Evolutionary and Ecological Genomics of Developmental Plasticity: Novel Approaches and First Insights From the Study of Horned Beetles

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Abstract

Phenotypic plasticity pervades organismal development and physiology where it facilitates an enormous range of adaptive responses to novel or stressful environments. Plasticity also impacts evolutionary processes, reducing the probability of population extinction in the face of environmental changes and sometimes increasing speciation rates in developmentally plastic lineages. Despite the adaptive significance of plasticity, organisms are not infinitely plastic; rather they are constrained in the kinds and ranges of environmental changes to which their body parts, organs, and tissues can respond. Understanding the nature, costs, and limits of developmental plasticity requires insight into (i) the developmental-genetic and genomic mechanisms underlying plastic responses as well as (ii) their interplay with ecological and social conditions. In this chapter we review and summarize recent progress in the development of horned beetles as a study system with which to explore the interactions between changing ecological conditions and plastic, genome-wide responses in gene expression and developmental function. In particular, we focus on plastic responses to nutritional variation, which in horned beetles differ widely as a function of body region, sex, and species. We begin by introducing the study system and summarize the developmental-genetic and genomic tool set currently available for horned beetles. We then present recently developed statistical approaches that can be used to guide

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the design of multi-factorial genome-wide transcriptional comparisons when circumstances prohibit a fully balanced design. We present an example of such an approach in the horned beetle *Onthophagus taurus* and end by highlighting the growing opportunities for future ecological-genomic studies in horned beetles.

Keywords
Developmental plasticity • Nutrition • Microarray • Horned beetle • Sexual dimorphism • Onthophagus • Polyphenism • Doublesex

7.1 Introduction

Developmental plasticity is taxonomically widespread and believed to be of major ecological and evolutionary significance. Yet developmental plasticity and its emergent properties are difficult to study in traditional molecular-genetic model systems given their general lack of pronounced plastic responses and a solid ecological context. Hence, a deeper understanding of the causes, mechanisms, and consequences of plasticity requires the development of more appropriate model taxa and corresponding experimental tools and resources. In this chapter we review recent progress in the development of horned beetles as a study system with which to explore the interplay between ecological conditions and plastic, genome-wide responses in gene expression and developmental function. In the first part we provide a brief overview of the current critical questions in the field of developmental plasticity as they relate to long-standing frontiers in evolutionary ecology. We then introduce the biology of horned beetles, alongside key genetic, developmental, and genomic tools and resources that have been developed in recent years to investigate the multifactorial nature of horned beetle plasticity. In the second part we focus on an ecological variable of key relevance to most heterotrophic organisms – variation in nutrition. Here, we present recently developed statistical approaches that can be used to guide the design of multi-factorial genome-wide transcriptional comparisons of nutritional plasticity when circumstances prohibit fully balanced designs, and present the first results of such an effort in the horned beetle *Onthophagus taurus*. We end by discussing the growing opportunities for future ecological-genomic studies in horned beetles.

7.1.1 Plasticity's Significance in Development and Evolution

We define *developmental plasticity* as the ability of an individual or a genotype to adjust their development in response to the environment (see the Glossary for definitions of italicized terms). Developmental plasticity is taxonomically widespread, and virtually all organisms as well as developmental processes exhibit some degree of plasticity (Newman and Muller 2000; West-Eberhard 2003; Whitman and Ananthakrishnan 2009). Developmental plasticity ranges from simple responses to changes in ambient abiotic conditions such as temperature or pH, to highly choreographed adjustments of entire syndromes of traits, such as nutrition-dependent caste determination (Smith et al. 2008) or seasonal reproduction (Piersma and Drent 2003). Developmental responses may be gradual or discrete, reversible or not, and may or may not always be adaptive. In fact, *incorrect* developmental responses to the environment are at the heart of many human diseases, such as allergies, asthma, diabetes and obesity (Gilbert and Epel 2009; Gluckman et al. 2009).

In an extraordinarily wide range of circumstances, however, developmental plasticity

is adaptive, allowing organisms to maintain high performance in the face of environmental variability. As such, plasticity plays a key role in enabling individuals and populations to respond adaptively to environmental fluctuations, be they changes in climate, nutrient availability, social conditions, or predators (Charmantier et al. 2008; Sol 2009; Sih et al. 2011). In the process, developmental plasticity has significant consequences for evolutionary processes, on several levels (reviewed in West-Eberhard 2003; Pfennig et al. 2010; Moczek et al. 2011).

First, phenotypic plasticity promotes survival in novel and changing environments, which increases the chances that a population will eventually adapt to that environment (Price et al. 2003; Lande 2009). For instance, in vertebrates, variation in behavioral plasticity has been linked to differences in survival in new environments and subsequent diversification (Sol et al. 2005, 2008; Sol and Price 2008). Second, the developmental architecture underlying a plastic response can be utilized in the evolution of a fixed trait. For instance, the genes involved in a plastic aggressive response overlap with those that have diverged in expression between more or less aggressive honeybee subspecies (Alaux et al. 2009). Similarly, pathways involved in plasticity in skeletal development are similar to those that are responsible for skeletal divergence across species (Young and Badyaev 2007). Third, developmental plasticity can foster the accumulation of genetic variation because new mutations are more likely to be hidden from selection. Such cryptic genetic variation may be revealed in novel environments, leading to subsequent rapid adaptation (Snell-Rood et al. 2010; Draghi and Whitlock 2012). Fourth, the developmental machinery that enables plastic responses, once evolved, can be recruited to orchestrate plastic developmental responses in other, and possibly very different, contexts. A spectacular example of the repeated cooption of environment-sensitive development can be seen in holometabolous insects, where the same endocrine machinery plays a critical role in coordinating alternative reproductive decisions (e.g. whether to invest in growth/maintenance or reproduction), alternative developmental decisions (molting to larva, pupa, or adult) and polyphenic development (facultative diapause, host switch, caste and morph expression; reviewed in Stansbury and Moczek 2013). Fifth, plasticity enables diversification through *genetic assimilation*, a process whereby a trait that was initially induced by the environment becomes constitutive in expression through genetic changes in underlying developmental pathways (Pigliucci and Murren 2003; West-Eberhard 2003; Bateson and Gluckman 2011; Renn and Schumer 2013). Assimilation of induced traits is particularly likely if plasticity is costly (Lande 2009; Bateson and Gluckman 2011) as it is generally assumed (but see below). Lastly, plasticity can fuel diversification if the induction of alternate developmental pathways results in assortative mating or pre-zygotic isolation, for instance through changes in ornamentation, sensory systems or the timing or location of mating (Pfennig et al. 2010). In summary, while there are clearly some situations where plasticity can impede diversification through buffering of selection pressures (Huey et al. 2003; Pfennig et al. 2010), much emerging evidence suggests that in many cases, plasticity is capable of promoting adaptation, diversification, and innovation in response to novel and changing environments (West-Eberhard 2003; Pfennig et al. 2010; Moczek et al. 2011).

