that the most parsimonious explanation for the origin of thoracic horns in *Onthophagus* may be as simple as a single gain, followed by diversification of adult horn expression in different species via pupal resorption in one or both sexes. If this scenario is correct, this presumed origin of adult thoracic horns from ancestral moulting devices would illustrate well how convoluted developmental evolution can be, and how it can yield evolutionary novelty from well within the confines of ancestral diversity and homology.

The same complexity in the interactions between development, morphology, and ecology emerges when we shift our emphasis away from the origin and diversification of horns *per se* and toward the diversification of shape and scaling in general, as illustrated in the next section.

## 7.5 The regulation and evolution of scaling

How organisms and their parts ‘know’ to what size to grow is a fundamental, yet still poorly understood, question in developmental biology (Huxley, 1932; Thompson, 1942; Gilbert, 2006). Similarly, how the mechanisms by which organisms and their parts regulate their growth evolve and contribute to organismal diversification also remains poorly understood (e.g. Stern & Emlen, 1999; Emlen *et al.*, 2007; Shingleton *et al.*, 2007; Frankino *et al.*, 2005; 2008). Scaling relationships, used here synonymously with allometries, can be depicted in their most simple forms as bivariate plots, correlating size of one trait against, typically, some measure of body size. Comparing these so-called ‘static allometries’ of different traits measured in the same group of individuals can begin to reveal some of the complexities of scaling relationships (Figure 7.3A).

Allometries range from linear and proportional (where larger animals are essentially proportionally enlarged versions of smaller animals, e.g. tibia length in Figure 7.3A) to flat and largely body size independent (large individuals have a trait with the same absolute size as smaller animals, e.g. paramere size in Figure 7.3A) to sigmoidal (a threshold body size separates two different trait sizes, e.g. horn length in Figure 7.3A). Much the same applies to scaling relationships of the same trait measured in different populations (Figure 7.3B) or species (Figure 7.3C). Intriguingly, even though scaling relationships can vary dramatically for different traits, populations or species, the variance around a given allometry is usually rather small. In other words, trait size tends to scale with body size in a highly



**Fig. 7.3** Examples of static allometries and allometric diversity in *Onthophagus* beetles. (A) Scaling relationships between body size (x-axis) and the length of the horn, fore tibia and paramere, respectively, in male *O. taurus*. The paramere is part of the male copulatory organ. (B) Scaling relationships between body size and horn length in male *O. taurus* collected in North Carolina and Western Australia. (C) The same scaling relationship for three *Onthophagus* species. Data in panels (B) and (C) are from Moczek, 2006a.



predictable manner for a given trait and population or species. This begs a number of questions, such as:

- How does each part ‘know’ to what size to grow?
- How do parts ‘know’ the size of the remainder of the organism?
- What does it take developmentally, and evolutionarily, to change the relative sizes of parts?

Preliminary answers to these questions are being provided by a growing understanding of the hormonal, genetic and environmental regulators of growth and scaling (e.g. Oldham *et al.*, 2000; Nijhout & Grunert, 2002; Nijhout, 2003a; 2003b; Emlen *et al.*, 2006; Shingleton *et al.*, 2007; 2009). Research on dung beetles, and in particular *Onthophagus*, has made several relevant contributions, which are briefly summarized in the following two sections. The more recent development of genetic and genomic resources now offers the opportunity to deepen substantially our understanding of the regulation and evolution of scaling in dung beetles, as will be discussed subsequently.

### 7.5.1 Onthophagine scaling relationships: the roles of nutrition and hormones

Larval feeding conditions play a pivotal role in determining the size of adult traits (Emlen, 1994; Moczek & Emlen, 1999). Larvae with access to optimal feeding conditions grow longer, attain larger mass and moult into larger pupae and adults. Moreover, in species in which males are separated by a body size threshold into hornless (minor) and horned (major) morphs (see Chapter 6), nutrition also determines which morph a given male develops into.

Exactly how variation in nutrition is translated into variation in growth is still largely unclear, but several likely important aspects are beginning to emerge (reviewed in Hartfelder & Emlen, 2005; Shelby *et al.*, 2007; Emlen *et al.*, 2007). For instance, juvenile hormone (JH) is an important regulator in insect metamorphosis, but it also appears to play important roles in the determination of alternative morphs such as phase polyphenism in aphids (Hardie & Lees, 1981) and castes in social insects (bees: Rachinsky & Hartfelder, 1990; ants: Wheeler, 1986; 1991; Wheeler & Nijhout, 1983).

