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INVITED REVIEW

Finding the Way in Phenotypic Space: The Origin and Maintenance of Constraints on Organismal Form

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• *Background* One of the all-time questions in evolutionary biology regards the evolution of organismal shapes, and in particular why certain forms appear repeatedly in the history of life, others only seldom and still others not at all. Recent research in this field has deployed the conceptual framework of constraints and natural selection as measured by quantitative genetic methods.
• *Scope* In this paper I argue that quantitative genetics can by necessity only provide us with useful statistical summaries that may lead researchers to formulate testable causal hypotheses, but that any inferential attempt beyond this is unreasonable. Instead, I suggest that thinking in terms of coordinates in phenotypic spaces, and approaching the problem using a variety of empirical methods (seeking a consilience of evidence), is more likely to lead to solid inferences regarding the causal basis of the historical patterns that make up most of the data available on phenotypic evolution.

Key words: Quantitative genetics, phenotypic space, inference, natural selection, constraints.

THE PROBLEM OF PHENOTYPIC SPACE

Why do living organisms look the way they do, and not in some other way? This is perhaps one of the broadest, and most challenging, questions in the biological sciences, and has attracted attention both within (Darwin, 1859) and without (Thompson, 1917) an evolutionary framework. In its broad sense this is much too general a question actually to lead to meaningful empirical investigations, but it can be used as a framework to provide a rich source of inspiration for empirical research and theoretical analyses alike. Although the outlines of an answer have been worked upon ever since Darwin, and expanded during the Modern Synthesis of the 1930s and 1940s (Mayr and Provine, 1980), some recent conceptual and technical advances are beginning to make it possible for us to think in novel directions about the causes and patterns of phenotypic evolution. In this paper I will lay out a general framework using the concept of 'phenotypic space', discuss some of the most challenging theoretical problems arising within such framework, and examine some empirical evidence gathered over the years in my laboratory while working on a model system. I will end with some general considerations on the relationship between theory and empirical research on phenotypic evolution.

One way to begin to think about the issue of organismal form is to ask ourselves a deceptively simple question: why, for example, do we have horses, but not, say, unicorns, pegasi (mythological flying horses) or hippogryphs (mythological chimeras, part horse part bird)? A moment's reflection actually makes clear that the answer is likely to require several lines of explanation: for instance, although there is nothing inherently inconceivable about a mammal with a horn on the head (given the appropriate genetic variation and selective pressures), and indeed we do know of examples outside the horse clade, it seems developmentally much more difficult (perhaps even impossible) to attach a pair of wings to the body of a vertebrate, unless one uses the well-known 'trick' of transforming the forelegs into wings. Finally, hybrids between so phylogenetically disparate groups as birds and mammals are made impossible by the divergence in the behaviours, genetic architectures and developmental systems of the animals in question.

More rigorously, palaeontologist David Raup long ago proposed the idea of a 'cube of life' quantifying the possible phenotypic space of shelled animals (Raup and Michelson, 1965; Raup and Gould, 1974). As a simple equation with three parameters can generate any conceivable shell shape, one can plot the values of these parameters in the appropriate three-dimensional space and ask which parts of the resulting phenotypic map are actually occupied by living or extinct species of shelled animals. It turns out that there are crowded areas as well as major gaps in the cube of life: why?

The classical answer to this sort of question has been: 'constraints' (e.g. Antonovics, 1976; Gould, 1980; Cheverud, 1984; Maynard-Smith *et al.*, 1985; Arnold, 1992; Hall, 1992; Thomas and Reif, 1993; Hodin, 2000; Wagner and Schwenk, 2000; Breuker *et al.*, 2006). But this immediately leads to the obvious question: what, in fact, is a constraint? The literature is full of definitions of constraints, and the terminology has become so baroque as to be comical (Antonovics and van Tienderen, 1991). Schlichting and I have argued, however, that – when all is said and done – there are essentially two broad sources of limits to phenotypic evolution (Schlichting and Pigliucci, 1998): genetic constraints and natural selection [with two additional exceptions,

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which I will not discuss here because they apply uniformly to all living beings: constraints imposed by the laws of physics, such as those discussed in Shipley *et al.* (2006), and constraints that are inherently mathematical, as when eliminating one factor from consideration in an analysis of variance by necessity increases the variance associated with one or more of the remaining factors]. Each of these can, of course, be further subdivided into more specific causes; for example, one can have genetic constraints due to lack of genetic variation for a trait, or to trait–trait covariation induced by pleiotropy or epistasis, and so on. Similarly, selection can be understood within the classic categories of stabilizing, directional or disruptive, or – alternatively – it can be conceptualized ecologically, as imposed by a host of biotic and abiotic factors.

Two considerations need to be made within this context, as they may clear common misconceptions about constraints. First, developmental constraints are in fact a highly heterogeneous category that is best thought of as the combination of genetic constraints (i.e. limits imposed by the genetic architecture on the developmental system) and natural selection, in turn usefully distinguished into external (i.e. imposed by ecological factors) and internal (i.e. resulting from the necessity for the interacting parts of developmental systems to unfold in a coherent fashion). Second, phylogenetic constraints are no such thing. Phylogenies are hypothetical descriptions of the historical sequence of certain biological events, and historical sequences require causal explanations, they do not provide them. Therefore, phylogenetic patterns of phenotypic change are best understood as explananda (the things that need to be explained), not as explanans (the things that do the explaining), and we need to keep in mind that different combinations of causal factors can well produce similar phylogenetic patterns.