In order to understand how plasticity may impact diversification, we must also understand why plasticity varies within and across species. Despite the benefits of plasticity, organisms are not infinitely plastic; rather, plastic responses during development are limited in range and kind, and many environmental challenges result in no, or non-adaptive, responses. Biologists have long been interested in the costs and constraints that limit the evolution of plasticity (DeWitt 1998; Schlichting and Pigliucci 1998). Yet the costs of plasticity remain elusive (Pigliucci 2005; Van Buskirk and Steiner 2009) and the forces that constrain the evolution of plasticity remain

poorly understood. A major stumbling block to understanding the evolution of plasticity is an incomplete knowledge of the developmental mechanisms underlying plasticity, given that such mechanisms will determine the types of costs and constraints associated with plasticity (Snell-Rood et al. 2010; Snell-Rood 2012). For example, theoretical considerations predict that the evolution of alternate developmental pathways may be limited by *relaxed selection* relative to a specialized, less plastic genotype (Kawecki 1994; Whitlock 1996; Van Dyken and Wade 2010). In contrast, forms of plasticity that rely less on evolved switches and more on learning-like mechanisms (Frank 1996) come with substantial individual-level costs and life history tradeoffs, such as delayed reproduction and reduced fecundity (Mayr 1974; Johnston 1982; Snell-Rood 2012). However, the developmental genetic basis of many forms of plasticity remains unclear. Even for environment-induced differences in gene expression, we know that many plastic responses are a result of conserved, environmentally responsive pathways (like insulin signaling (Nijhout 2003; Shingleton et al. 2007)), but many other responses rely on stochastic processes (Eldar and Elowitz 2010; Feinberg and Irizarry 2010; Wang and Zhang 2011). Each of these mechanisms comes with distinct costs and evolutionary consequences.

A key hurdle in this process of determining the mechanisms underlying plasticity and their evolutionary consequences has been a general lack of model systems with pronounced plastic responses that also possess the relevant genomic and developmental tools. Horned beetles, most notably in the genus *Onthophagus*, have emerged as a valuable model system in this regard, combining rich diversity of plastic responses over a range of phylogenetic distances with an increasing array of developmental genetic and genomic tools and resources (reviewed in Kijimoto et al. 2012b). In the next section we provide a brief overview of the biology of horned beetles, and then summarize key techniques and resources available to investigate the multifactorial nature of horned beetle plasticity.

7.1.2 The Plastic Biology of Horned Beetles: A Primer

Horned beetles are not a monophyletic group; rather, species in at least seven, partly quite distantly related beetle families have evolved horns or horn-like structures (Fig. 7.1) (Snell-Rood and Moczek 2013). However, the majority of horned beetle species as well as diversity in horn structures are concentrated in two subfamilies within the Scarabaeidae: the Dynastinae (rhinoceros beetles) and Scarabaeinae (true dung beetles) (Arrow 1951). In both subfamilies, thousands of species develop horns, including many cases of extreme elaboration (Fig. 7.1). Where they exist, and no matter how diverse in shape and size, horns are used as weapons in male combat over access to females.

Horned beetles exhibit plasticity on a variety of levels of biological organization and developmental time scales (Fig. 7.1) (reviewed in Valena and Moczek 2012). Most obvious is the development of horns, which in most species is limited to males, and among conspecific males, is closely tied to the availability of food during larval development (Fig. 7.2). Nutrition-dependent development of horns can be isometric (i.e. large males are essentially proportionally enlarged versions of small males in all respects including horns), positively allometric (large males develop disproportionally larger horns) or discretely dimorphic: in this case males below and above a certain size threshold develop into alternative hornless and horned morphs, akin to the development of alternative worker and soldier castes in social insects. Male morphs also diverge plastically in other morphological traits, such as the relative sizes of wings, mouthparts and antennae (possibly due to resource allocation tradeoffs arising from investment into horns; Emlen 2001) as well as the relative sizes of testes, which play an especially important role in the behavioral ecology of individual, competing males. Whereas large, horned males rely on aggressive fighting behavior and the use of horns as weapons to secure mating opportunities, small, hornless males utilize non-aggressive sneaking behaviors to access females on the sly and rely greatly on

Fig. 7.1 Examples of the exuberance of horned beetle diversity. From *top* to *bottom*: *Phanaeus imperator* (South America), *Onthophagus watanabei* (Borneo), *Eupatorus gracilicornis* (Southeast Asia), *Trypoxylus* (*Allomyrina*) *dichotoma* (East Asia), *Golofa claviger* (South America)

enhanced sperm competition through enlarged ejaculate volumes (Simmons et al. 1999; Simmons and Emlen 2006). Alternative male morphs therefore reflect divergent syndromes of morphological, physiological, and behavioral traits, adapted to suit alternative, sexually selected competitive niches. Male dimorphisms are extremely common and can be so elaborate that alternative morphs have on occasion been described as belonging to separate species (Paulian 1935).

A second major level of variation in horned beetle development is found between sexes, which in many ways parallels the differences just described for male morphs (Fig. 7.2; Kijimoto et al. 2012b). Like the hornless, or *minor* male morph, females typically exhibit greatly reduced horn development or no horns altogether. Sexual dimorphisms are not due to plasticity in the strict sense and instead result from sex-specific development, most likely following XY sex-determination (Angus 2008). However, recent work has shown that male (morph)– and sex-specific horn development are underlain by the same developmental machinery, and that their co-evolution has greatly influenced the radiation of horned beetles (Kijimoto et al. 2012a). Lastly, enormous variation exists among horned beetle species in the precise location, number, and shape of horns, as well as degree of male– and sexual dimorphism, reflecting evolved modifications in the developmental mechanisms underlying plastic and *canalized* aspects of horn formation (Figs. 7.1 and 7.2; reviewed in Kijimoto et al. 2012b).

In recent years the horned beetle genus *Onthophagus* has emerged as a particularly accessible study system with which to examine the evolutionary and developmental genetics of plasticity, as well as the role of plasticity in diversification and innovation (e.g. Emlen et al. 2005; Moczek 2005; Kijimoto et al. 2012b). *Onthophagus* is home to over 2,000 extant, and highly diverse species, many of which are widely accessible and easy to maintain, observe, and rear. Moreover, a subset of species has been introduced to exotic locations either on purpose as part of bio control programs or by accident, providing rare opportunities to study contemporary evolution (including of plasticity) in action.

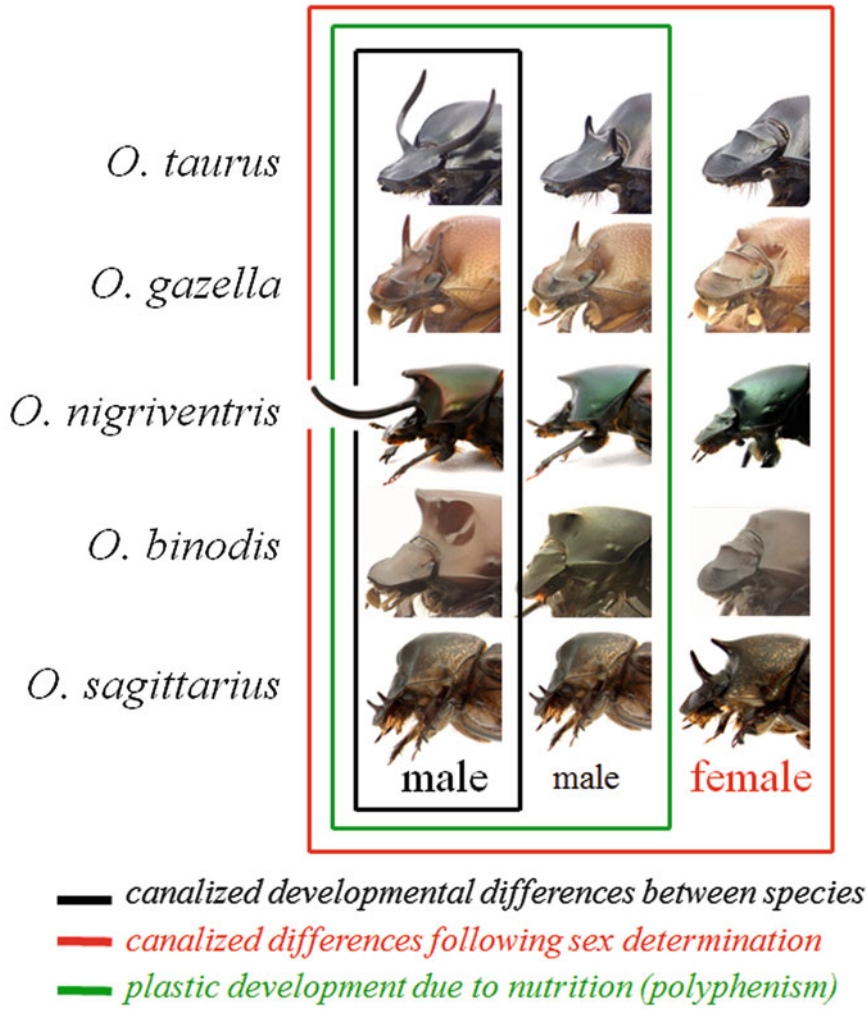

Fig. 7.2 Diversity in horned beetle morphologies and underlying causes, as illustrated by five *Onthophagus* species. Species differ in male and female morphologies due to evolved, canalized differences in developmental programs (*black box*, indicating species-specific differences in horn development in large males). Within each species, males and females exhibit more or less pronounced sexual dimorphisms due to canalized, sex-specific development following XX/XY sex-determination (*red box*). Lastly, males within each species exhibit more or less pronounced, facultative male dimorphisms, cued entirely by larval nutrition. All five species are widely available and easily maintained in captivity