In *Onthophagus* beetles, JH applications induce horn development in larvae otherwise fated to develop into small, hornless males, suggesting that JH may also play a role in the nutritional determination of horn expression (Emlen & Nijhout, 1999). Moreover, males from populations that have diverged in the exact body size threshold that separates hornless and horned morphs are differentially sensitive to the same JH manipulation (Moczek & Nijhout, 2002). Similarly, different species and sexes also respond differently to JH perturbations (Shelby *et al.*, 2007). Separate work also provides some evidence that ecdysteroids, a second important class of insect hormones most known for their role in the regulation of moulting, may also regulate aspects of horn expression (Emlen & Nijhout, 1999; 2001; but see Shelby *et al.*, 2007 for a critical evaluation).

Thus far, however, all data available on the endocrine regulation of size and scaling are correlational at best, and mostly derived from relatively crude manipulations. Moreover, only a very limited understanding of natural hormone titre profiles exists for ecdysteroids, and none does for JH. A deeper understanding of the evolutionary endocrinology and its role in the regulation of size and scaling in horned beetles will therefore depend on our ability to document and manipulate hormonal regulation in these organisms in a more quantitative fashion, and to identify the nature of interactions between endocrine mechanisms and their upstream regulators and downstream targets during development.

Toward this end, studies are needed to obtain JH titres as well as titres of the most relevant JH metabolizing enzyme, JH-Esterase, for several *Onthophagus* species. Comparing natural and manipulated titre profiles to transcription profiles compiled through the application of microarrays could then be used to identify putative endocrine-responsive genes, the most promising of which could be analysed functionally via RNAi. A first step in this direction has recently been taken by Kijimoto *et al.* (2010), who first documented programmed cell death (PCD) during early pupal development using standard bioassays, and then used expression profiles for the same developmental stage to identify a number of ecdysteroid-signalling genes that may regulate PCD in a sex-specific manner. This resulted in a first testable model for the developmental genetic regulation of sex-specific PCD in *Onthophagus* beetles.

Another canonical pathway involved in translating variation in nutrition into variation in growth in animals, including insects, is insulin signalling. In an important review, Emlen *et al.* (2006) presented preliminary data implicating differential expression of the insulin receptor as a possible regulator of nutrition-mediated development of alternative morphs in *O. nigriventris*. No validation of these preliminary findings has yet been published, but these results suggest an important and likely pathway as a potential interface between nutritional variation, as it is experienced by larvae, and differential growth of structures as it occurs during the prepupal stage.

Recent microarray studies further support the notion that insulin-signalling genes are differentially expressed in the context of horn development (Snell-Rood *et al.*, in press). Preliminary functional analysis of one such gene, the growth inhibitor *FoxO*, further corroborates this hypothesis (Snell-Rood & Moczek, in review). Insulin signalling may be particularly important in horned beetles, because studies in other insects suggest interesting interactions between insulin signalling and juvenile hormone (Tu *et al.*, 2005).

### 7.5.2 Onthophagine scaling relationships: the role of trade-offs during development and evolution

All growth requires resources and, if resources are in limited supply, structures that compete for them may find themselves locked in a trade-off (Klingenberg & Nijhout, 1998; Nijhout & Emlen, 1998). In such a situation, enlargement of one structure, whether during the development of an individual or the evolution of a lineage, may only be possible at the expense of another. The notion that resource allocation trade-offs may bias developmental outcomes and evolutionary trajectories is an old one, and a growing number of studies both in the laboratory and in natural populations have now shown that trade-offs are real and potentially

widespread (reviewed in Roff & Fairbairn, 2007). However, the underlying mechanisms have remained largely elusive.

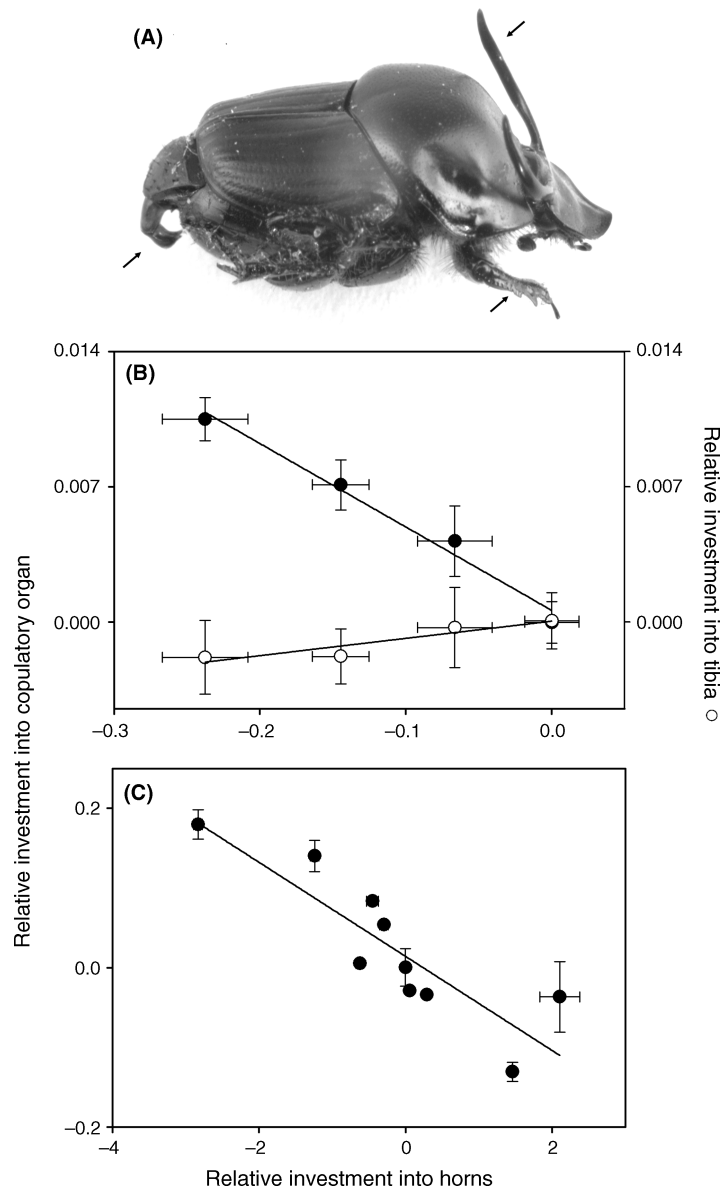
Dung beetles have provided some of the most compelling evidence for the power and scope of developmental trade-offs. For example, studying several *Onthophagus* species, Emlen (2001) showed that scaling relationships between body size and traits such as eyes, antennae or wings are affected by the relative amount of horn expression that occurs in their proximity, suggesting that structures that grow in closer proximity to each other may be more likely to engage in a trade-off. Moczek & Nijhout (2004) later expanded this notion by showing that timing of growth may be the main determinant of trade-off intensity. Using experimental manipulations of development in the laboratory, this study showed that structures growing as far apart as head horns and abdominal copulatory organs can still engage in a trade-off, provided they overlap in the exact timing of their respective growth periods. Specifically, males whose copulatory organs were prevented from developing expressed relatively larger horns than untreated or sham-treated males.

Recent comparative studies showed that this developmental trade-off may also bias evolutionary trajectories (Figure 7.4). Studying four recently diverged populations of one species (divergence < 50 years) and an additional ten more distantly diverged species (10,000–38 million years), Parzer & Moczek (2008) showed that in both cases, increased investment into horns was correlated with decreased investment in copulatory organs, and vice versa. A study by Simmons & Emlen (2006) presented at least partly complementary data (see Chapter 4). Experimental inhibition of horn development in *O. nigriventris* resulted in males producing relatively larger testes. While the authors did not find a general relationship between the relative sizes of horns and testes across species, they did observe a negative correlation between the steepness of the body size-horn length allometry and the steepness of the body size-testes size allometry, i.e. species with the steepest horn allometry had the shallowest testes allometry, and vice versa (Simmons & Emlen, 2006). Combined, these data underscore the power of developmental trade-offs over both short and long timescales and the diversity of structures that might engage in trade-offs.