To this end, a growing set of experimental tools and resources has become available over the past decade (reviewed in Kijimoto et al. 2012b). Next-generation transcriptomes of multiple species and populations and the use of custom microarrays or RNA sequencing now enable comprehensive, genome-wide comparisons of sequence and expression data, while RNA *interference* mediated transcript-depletion provides an effective and reliable means by which to examine the function of candidate pathways in a comparative, phylogenetic framework. In the next section we focus on recent efforts to utilize a subset of these resources to gain a better understanding of developmental-genetic underpinnings of sex– and body region-specific plasticity in one particular species: *Onthophagus taurus*.

7.2 Developmental Plasticity in Horned Beetles: Challenges and Approaches

Understanding the nature, costs and limits of plastic responses to environmental changes, and how and why plasticity evolves the way it does, requires a thorough understanding of the developmental genetic mechanisms that underlie the diversity of plastic responses seen in nature. In this section we focus on a study that aimed to compare and contrast the developmental-genetic mechanisms underlying diverse growth responses to nutritional variation in different body regions and sexes in *O. taurus*, a species with an extreme sexual and male dimorphism (Fig. 7.2). We begin by providing a brief background behind the rationale for this approach.

7.2.1 The Multifactorial Nature of Horned Beetle Plasticity

As introduced above, horned beetle plasticity occurs on different time scales and levels of biological organization. For example, male larvae initiate the development of future alternative hornless and horned morphologies in mid- to late larval development, with different tissues and body regions growing and differentiating at different rates. This process then gives rise to adult individuals which weeks later need to engage in facultative, morph-appropriate sneaking or fighting behaviors, etc.

Even at the same level of organization and time scale, such as growth responses to a shared nutritional gradient, plastic responses can vary dramatically. A case in point is the bull-headed dung beetle *Onthophagus taurus*, in which males and females differ substantially in body region-specific growth responses to nutritional variation. We chose to explore the regulation and diversification of plastic development by focusing on the diversity of growth responses seen among four such body regions, all of which derive from epidermal tissue: abdominal epidermis, legs, thoracic horns and head horns, for the following reasons.

In female *O. taurus*, all four body regions exhibit roughly proportional growth increases in response to increased nutrient availability during larval development. In males, in contrast, only abdominal epidermis shares the same growth response as seen in females, whereas male legs grow slightly – and male thoracic horns grow substantially – larger than their female counterparts when exposed to the same nutritional gradient. Finally, male head horn epidermis shows the most extreme growth response and exhibits explosive, non-linear growth once a certain nutrition threshold is exceeded. Combined, these sex- and body-region-specific responses to nutrient availability result in females expressing a continuous range of adult body sizes, such that large adult females represent proportionately enlarged versions of small females. Adult males exhibit the same range of nutritionally determined body sizes as do females, but instead metamorphose into two relatively discrete horned and hornless morphs.

Understanding the developmental mechanisms that enable plastic responses and their modification, be it during development as a function of sex and body region, or during evolution as a function of population- and species, requires that we realistically incorporate the complexities of plasticity into experimental designs. Focusing on *O. taurus*, we sought to execute a transcriptome-wide comparative study that would be able to robustly disentangle and analyze the transcriptional response associated with nutrition-dependent differential growth of different body regions in males and females. Before doing so, however, we had to overcome several experimental design limitations. In the next section we present a case study that hopes to demonstrate how careful experimental design can help overcome constraints imposed on transcriptional comparisons by limited resources or more generally, incomplete data, issues that will likely be common in ecological genomic studies in the future.

7.2.2 Robust Variance Estimation When Circumstances Preclude Balanced Designs

In this study we sought to characterize the nutritional responses of four different body regions in male and female *O. taurus*. Our experimental design therefore had to enable robust characterization of 16 conditions: two nutritional levels [large "L", small "S" x 2 sexes (male "M", female "F") x four body regions (abdominal epidermis "A", leg "L", thoracic horn "T" and head horn "H")]. We employed a microarray approach involving custom-made NimbleGen© arrays developed for *O. taurus* based on a comprehensive 454-transcriptome (Choi et al. 2010) to estimate the effects of nutrition, sex, and body region on gene expression. However, as explained in further detail below, executing this study using a traditional design was not possible due to cost limitations, which instead limited us to the use of only four arrays, or 48 subarrays. In the next section we first describe the general consideration that led to the final experimental design, which allowed us to greatly reduce the number of sample comparisons in our experiment without sacrificing statistical power for those contrasts we considered most relevant. We conclude this section by presenting measures that document the effectiveness of our approach.

7.2.2.1 Experimental Design: General Considerations

Completely balanced experimental designs were developed for precisely the kind of factorial experiment that is needed for studies of effects of different factors (e.g., nutrition, sex, body region) on responses of interest (e.g., gene expression levels; Box et al. 2005). Such designs aim primarily to estimate, with as little uncertainty as possible, main effects of these factors – e.g., the effect of nutrition (high vs. low) for all levels of sex and body region, the effect of sex for all levels of nutrition and body region, and the effect of body region (e.g. head horn vs. abdomen) for all levels of sex and nutrition. Secondary interest applies to two-way interactions among these main effects – e.g., the effect of nutrition for males and for females averaged over all body regions (N × G), the effect of nutrition for different tissues averaged over both males and females (N × T), and the effect of sex for different tissues averaged over both nutrition levels (G × T). In the present circumstance, as well as in many other studies in ecological genomics, the focus was not on these main effects and two-way interactions; rather the primary interest lay in a 3-factor interaction: the effect of nutrition (N) on body-region and sex classes.

The technology of microarray experiments adds further complications: first, not all 16 treatment conditions can be examined in a single subarray. Instead, array technology limits comparisons to be executed only 2 at a time. The resulting design would need to take account of the fact that only 2 of the 16 conditions can be run in a single subarray. This situation arises in many other fields of experimentation as well, prompting the development of experimental designs with incomplete blocks (here, the "block" is the subarray). Second, array-based experiments pose the additional challenge of "dye bias" – i.e., the difference in the responses when conditions are tagged "red" vs. "green" may not be the same as when they are tagged "green" vs. "red". Taken together, these three features – interest in 3-way interaction, incomplete blocks of size 2, and dye bias – therefore challenge the classical experimental design paradigm to provide accurate, precise estimates of direct interest.

7.2.2.2 Possible Experimental Designs

To compare all 16 treatments, including dye-flips (also known as dye reversals), would require $16 \times 15 = 240$ subarrays, or 20 12-plex arrays, about five times the resources available to us at the start of this study. If we assume that the dye-bias effect does not interact with any of the other (main, 2-way, 3-way) effects of interest, then we could infer the effects of dye bias by a sensible labeling of treatments in the design (e.g., label a given treatment with Cy-3 on one subarray but with Cy-5 on another subarray), reducing the need for dye-flips among all 120 comparisons

among the 16 treatments. This assumption would enable us to reduce the number of 12-plex arrays to only ten, which is still 2.5 times the size of the study permitted with available resources. Thus, a balanced incomplete block design was considered infeasible.