Unanswered still, however, are the developmental mechanisms underlying trade-offs. For instance, it is entirely unclear why the trade-off described by Parzer & Moczek (2008) only extends to horns and copulatory organs, but not legs, even though all three structures overlap in their growth periods (Figure 7.4B). Here again, a combination of more sophisticated endocrine and genetic manipulations, combined with careful quantifications of endocrine and transcription profiles, may provide important hints as to when, where and on what level of biological organization trade-offs may arise. Some of the beginnings of such attempts are described next.

### 7.5.3 Onthophagine scaling relationships: developmental decoupling versus common developmental programme

The development of alternative phenotypes, such as hornless and horned male morphs in dung beetles, is thought to play important roles in the evolution of organismal diversity, including speciation and the origins of novel traits



**Fig. 7.4** Trade-offs between primary and secondary sexual characters in populations and species of *Onthophagus* beetles. **(A)** Horned male *O. taurus*. Arrows highlight horns, copulatory organ and fore tibia. **(B)** Relative investment into copulatory organ size (left, solid symbols) and fore tibia size (right, open symbols) as a function of relative investment into horn size in four different 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. Modified after Parzer & Moczek, 2008. Note that in the original figure, residual horn lengths were calculated as residual = expected – observed horn length. The present figure follows the more conventional way of calculating residuals (residual = observed – expected). Main results and conclusions of this study remain the same.

(West-Eberhard, 1989; 2003; Pfennig *et al.*, 2007). One central issue is the notion that alternative phenotypes are discrete developmental products resulting from genetic reprogramming, or decoupling, of development across an environmental threshold. Alternative phenotypes should therefore be able to respond to selection independent of one another, thereby increasing the evolvability of polyphenic, compared to monophenic, lineages.

However, much debate exists about the actual degree of genetic, and by extension evolutionary, independence of alternative morphs. In fact, as detailed in Chapter 6 of this volume, several allometric modelling studies argue against the evolutionary independence of alternative morphs and suggest instead that alternative forms may be the product of a common developmental programme which, through extreme positive allometry (*sensu* Tomkins *et al.*, 2005), may be able to generate discrete morphs without needing a developmental threshold to dissociate alternative developmental pathways (Nijhout & Wheeler, 1996; Tomkins *et al.*, 2005; Tomkins & Moczek, 2009).

It is important to recognize here that much of the evidence in support of genetic re-programming during polyphenic development has been generated using methodologies and taxa that may bias results toward the identification of morph-specific gene expression. For instance, direct tissue hybridizations on microarrays (see Box 7.1) or candidate-gene studies are designed specifically to detect very small differences in gene expression rather than to quantify patterns of shared expression across morphs. Furthermore, the majority of studies of morph-specific gene expression have been conducted in eusocial insects (ants, bees, termites) which may be under unique constraints in the evolution of developmental reprogramming (Snell-Rood *et al.*, 2010).

Consequently, while we can be confident that alternative morphs differ in the expression of at least some genes, we know little about the nature, extent and consequences of developmental reprogramming. More generally, we lack the ability to formulate expectations about how much differential expression may be indicative of re-programming. For example, is the development of alternative phenotypes comparable to classic examples of developmental reprogramming such as sex-specific development (Bull, 1983; West-Eberhard, 2003)? Recent transcriptional profiling of alternative male morphs and sexes in two *Onthophagus* species has provided the first tentative answers to these questions.

Contrasting transcription profiles of developing head and thoracic horns, legs and brains in *O. taurus* and *O. nigriventris*, Snell-Rood *et al.* (in press) found that patterns of expression in developing beetle morphs were generally just as divergent as between the sexes. For instance, in the developing head epidermis of *O. taurus*, which gives rise to horns in major males only, overall patterns of gene expression were more similar between females and hornless males than between the two male morphs. In contrast, in the developing brain, patterns of expression in horned males were more similar to those in females rather than the hornless male morph.

It is intriguing to speculate whether the latter similarity may arise from biparental care behaviour shared between females and horned, but not hornless, males. Thus, while differences in gene expression detected between morphs were similar in magnitude to those detected between the sexes, the development of the hornless morph appeared not to be simply due to a ‘feminizing’ of horned male expression

patterns; instead, the nature of differential gene expression across morphs and sexes depended very much on tissue type, body region and species (Snell-Rood *et al.*, in press).