Alternatively, we considered a specific type of partially balanced incomplete block design, known as a cyclic (or loop) design, in which each treatment occurs the same number of times and some pairs occur together zero, one, or two times. With a limit of only 48 subarrays, a balanced cyclic design would allow each of the 16 treatments to occur with six other treatments. Such a design initially represented a viable option. However, when compared with the design presented in the next section it became clear that a cyclic design would result in lower precision for the eight nutrition contrasts that were of primary interest in this study.

7.2.2.3 Alternative Experimental Design Options Through Application of the Square Combining Table

The *square combining table* (SCT) is a type of analysis of variance, specifically developed for data arising from pairwise comparisons where the relevant data can be computed as the difference between two states (Godfrey 1985). The SCT was originally developed as an analytical tool to enable robust variance decomposition in instances in which conventional analysis of variance approaches become unreliable, for instance when data are missing (Godfrey 1985). The effectiveness of the SCT in fitting tables with missing data values is a function of the difference data that do exist, and the degree to which they allow repeated, independent estimation of missing data. When applied correctly, the SCT enables a standard least-squares analysis of variance in the face of missing data and maintains formal orthogonality among row and column combinations, enabling simple comparisons among independent contrasts. Development, application, and limits of the SCT are discussed in detail in Godfrey (1985). While the SCT was developed originally as an analysis rather than as a design tool, it can be used to create experimental designs for comparing treatments within a block such as those that arise with microarray experiments and more generally any experimental approach where data are missing due to design constraints or partial experimental failure. Specifically, by arranging effect types *a priori* from most to least interesting from the viewpoint of the investigator, the SCT can be used to prioritize direct and indirect contrasts, as well as to determine the appropriate number of replicate observations for specific effects. Below we explain how we used the SCT to guide design and analysis of our experiment.

7.2.2.4 Prioritizing Direct Comparisons

Cost considerations limited our experimental design to four 12-plex *NimbleGen*© arrays, i.e. a total of 48 identical subarrays, for a maximum of 48 pairwise comparisons. At the same time, not all pairwise comparisons were considered equally biologically meaningful. Instead, the primary focus of this experiment was to estimate the effect of nutrition (i.e. comparing "L"(= "High nutrition") and "S"(= "low nutrition")) in each of eight sex x treatment conditions. Specifically, comparisons of the form LM-SM and LF-SF for all four body regions were of primary interest. Within this group, comparisons for head horns (LMH-SMH) and thoracic horns (LMT-SMT) were considered especially relevant, given the elevated and extreme levels of nutritional plasticity seen in developing thoracic and head horns, respectively. In contrast, characterization of the effect of sex in large (high nutrition) and small (low nutrition) individuals (i.e. LF-LM and SF-SM for all four body regions) were considered of secondary importance. Lastly, of least importance were comparisons among different sizes and sexes, i.e. comparisons of the form LM-SF and SM-LF for all four body regions.

With these considerations in mind we prioritized pairwise, direct comparisons (i.e. hybridizations onto the *same* subarray) such that (a) all possible body region comparisons would be executed for LM (i.e. high nutrition = large males), *including* dye flips, (b) all possible body region comparisons would be executed for SM

Table 7.1 Comparing variances of nutrition differences for two designs (SCT, Cyclic) by sex (F = female, M = male) and body region (A =abdomen, H = head horn, L = leg, T = thoracic horn d = nutrition difference; for example dFA = nutrition difference in female abdomen = LFA-SFA)

	dFA	dMA	dFH	dMH	dFL	dML	dFT	dMT
SCT-design	0.19365	0.17322	0.14034	0.13004	0.19409	0.19612	0.14036	0.13247
Cyclic design	0.15699	0.15699	0.15699	0.15699	0.15699	0.15699	0.15699	0.15699
Ratio	1.23350	1.10337	0.89391	0.82828	1.23631	1.24923	0.89403	0.84381

(low nutrition males) and LF (high nutrition females) *excluding* dye flips, and (c) all possible large-small comparisons (i.e. LM-SM, LF-SF, LM-LF, SM-SF) for each body region, including dye flips for LM-SM and LF-SF, but not LM-LF and SM-SF comparisons. However, this design did not permit direct tissue comparisons in small (=low nutrition) females, and subsequent analyses showed that corresponding estimates would not be obtainable from linear combinations of subsets of other observations in this design. Because the overall design offered more than adequate estimates of dye bias, we thus reallocated six comparisons originally designated as replicate hybridizations (involving dye flip) to instead estimate body region differences in low-nutrition females. This adjusted design included a total of 33 red-green and 11 green-red hybridization among replicate hybridizations, which was deemed adequate for estimating dye bias while enabling at least some investigation into body region differences in low-nutrition females. Not surprisingly, the resulting final design was not perfectly balanced. Among 16 conditions, 12 are represented 5–6 times, while one each is represented four, seven, eight, and nine times respectively. This lack of balance had little effect on the precision of the estimates, and in fact enabled greater precision for some comparisons, as discussed below.

7.2.2.5 Effectiveness of SCT-Based Experimental Design

The effectiveness of our approach can be quantified by comparing the estimated variances in the effects of interest (the eight nutrition comparisons in the eight sex x body-region classes) that arise from both a conventional cyclic design (de Mendiburu 2013) and the SCT-based design. Both design matrices as well as details on variance computation are detailed in the Appendix. Using conventional least-squares estimates for the mean gene expression in each of the 16 conditions types, one can calculate not only the 16 means but also estimates of their precision, which then translate into estimates of precision for the contrasts of interest. In general, the higher the precision (i.e., the lower the variance of the estimated effect), the better the design. Table 7.1 compares the variances of the eight nutrition differences among the sex x body region categories, for both the SCT-based design and for a conventional cyclic design. The cyclic design results in similar variances for all pairs of differences, but many of which are of no interest for our study's purposes (e.g., LMA-SFA, LFH-SMT). In contrast, even though the SCT-based design gives slightly larger variances for the estimated nutrition differences among male and female abdomen and legs, the variance for the nutrition differences of greatest interest, i.e. head horns and thoracic horns for both males and females, are 11–18 % smaller compared to the cyclic design. Thus, although the cyclic design has better balance in the variances across all pairs, the SCT-based design has lower variances for the contrasts of greatest interest.

More generally, the SCT-based design allowed us to fit our experimental objectives within the constraints imposed by array technology as well as the limitations of our budget, while maximizing our ability to investigate the transcriptional response to nutritional variation across a diversity of traits. Our study illustrates the need for the design to take into consideration numerous sources of variability that can arise in any study, though clearly the exact issues to consider will depend on the measurement technology. Derivations for these calculations, as well as the equations for

estimating the contrasts and their estimates of precision, are detailed in the Appendix. Additional analyses that assess significance of the nutrition differences for all 42,010 contigs and their distribution across body regions and sexes are currently being conducted and will be reported elsewhere.

7.3 Genomics of Horned Beetle Plasticity: Recent Insights and Future Opportunities

In this last section we would like to summarize important recent studies and highlight several additional opportunities that exist in the study of horned beetles that would enable a further integration of developmental genetic and ecological genomic perspectives of the mechanisms and consequences of plasticity, while taking advantage of some of the statistical methodology discussed above.

7.3.1 Microevolution of Plasticity: Integrating Ecological Genomics and Behavioral Ecology

A major goal in ecological and evolutionary genomics is the identification of genes underlying ecologically important phenotypes. In the horned beetle system described above, both sexual and male dimorphisms involve marked differences in morphology, behavior, and reproductive tactics. Recall that while sexual dimorphisms are the product of canalized sex-specific differentiation, male dimorphisms are the product of nutritional variation experienced during larval development. In other words, genetically related males, depending on the nutritional conditions experienced during larval development, may develop into a large, horned (major) morph that fights for access to females or a small, hornless (minor) morph that sneaks copulations. The precise scaling relationship between body size and horn length is generally species specific and diagnostic, suggesting that this nutritional *polyphenism* is under tight genetic control (Emlen et al. 2005).

Importantly, species readily diverge in important components of this male nutritional polyphenism and the corresponding relationship between body size and horn length. For example, considerable differences exist among species in the morphological disparity between alternative morphs, manifest in species-specific differences in the average horn length of minor and major morphs, or the *amplitude* of the body size – horn length *allometry* (Valena and Moczek 2012). In Fig. 7.3a, for example, *O. nigriventris* males exhibit the most dramatic disparity between large and small morphs, followed by *O. taurus* and *O. gazella*. *O. sagittarius*, in contrast, has secondarily lost the male dimorphism; in this species male horns are overall rather small and scale linearly with body size. These differences among closely related species provide a rich context for comparative studies on the evolution of scaling relationships. Comparative genomics utilizing whole-transcriptome sequences of at least three *Onthophagus* species are currently being executed to explore the genetic underpinnings of horn polyphenisms and their diversification. Such genome-wide studies have the power to discover unexpected targets of selection as well as test predictions based on previous work.

For example, several developmental and physiological mechanisms have the potential to drive differences in scaling relationships, e.g. by altering the rate or duration of cell proliferation during growth (Emlen and Allen 2003) via changes in the expression and/or function of signaling molecules and transcription factors that coordinate outgrowth formation. A number of studies have already demonstrated that a diversity of patterning mechanisms and growth regulators normally involved in appendage formation have been co-opted to function in horn development (reviewed in Kijimoto et al. 2012b). Alternative, or additional mechanisms involve evolutionary changes in the function of endocrine regulators, such as juvenile hormone (Moczek and Nijhout 2002, see below) and in particular insulin signaling (Shingleton et al. 2005; Snell-Rood and Moczek 2012; Emlen et al. 2012). Two recent studies on species belonging to two distinct groups of horned beetles, which

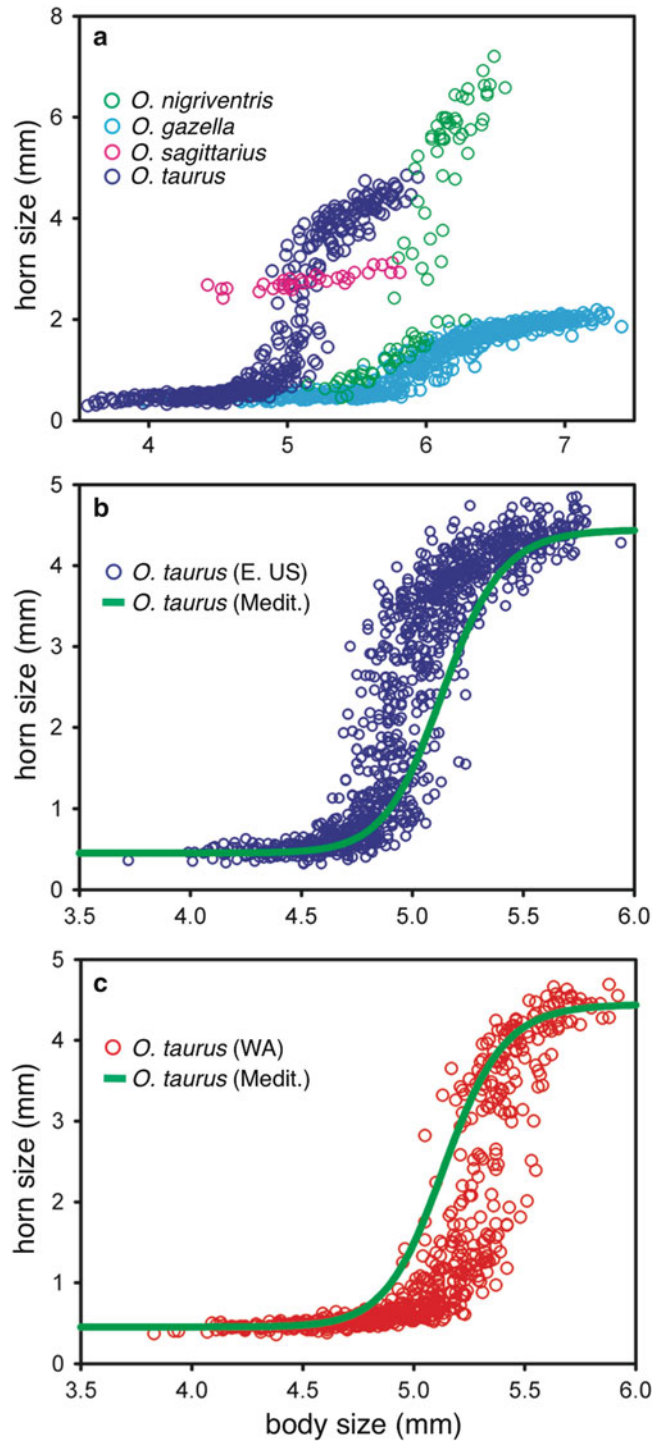

Fig. 7.3 Macro- and microevolutionary divergences in body size-horn length scaling relationships. (**a**) Body size – horn length scaling relationships highlight distinct degrees of male polyphenism among four *Onthophagus* species. (**b**, **c**) Diversity in body size thresholds separating hornless and horned male morphs in rapidly evolving introduced populations of *O. taurus* in the Eastern United States (**b**, *blue circles*) and Western Australia (**c**, *red circles*) relative to the average scaling relationship seen in ancestral Mediterranean populations (**b** and **c**, *green line*)

evolved horns independently (Dynastinae; *Trypoxylus* (*Allomyrina*) *dichotoma*; Emlen et al. 2012; Scarabaeinae; *Onthophagus nigriventris*; Snell-Rood and Moczek 2012), both implicate aspects of insulin signaling in the regulation of organ-specific sizes across nutritional gradients. For instance, results suggest that the insulin receptor may be particularly important in

disproportionate growth responses in plastic traits such as horns (Emlen et al. 2006, 2012), while the gene FOXO acts as a potential repressor of growth in traits such as genitalia (Snell-Rood and Moczek 2012). Together, these studies complement developmental work being performed in more traditional models systems such as *Drosophila* (Kopp 2011; Tang et al. 2011) because they find comparable results, but also show how these mechanisms may be co-opted to regulate differences between organs in their sensitivity to variation in nutritional conditions.

Similarly, horned beetles are beginning to provide important complementary insights into the epigenetic basis of developmental plasticity. Adaptive plastic responses to nutrition have been hypothesized to be regulated by environmentally-induced, heritable changes "above" the level of DNA, such as DNA methylation or histone acetylation (Junien et al. 2005; Burdge et al. 2007; Gilbert and Epel 2009). Recent work on horned beetles has shown that, like honey bees, (but unlike *Drosophila* and *Tribolium*), horned beetles possess the complete methylation machinery (Choi et al. 2010; reviewed in Valena and Moczek 2012), that methylation occurs, and that a fraction of it may underlay adaptive plastic responses to nutritional variation experienced during development (Snell-Rood et al. 2013).