Whether the fraction of differentially expressed genes detected in this study is sufficient to support a genetic decoupling or reprogramming metaphor over a common developmental programme model for beetle horn polyphenisms is a different matter, but one that may be largely semantic rather than biologically meaningful. Alternative phenotypes, no matter how divergent, nevertheless remain similar, and thus a significant degree of shared development, including gene expression, is to be expected. Whether the residual deserves to be taken as evidence for decoupling and re-programming lies largely in the eye of the beholder. The decoupling and re-programming metaphors, however, remain useful if applied to individual genes, their products and their regulators. Genes either do or do not share similar expression levels across morphs, and thus their products do or do not share similar exposure to selection (Demuth & Wade, 2007).

#### 7.5.4 *Onthophagine* scaling relationships: the developmental genetics of size and shape

The diversity of traits, shapes and sizes within and between species, and the importance of both environmental and heritable contributions to phenotype determination, make *Onthophagus* beetles a promising microcosms for exploring development and diversification of size and form. What has stood in the way until recently has been the absence of powerful genetic and developmental tools. As we have seen, this is now changing, and we are beginning to acquire new insights into evolutionary developmental genetics of size and shape. For example, as highlighted above, preliminary data implicate differential expression of the insulin receptor as a possible regulator of nutrition-mediated development of alternative morphs in *O. nigriventris* (Emlen *et al.*, 2006). Additional support for the notion that insulin-signalling genes are differentially expressed in the context of horn development comes from recent microarray studies (Snell-Rood *et al.*, in press) as well as functional analysis of the growth inhibitor *FoxO* (Snell-Rood & Moczek, in review).

Similar progress has been made toward a better understanding of the regulation and diversification of pupal remodelling of horn size and shape, and its contribution to phenotypic diversification. Previous sections have already introduced the role of programmed cell death (PCD) and its putative regulation through ecdysteroid signalling (Kijimoto *et al.*, 2010). Recent work suggests that alternatively, or in addition, *Hox* genes may play a critical role in determining adult horn size and shape through the segment-specific activation of PCD. Specifically, the *Hox* gene *sex combs reduced* (*Scr*), alters the magnitude of sex-specific pronotal horn resorption in *Onthophagus*, suggesting that PCD genes may be among the targets of *Scr* during pronotal horn development (Wasik *et al.*, 2010). This would not be unexpected, as other *Hox* genes are known to regulate PCD in a segment- or organ-specific manner. For example, the *Hox* gene *deformed* (*dfd*) directly controls the expression of *reaper*, an upstream mediator of PCD, during *Drosophila* mouthpart formation (Lohmann *et al.*, 2002).

Most of the developmental genetic regulators of growth and differentiation in dung beetles, and insects in general, remain to be identified and functionally characterized, and the same goes for their interactions as well as their role, if any, in organismal diversification. What is exciting and encouraging, however, is that in *Onthophagus* beetles, the most critical resources that will permit eventually reaching this goal appear to be in place, and that the first preliminary applications of these resources have already yielded many exciting insights. Much of the same can be said for the last focus of this chapter – the evolutionary developmental genetics of plasticity.

## 7.6 The development, evolution, and consequences of phenotypic plasticity

Phenotypic plasticity, the phenomenon by which a genotype gives rise to different phenotypes in response to changes in environmental conditions, is a ubiquitous property of all organisms (West-Eberhard, 2003). At one extreme, responses to changes in environmental conditions may be thought of as more passive, arising from the many biochemical and biophysical dependencies of biological processes. At the other extreme, such responses may arise through complex, highly choreographed and integrated adjustments of a wide range of traits. Examples of the latter extreme abound among polyphenic insects, such as seasonal or phase polyphenisms, social caste or alternative male reproductive phenotypes (reviewed in Nijhout, 1999; 2003c; Hartfelder & Emlen, 2005; Moczek, 2010).

Phenotypic plasticity has been integrated into quantitative and population genetics as genotype  $\times$  environment interactions and visualized as reaction-norms across environmental gradients (Schlichting & Pigliucci, 1998). An intense debate dominated this field into the 1990s, centring in part around whether or not ‘genes for plasticity’ are needed to properly model the evolution of plasticity (Via *et al.*, 1995). This was an important issue, because the answer would determine whether plasticity could evolve independent of trait expression within each environment. While both camps vigorously argued their case, neither spent much effort actually exploring the genetic, cellular or developmental basis of plasticity.