Another divergence pattern common among species, as well as populations, involves the point of inflection of the body size-horn length allometry, or the threshold body size that separates small, minor (sneaking) males from large, major (fighting) males. A case in point are exotic populations of the beetle *O. taurus* introduced to Western Australia and the Eastern United States, which in less than 50 years have evolved highly divergent threshold body size (Fig. 7.3b, c). Threshold sizes have diverged in *opposite* directions relative to the ancestral, Mediterranean population, and to a degree that rivals divergences seen among closely related species (Fig. 7.3b, c) (Moczek and Nijhout 2003). Comparative ecological and behavioral studies suggest that threshold divergences have been driven by differences in the intensity of intra– and interspecific competition for breeding opportunities, which resulted in relatively low levels of male-male competition for females in the Eastern US but extremely high levels in Western Australia. In turn, these ecological differences may have resulted in selection for genotypes that express horns at relatively small body sizes to be favored in the US, but to be selected against in Western Australia.

Past as well as ongoing studies suggest that divergences among exotic *O. taurus* populations are not limited to male horn development, but also include larval physiology (Australian larvae require much longer to complete larval development and exhibit reduced sensitivity to hormonal manipulations; (Moczek and Nijhout 2002)) and female fertility and fecundity (Beckers and Moczek, unpublished). This differentiation among populations presents an excellent opportunity to study the early stages of polyphenism evolution, its developmental underpinnings, ecological causes, and its interactions with other diversifying traits. Integrating genome-wide data on genetic diversity (from next-gen sequencing efforts on W-Australian and Eastern US populations currently underway), gene expression (such as those described above for the multifactorial array experiment), and gene function (from a growing body of RNAi screens) aims to reveal mechanisms underlying such rapid morphological and behavioral evolution. Further, integrating data on patterns observed among populations with those observed among species can reveal processes that occur in parallel on micro- and macro-evolutionary scales. We predict similar functional targets related to cell growth and proliferation described above to also underlie trait diversification between rapidly evolving populations within a species. Such mechanisms may further interact with pheromones or other chemical cues that signal local densities of competing individuals, and thus act as a proxy of the degree of intra- and inter-specific competition (Butcher et al. 2007). Similarities in the genetic targets underlying trait diversification within and between species will reveal the genetic machinery that is repeatedly accessed at multiple evolutionary time-scales, while differences may indicate potentially novel evolutionary targets.

7.3.2 Macroevolution of Plasticity and the Diversification of Male and Sexual Dimorphisms

Male horn dimorphism is cued by variation in nutrition, causing well-fed male larvae to develop into horned males, whereas larvae subject to suboptimal feeding conditions metamorphose into hornless males (Emlen 1994; Moczek 1998). In contrast, sex-specific development of horned males and hornless females is strictly tied to somatic sex determination following – most likely- a traditional XX/XY sex determination scheme (Angus 2008). However, on a more general level, both processes have much in common: in both cases the same genome (or nearly same genome if one includes the modest contribution of the Y chromosome) is used to allow developmental processes to generate very different phenotypic outputs depending on cues experienced either late in larval development (such as nutrition in the case of male dimorphisms) or very early during embryonic differentiation (as is the case for the somatic sex-determination cascade). Remarkably, recent work (Kijimoto et al. 2012a) has shown that these general similarities also extend to the molecular and developmental genetic level.

In insects, somatic sex determination involves the gene *doublesex* (*dsx*) as the terminal gene in the sex determination pathway that regulates the sex-limited expression of downstream target genes, which in turn enable sexually dimorphic development and behavior across diverse insects (Fig. 7.4a; Sanchez 2008). Even though the sex-determination pathway upstream of *dsx* is divergent across insect orders, the basic genetic architecture and function of *dsx* are highly conserved (Shukla and Nagaraju 2010). In particular, in all insects examined so far *dsx* structure and function involve the expression of male- and female-specific *Dsx* isoforms generated through alternative splicing (Fig. 7.4a).

Recent microarray-based transcriptional profiling of *Onthophagus* development suggested that, in line with previous studies, differential expression of male and female *dsx*-isoforms may underlie sex-specific differentiation in horned beetles. Unexpectedly, however, the same studies also raised the possibility that aspects of the same machinery have become co-opted to generate morph-specific development within males (Kijimoto et al. 2012a).

A subsequent analysis of the expression and function of alternate *dsx* isoforms yielded three major conclusions: first, alternative *dsx* transcripts indeed promote the presence of horns in males but inhibit their formation in females (Fig. 7.4b). As such, beetle horn development joins a growing list of secondary sexual traits whose sex-specific expression is regulated by *dsx*. Second, within males, the *level* of *dsx* expression appears to have evolved to function as a regulator of relative horn size, regulated in turn by larval nutrition. If the expression of the male *dsx* isoform is knocked down in *O. taurus*, nutrition-sensitive horn development is greatly reduced (Kijimoto et al. 2012a; Fig. 7.4b). Lastly, when these studies were replicated in a second species, *Onthophagus sagittarius*, it became clear that *dsx* represents a nexus in the evolution and diversification of both sex- and morph-specific development: *O. sagittarius* is an unusual, closely related and recently derived, species that exhibits a *reversed* sexual dimorphism: males have lost the ancestral male dimorphism and only develop small paired horns in front of their heads, whereas females have gained conspicuous medial head and thoracic horns. Sequencing experiments revealed that *O. sagittarius* expresses male- and female-specific *dsx* transcripts with splicing patterns and translated protein sequences highly similar to those in *O. taurus*, i.e. *dsx* expression appeared conserved across both species. However, comparative functional studies showed that *O. sagittarius dsx* functions have expanded beyond their conserved roles to include both modified as well as novel functions in the regulation of horn position, shape, and size (Kijimoto et al. 2012a).

Manipulations of *dsx* function are highly robust across species, have high penetrance, and yield long-lived adults, which offers interesting opportunities to further explore the developmental genetic mechanisms of plasticity, morphological integration, and plasticity evolution in

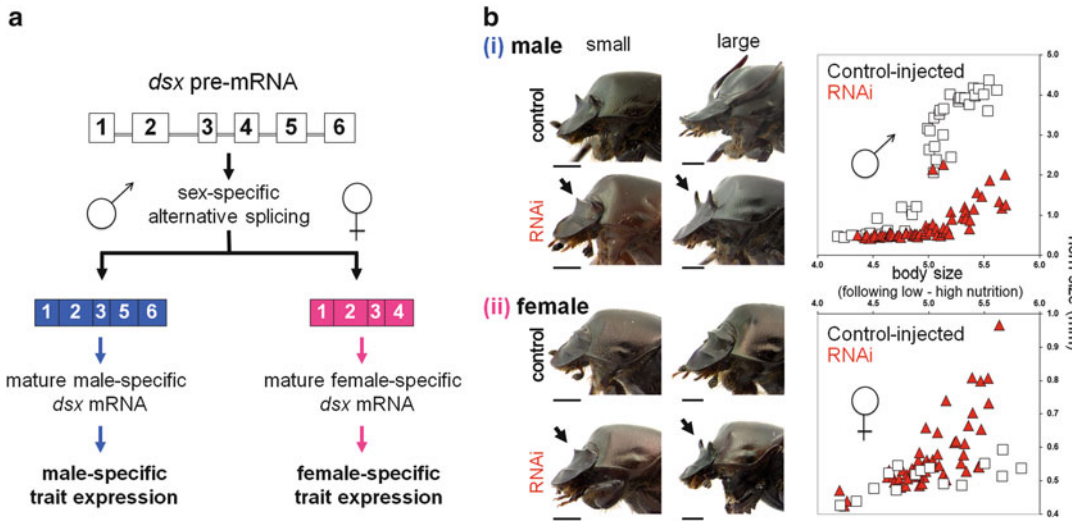

Fig. 7.4 Structure and function of the gene *doublesex* (*dsx*) and its role in the development of sexual and male dimorphisms. (**a**) In all insects examined so far *dsx* structure and function involve the expression of male- and female-specific Dsx isoforms generated through alternative splicing. Shown here is a schematic representation of *dsx* structure in *Drosophila melanogaster*. White-numbered boxes represent exons, whereas blue and pink-numbered boxes represent gene products in males and females, respectively. Sex-specific alternative splicing (presence or absence of exon 4) results in sex-specific trait expression. (**b**) Effects of *dsx* double-stranded (ds) RNA injection (RNAi) on horn development in adult *O. taurus* (i) males and (ii) females. Left: Representative animals obtained after mock (control) injections (*top row*) and *dsx* dsRNA injections, respectively (*bottom row*). Small individuals are shown on the *left* and large individuals on the *right*. *Filled arrows* indicate locations of head horn development in RNAi individuals. Right: Bivariate plots of body size (x-axis) and head horn length (y-axis) for (i) male and (ii) female *O. taurus*. Control and RNAi individuals are plotted as white squares and red triangles, respectively. *dsx* dsRNA injections substantially reduced nutrition-responsive horn development in males but induced it in females (Modified after Kijimoto et al. 2012a)

nature. For example, efforts are under way to investigate the degree to which morphological plasticity (horned vs. hornless morphs), behavioral plasticity (sneaking vs. fighting) and sex-specific behaviors (e.g. courting) are co-regulated by *dsx* via a detailed behavioral analysis of *dsx*-deficient males and females. Similarly, the conservation of *dsx* expression across species on one side, and the diversification of *dsx* function on the other, invite a comparative analysis of *dsx*'s target repertoire. Specifically, experiments are being conducted utilizing next-gen sequencing approaches to identify which genes change expression following *dsx*-knockdown as a function of body region, sex, nutritional conditions, and ultimately, species. Similar to the analysis of nutrition-dependent gene expression across diverse conditions described above, this effort will make use of the same statistical toolbox to generate robust results in the face of incomplete data.

Lastly, the same approaches may allow us to investigate the possible involvement of *dsx* and its target repertoire in the context of *threshold evolution*, for instance as detailed for exotic *O. taurus* populations in the preceding section. Recall that exotic populations have diverged heritably with respect to the body size (= larval nutrition) threshold that separates hornless from horned developmental fates, a developmental decision we now know is at least in part regulated via the differential expression of male-specific *dsx*-isoforms. Collectively, these efforts will help inform our understanding of the similarities and differences in the mechanisms by which diversity is generated within and across sexes, and the evolutionary lability or conservation of these mechanisms.

7.4 Conclusion

Developmental plasticity mediates the expression of a rich diversity of morphological, physiological, and behavioral phenotypes in horned beetles and thus plays a central role in the evolutionary and behavioral ecology of these organisms. The biology of horned beetles, including that of developmental plasticity, is increasingly experimentally accessible, representing growing opportunities with which to explore the causes, mechanisms and consequences of plasticity and plasticity evolution over a range of phylogenetic distances. For example, horned beetles allow us to address whether certain genes or pathways are biased or specific in their expression to different nutritional conditions, whether they are subject to relaxed selection, whether these patterns are shared across species and/or types of plastic responses, and whether the developmental-genetic underpinnings of plastic responses have enabled phenotypic diversification or innovation in other developmental contexts. At the same time, many horned beetle species are easily and inexpensively maintained in captivity, and several of the most interesting species are broadly distributed geographically. Research conducted thus far has only begun to scratch the surface of what horned beetles can teach us about the interplay between development, environment, phenotypic variation, and evolution, and we hope that this chapter will encourage future research efforts into the biology of horned beetles and beetle horns.

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Glossary: Important Concepts at the Interface of Ecological Genomics and Developmental Plasticity

Phenotypic plasticity The capacity of a genotype or individual to express different phenotypes across a range of environments. Adaptive plasticity results in maintenance of high performance across these conditions.

Canalization The capacity of a developmental system to produce the same phenotype despite variation in the external or internal environment. Canalization in a given trait (e.g., fitness) may be underlain by plasticity in other traits (e.g., horn expression).

Genetic assimilation Evolutionary changes to an underlying developmental system whereby a phenotype that was initially environmentally induced becomes constitutively expressed.

Polyphenism A specific form of developmental plasticity that results in the development of discrete alternate phenotypes in response to an environmental cue.

Relaxed selection Any form of lower selection intensity relative to a population, trait or ancestral state subject to higher selection intensity. Relaxed selection encompasses both reduced purifying selection and positive selection, leading to a lower likelihood of loss of deleterious alleles and fixation of beneficial alleles, respectively. Also referred to as relaxed selective constraint.

Allometry The relationship of traits to body size. Changes in scaling or allometric relationships can be a method of describing a plastic response of a trait in response due to variation in nutritional effects on body size.

Square combining table A statistical method for pairwise comparisons that allows researchers to focus on specific comparisons of interest and cope with an unbalanced design.

Appendix: Effectiveness of SCT-Based Experimental Design: Computational Analysis

As indicated in Sect. 7.2.2.5, the effectiveness of our approach can be quantified by comparing the estimated variances in the effects of interest (the eight nutrition comparisons in the eight sex x body-region classes) that arise from both a conventional cyclic design (de Mendiburu 2013)

and the SCT-based design (developed with reference to the Square Combining Table, described in Godfrey 1985). Both design matrices, shown at the end of this appendix, can be expressed with 48 rows (corresponding to the 48 subarrays) and 16 columns (for the 16 body region x sex x nutrition types), whose elements x_{ik}, $i = 1, \ldots, 48$ and $k = 1, \ldots, 16$, are 0 (type not on subarray), 1 (type is labeled "red"), or –1 (type is labeled "green"). The log2-response on the first contig from the first array and first subarray reflects $\log_2(LFL/LFS) = \log_2(LFL) - \log_2(LFS)$ or $\log_2(AML/AMS) = \log_2(AML) - \log_2(AMS)$, depending on whether X is the SCT-based design, X_{SCT}, or the cyclic design, X_{cyc}, respectively. With multiple observations on LFL, LFS, AML, AMS (etc.), we need to estimate a reliable summary of the observations for each of the 16 conditions, taking into account that LFL may be paired with LFS for one subarray but with LML on another subarray (and likewise for all beetle types). If we denote the 48 gene responses (on the 48 subarrays) for a single contig (gene) by the vector y_j (here, $j = 1, \ldots, 42{,}010$), the design matrix by X (same design matrix for all contigs), and the vector of the 16 means of the responses from the 16 types (two nutrition levels × 2 sexes × 4 body regions) for contig j by M_j, then we can express the observed responses y_j as a linear model as follows:

$$y_j = X_{SCT} M_j + (error) \text{ or } y_j = X_{cyc} M_j + (error)$$

depending upon whether the design was the SCT-based design or the cyclic design. Whichever X design is used, the least-squares estimate of the vector of the 16 means, denoted as m_j, is calculated in the usual fashion:

$$m_j = \left(X'X\right)^{-1} X' y_j$$

In fact, both design matrices have a redundancy in them, in that the last column can be obtained from a linear combination of the first 15 columns, so the matrix inverse $(X'X)^{-1}$ is actually calculated as $\left(\tilde{X}'\,\tilde{X}\right)^{-1}$ where $\tilde{X}$ is the design matrix X without the last column. (This problem formulation is equivalent to setting the last mean, SMT, equal to zero. Because we are interested in differences between two type means at a time, the actual value for SMT will be irrelevant.) Conventional least squares analysis (i.e., m_j is the vector of 16 means that minimizes the sum of the squared error terms from this linear model) allows us to calculate the variances of the estimated means m_j as the diagonal of the matrix $\left(\tilde{X}'\tilde{X}\right)^{-1} s_j^2$, where s_j^2 provides a measure of the variability in the error terms from the linear model and is estimated from the mean of the squared error terms:

$$s_j^2 = \sum_{k=1}^{48} \left(y_{jk} - \sum_{i=1}^{15} x_{i=1} m_{jk} \right)^2 / 33$$