This debate is now largely behind us, in part because it has become abundantly clear that many plastic responses to environmental change indeed involve a complex developmental machinery, the genetic basis of which can evolve and diversify on its own, independently of other aspects of trait expression (Moczek, 2009; 2010; Pfennig *et al.*, 2010). Instead, focus has now shifted toward characterizing the exact nature of the genetic basis of plasticity and whether different phenotypic manifestations of plastic responses, from graded to step-wise and threshold dependent phenotype adjustments, share similar genetic underpinnings.

Dung beetles have contributed to this change of focus by providing many examples of plasticity evolution in the context of the expression of alternative male phenotypes (reviewed in Moczek, 2009; and see Chapter 6 of this volume). Specifically, artificial selection experiments (Emlen, 1996) and common garden rearing of divergent populations (Moczek *et al.*, 2002) have provided evidence that body size thresholds separating alternative male morphologies can diverge genetically

both in the laboratory and among natural populations. Subsequent developmental studies have offered the first hints that at least some of these divergences may be mediated by heritable changes in juvenile hormone signalling.

Overall, however, the developmental and genetic mechanisms underlying plastic responses remain poorly understood, including in insects generally and dung beetles in particular (Nijhout, 1999, 2003c; Hartfelder & Emlen, 2005; Moczek, 2010). Most importantly, the means by which these mechanisms are able to contribute and bias evolutionary diversification are only now beginning to be grasped. Recent developments in these directions are briefly discussed in the last section of this chapter.

### 7.6.1 Developmental mechanisms and the evolutionary consequences of plasticity

A growing number of studies have now shown that plasticity, whether polyphenic or otherwise, is often underlain by modularity in gene expression. In other words, different environmental conditions are associated with the expression of different suites of genes (Evans & Wheeler, 1999; 2001a; 2001b; Donnell & Strand, 2006; Hoffman & Goodisman, 2007). In such cases, the frequency of the inducing environment should determine the frequency by which a given suite of genes, or module, becomes expressed in a population within a given generation, and thus becomes visible to selection (Snell-Rood *et al.*, 2010). Rare conditions affecting gene expression only in a subset of individuals within a population should result in genes whose expression is specific to such rare conditions becoming hidden from selection and thus free to accumulate a larger number of mutations relative to genes expressed in every individual and in every generation. In turn, relaxed selection resulting from modularity in gene expression may bring about a fundamental trade-off between mutation accumulation on one side, and the degree of modularity in gene expression underlying plastic responses to environmental changes on the other (Snell-Rood *et al.*, 2010). If this is correct, such a trade-off would have far-reaching implications for defining the costs and limits, as well as the evolutionary consequences, of plasticity.

The notion that restricting gene expression to a subset of the population per generation can result in relaxed selection that may permit mutation accumulation has been examined previously in contexts outside phenotypic plasticity (e.g. evolution of senescence: Charlesworth, 1994; niche conservatism: Holt, 1996). Recent work on maternal effect genes has brought this concept closer to developmental genetics and, in particular, the modularity in gene networks (Cruickshank & Wade, 2008). Maternal effect genes are genes transcribed only by mothers. Mothers then incorporate transcripts, or their protein products, into their eggs. Strict maternal effect genes are only expressed by females and only function during early embryogenesis. The corresponding genes exist, but are not expressed, in fathers. Mutations that occur in paternal copies are therefore passed on to the next generation without being screened by selection.

Assuming equal frequencies of males and females in a population, the strength of selection operating on such genes is half that of the strength of selection operating on comparable genes expressed in every individual, or so-called zygotic genes.



Population-genetic theory consequently predicts that maternal effect genes should accumulate twice the mutation load within populations compared to similar zygotic genes (Wade, 1998). Similarly, theory predicts that, assuming nucleotide substitutions are at least mildly deleterious, maternal effect genes have the potential to diverge many times faster between species than corresponding zygotic genes (Demuth & Wade, 2007).