(the denominator "33" arises from 48 differences less 15 means being estimated; recall that one mean, SMT, is set to zero). In fact, we are not interested in the variances of the 15 means (SFA, ..., LMT), but rather in the variances of the eight nutrition differences LFA-SFA, ..., LMT-SMT, which can be expressed in matrix terms as Cm_j, where the (8 rows × 16 columns) matrix C is:

	LFA	SFA	LMA	SMA	LFH	SFH	LMH	SMH	LFL	SFL	LML	SML	LFT	SFT	LMT	SMT
LFA-SFA:	1	−1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LMA-SMA:	0	0	1	−1	0	0	0	0	0	0	0	0	0	0	0	0
LFH-SFH:	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
LMH-SMH:	0	0	0	0	0	0	1	−1	0	0	0	0	0	0	0	0
LFL-SFL:	0	0	0	0	0	0	0	0	1	−1	0	0	0	0	0	0
LML-SML:	0	0	0	0	0	0	0	0	0	0	1	−1	0	0	0	0
LFT-SFT:	0	0	0	0	0	0	0	0	0	0	0	0	1	−1	0	0
LMT-SMT:	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	−1

Table 7.2 Cyclic design

	LFA	SFA	LMA	SMA	LFH	SFH	LMH	SMH	LFL	SFL	LML	SML	LFT	SFT	LMT	SMT
1	0	0	1	−1	0	0	0	0	1	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	1	−1	0	0	0	0	0	0
3	−1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
4	0	0	0	0	0	0	0	0	0	0	0	0	0	1	−1	0
5	0	0	0	0	0	0	0	0	0	0	1	−1	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	1	−1	0	0
7	0	0	0	0	0	0	0	1	−1	0	0	0	0	0	0	0
8	1	−1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	1	−1	0	0	0	0	0	0	0	0	0	0	0
10	0	1	−1	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	1	−1	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	1	−1	0	0	0
13	0	0	0	0	1	−1	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	1	−1	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	1	−1	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	−1
17	0	1	0	0	0	−1	0	0	0	0	0	0	0	0	0	0
18	−1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
19	0	0	0	1	0	0	0	−1	0	0	0	0	0	0	0	0
20	0	0	0	0	1	0	0	0	−1	0	0	0	0	0	0	0
21	0	0	1	0	0	0	−1	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	1	0	0	0	−1	0	0	0	0	0	0
23	1	0	0	0	−1	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	−1
25	0	0	0	0	0	0	1	0	0	0	−1	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0	1	0	0	0	−1	0	0
27	0	0	0	0	0	0	0	0	0	0	1	0	0	0	−1	0
28	0	−1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
29	0	0	0	0	0	0	0	1	0	0	0	−1	0	0	0	0
30	0	0	0	−1	0	0	0	0	0	0	0	0	0	0	0	1
31	0	0	0	0	0	0	0	0	1	0	0	0	−1	0	0	0
32	0	0	−1	0	0	0	0	0	0	0	0	0	0	0	1	0
33	0	−1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
34	0	0	0	1	0	0	0	0	0	0	−1	0	0	0	0	0
35	0	0	−1	0	0	0	0	0	0	0	0	1	0	0	0	0
36	0	0	0	0	1	0	0	0	0	0	0	−1	0	0	0	0
37	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	−1
38	0	0	1	0	0	0	0	0	0	−1	0	0	0	0	0	0
39	0	0	0	0	0	0	1	0	0	0	0	0	0	−1	0	0
40	0	1	0	0	0	0	0	0	−1	0	0	0	0	0	0	0
41	0	0	0	0	−1	0	0	0	0	0	0	0	0	1	0	0
42	0	0	0	0	0	−1	0	0	0	0	0	0	0	0	1	0
43	0	0	0	0	0	1	0	0	0	0	0	0	−1	0	0	0
44	0	0	0	0	0	0	0	1	0	0	0	0	0	0	−1	0
45	−1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
46	1	0	0	0	0	0	0	−1	0	0	0	0	0	0	0	0
47	0	0	0	−1	0	0	0	0	0	0	0	0	1	0	0	0
48	0	0	0	0	0	0	−1	0	0	0	0	0	0	0	0	

Table 7.3 Design based on square combining table

	LFA	SFA	LMA	SMA	LFH	SFH	LMH	SMH	LFL	SFL	LML	SML	LFT	SFT	LMT	SMT
1	0	0	0	0	0	0	0	0	1	−1	0	0	0	0	0	0
2	0	−1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	−1	0	1	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	1	−1	0	0
5	0	−1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
6	0	0	0	0	0	0	0	0	0	0	1	−1	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	−1
8	−1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	1	−1	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	−1	0	1	0	0	0	0	0
11	0	0	0	0	0	0	1	−1	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	−1	0	1	0
13	0	0	0	0	0	0	0	0	0	0	−1	0	0	0	1	0
14	1	0	0	0	0	0	0	0	−1	0	0	0	0	0	0	0
15	0	0	0	0	0	0	1	0	0	0	−1	0	0	0	0	0
16	−1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
17	0	0	−1	0	0	0	1	0	0	0	0	0	0	0	0	0
18	0	0	0	0	1	0	0	0	−1	0	0	0	0	0	0	0
19	0	0	1	0	0	0	0	0	0	0	−1	0	0	0	0	0
20	0	0	0	0	0	0	1	0	0	0	0	0	0	0	−1	0
21	0	0	0	0	0	1	0	0	0	0	0	0	0	−1	0	0
22	1	0	0	0	0	0	0	0	0	0	0	0	−1	0	0	0
23	0	0	0	0	0	0	0	0	−1	0	0	0	1	0	0	0
24	0	0	0	0	1	0	0	0	0	0	0	0	−1	0	0	0
25	0	0	0	0	0	−1	0	1	0	0	0	0	0	0	0	0
26	0	−1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	−1	1
28	−1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	1	0	0	0	−1	0	0
30	0	0	0	1	0	0	0	0	0	−1	0	0	0	0	0	0
31	0	0	0	0	0	0	0	0	0	0	0	0	0	−1	0	1
32	0	0	0	0	0	0	0	0	0	0	0	0	−1	1	0	0
33	0	0	−1	1	0	0	0	0	0	0	0	0	0	0	0	0
34	0	0	0	0	−1	1	0	0	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	−1	1	0	0	0	0	0	0	0	0
36	0	1	0	0	0	0	0	0	0	−1	0	0	0	0	0	0
37	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	−1
38	0	0	0	0	0	0	−1	0	0	0	0	0	0	0	1	0
39	0	0	0	0	0	0	0	1	0	0	0	−1	0	0	0	0
40	0	0	0	−1	0	0	0	0	0	0	0	0	0	0	0	1
41	0	0	−1	0	0	0	0	0	0	0	0	0	0	0	1	0
42	0	0	0	0	0	−1	0	0	0	1	0	0	0	0	0	0
43	0	0	0	0	0	0	−1	0	0	0	1	0	0	0	0	0
44	0	0	0	1	0	0	0	0	0	0	0	−1	0	0	0	0
45	0	0	1	0	0	0	−1	0	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	−1
47	0	0	0	0	0	0	0	0	0	0	1	0	0	0	−1	0
48	0	0	0	−1	0	0	0	1	0	0	0	0	0	0	0	0

(recall that SMT is set to zero so again we let $\tilde{C}$ denote the above matrix without the last column). The variances of these eight contrasts are on the diagonal of the matrix,

$$\tilde{C}\left[\left(\tilde{X}'\tilde{X}\right)^{-1}\right]\tilde{C}'s_j^2$$

Thus, we can compare the eight values on the diagonal of this matrix where $\tilde{X}$ is either $\tilde{X}_{SCT}$ or $\tilde{X}_{cyc}$, as shown in Tables 7.2 and 7.3.

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