Both predictions are now matched by empirical data (Barker *et al.*, 2005; Demuth & Wade, 2007; Cruickshank & Wade, 2008). In the most extensive study to date, Cruickshank & Wade (2008) examined sequence variation within and between *Drosophila* species for 39 genes critical for early embryonic development. This list included nine strict maternal effect genes and 30 zygotically expressed genes. Following predictions, they found sequence variation within species to be 2–3 times higher for maternal-effect genes than any other gene class. Similarly, sequence divergences between species (*D. melanogaster* and *D. simulans*) were 2–4 times higher in maternal effect genes than any other gene class. Both findings strongly support the notion that relaxed selection acting on maternal-effect genes causes increased sequence variation within species, which in turn fuels more rapid divergences between species.

Even though this body of work did not explicitly address the consequences of modularity of gene expression, its theoretical foundation and predictions can easily be applied to a developmental plasticity context. Genes whose expression is restricted to individuals experiencing rare environments should exhibit reduced selection and accumulate mutations and, thus, greater sequence diversity within species. This, in turn, should create the potential for more rapid divergence between species, relative to similar genes expressed in every individual in every generation (Snell-Rood *et al.*, 2010). Recent studies on quorum-sensing genes in bacteria, which are induced only in generations exposed to certain population densities, provide support for both predictions (VanDyken & Wade, 2010).

The recent development of genomic resources for *Onthophagus* beetles has now created the opportunity to address these questions also in the context of developmental plasticity underlying the expression of alternative male morphs. Specifically, a series of microarray and DIGE-studies (Box 7.1) has permitted the identification of genes whose expression is specific to horned or hornless male morphs, or shared between morphs or sexes (Kijimoto *et al.*, 2009; Snell-Rood *et al.*, in press). Surveys of sequence variation are now ongoing to determine whether morph-specific genes harbour greater levels of nucleotide diversity within species, and also diverge faster between species, than similar morph-shared genes. Preliminary data on a small number of gene pairs are thus far consistent with both predictions (Snell-Rood & Moczek, unpublished data).

If genes underlying modular plasticity would, indeed, evolve faster than constitutively expressed genes, this could have far-reaching consequences for our understanding of the costs, limits and consequences of modular plasticity. For instance, accelerated mutation accumulation may permit genes underlying modular plasticity to evolve new functions more easily than similar non-plastic genes, and plasticity genes may thus contribute disproportionately to sub- and neo-functionalization events during organismal evolution (Demuth & Wade, 2007; Cruickshank & Wade, 2008). By the same argument, however, accelerated mutation accumulation may

cause modular plasticity genes to be more likely to acquire deleterious mutations and devolve into pseudogenes.

The probability of acquiring a deleterious mutation should increase with the rarity by which a given gene is induced and, as such, may place an upper limit on the range of plasticity that can be accommodated through modular plasticity. Modules whose expression occurs very rarely may simply suffer too many mutations to be maintained within populations (Snell-Rood *et al.*, 2010). By the same token, even though relaxed selection may impose constraints on the range of plasticity that can be accommodated through modularity in gene expression, it may pave the way for the evolution of alternative genetic networks for plasticity to evolve, such as integrated networks where the same suites of genes, but via altered types of interactions, contribute to the expression of different phenotypes in different environments. Recent methodological and theoretical advances, including in *Onthophagus* dung beetles, promise that these speculations will soon be followed by empirical evaluation.

## 7.7 Conclusion

The increasing availability of genetic, developmental and genomic techniques and resources outside classic model organisms has permitted dung beetles to emerge as a promising group for investigating developmental evolution in nature and in the laboratory. In the process, studies on dung beetle evo-devo and eco-devo have begun to contribute to many fundamental and longstanding debates in evolutionary biology. Given the very recent nature of some of these developments, dung beetle evo-devo promises to be an exciting area for future research, with the potential for much discovery and much integration between development, evolution, ecology and behaviour.

## Acknowledgements

I would like to thank Leigh Simmons and James Ridsdill-Smith for the opportunity to contribute this chapter. Quinton Hutton, Joseph Tomkins, Leigh Simmons, James Ridsdill-Smith and two anonymous reviewers provided many helpful comments on earlier drafts. Research presented here was supported in part by National Science Foundation grants IOS 0445661 and IOS 0718522.