GENETIC CORRELATIONS AND CAUSAL EXPLANATIONS OF PHENOTYPIC SPACE

One of the problems historically affecting the whole field of research on constraints was that it was often perceived as a hotchpotch of *ad hoc* concepts without an underlying conceptual framework to make sense of it. This has changed over the past two decades or so because of the application of the quantitative genetic approach to the quantification, and theoretical and empirical study of constraints (e.g. Cheverud, 1984; Arnold, 1992; Kirkpatrick and Lofsvold, 1992; Shaw *et al.*, 1995; Begin and Roff, 2004). The basic idea is that genetic correlations between pairs of quantitative traits reflect the underlying genetic architecture of the phenotype, for example as a result of pleiotropy or epistasis. These relationships can usefully be summarized by the genetic variance–covariance matrix, **G**. This can then be estimated empirically by using a variety of experimental designs, and it can be used within the context of the multivariate breeder equation (Lande and Arnold, 1983) to predict the outcome of phenotypic evolution, given measurable selective pressures.

I have argued at length elsewhere (Pigliucci, 2006; Pigliucci and Kaplan, 2006) why this framework is actually inadequate and potentially misleading. For our purposes here a brief mention of two major theoretical results will be sufficient. Houle (1991) has explored the complex relationship between the (statistical) measure of genetic covariation between traits and the various possible genetic architectures that may have led to it. His conclusion was that genetic correlations do not necessarily identify constraints, because different patterns of epistasis may result in very different measures of apparent genetic covariation. In other words, just because one measures a given genetic covariance between two traits one is not authorized to infer that the traits in question are related by some sort of physiological trade-off. The second contribution is due to Gromko (1995), who showed mathematically why the vagaries of pleiotropic effects mean that one may observe no genetic correlation despite the existence of an underlying trade-off. If we combine Houle's and Gromko's results, we are forced to accept the rather disheartening conclusion that genetic correlations do not necessarily tells us anything at all about the genetic architecture of phenotypic traits. Given that the very reason for estimating such correlations is precisely to infer genetic architectures, this is indeed a serious problem. The point here is not that we should stop doing quantitative genetics, but rather that we should take correlation patterns for what they are: convenient summaries of complex data sets, which may hint at functional relationships whose existence needs to be tested by means other than the calculation of statistics, however sophisticated such calculations may be.

In practice, all of this amounts to taking seriously the old mantra that correlations are not the same thing as causation. Shipley (2000) has suggested that a good way to think about the problem is by considering an analogy with the 'shadow theatre' popular in Malaysia and surrounding countries. In it, three-dimensional objects are put behind a screen, and light is used to project two-dimensional figures. As the same three-dimensional object can project very different shapes, depending on its position and that of the light source, it is possible to predict which two-dimensional figure will emerge from a given configuration of three-dimensional objects, but we cannot just look at the projections and confidently determine the shapes of the objects used to generate them. The problem in biology is analogous: different underlying causal processes may generate the same statistical 'shadows', so that one cannot directly infer the former from the latter. [Shipley (2000) does propose an ingenious method, based on structural equation modelling, to make progress in casual inference when one cannot experimentally manipulate the system. The method is not a substitute for additional empirical data, and it does require a large number of variables, but it certainly represents a much better approach than the standard correlational methods I critique here.]

The upshot of the preceding discussion is that, although the concept of phenotypic space as instantiated by the work of Raup, for example, provides a qualitative framework to represent and understand the interplay between constraints and selection on phenotypic evolution, the limits intrinsic in the quantitative genetic approach leave us without a proper quantitative framework. Some would consider this a major lacuna in the theory of phenotypic

evolution, while others, such as mathematical biologist Sergey Gavrilets (1999), would point out that models in biology are to be understood as heuristic metaphors, not as generators of quantitatively precise predictions, so perhaps qualitative approaches are all that is necessary or possible to do in this arena.

A MODEL SYSTEM APPROACH TO STUDYING PHENOTYPIC SPACE

The use of model systems in biology has produced spectacular successes over more than a century and a half, from Mendel's peas to the rebirth of genetics using fruit flies. Of course, model systems have their limitations, for example the fact that the very characteristics that make them ideal experimental subjects also tend to make them rather exceptional organisms, not necessarily representative even of their own clade. Nonetheless, I think that much can be learned in the study of the factors shaping phenotypic space by focusing our efforts on a small number of model systems, particularly if these are also being studied at the molecular and developmental levels.

In this section I will briefly consider a few examples of research about a particular phenotypic space occupied by genotypes of the weedy mustard *Arabidopsis thaliana*, a well-characterized model system in molecular genetics (Jackson *et al.*, 2002), developmental biology (Jack, 2004) and evolutionary ecology (Pigliucci, 1998; Mitchell-Olds, 2001). I will attempt to show that one can in fact make significant progress by using a combination of empirical approaches, each yielding a different piece of the overall puzzle within the framework provided by the concept of a phenotypic space.

For this example I will concentrate a on very simple type of phenotypic space, defined by all possible combinations of just two variables, one related to life history, the other to ontogeny. The two variables in question are flowering time and leaf production, which have been extensively studied in *Arabidopsis* from both an ecological (Pigliucci, 2003; Donohue *et al.*, 2005) and a molecular perspective (Simpson and Dean, 2002; Jack, 2004).

The logical starting point, as in Raup's original work on shell shapes, is to map the available phenotypic space and see which areas are occupied and which are relatively empty: observations of patterns often are the first step toward generating testable hypotheses concerning underlying causes. Figure 1 shows a composite of data obtained from the public *Arabidopsis* database (http://www.arabidopsis.org), including so-called 'early flowering' and 'late flowering' populations, which in reality correspond to spring and winter annuals, respectively, and flower at about the same time under field (as opposed to growth chambers) conditions (Napp-Zinn, 1985). Some patterns are obvious, and in part they immediately suggest causal mechanisms, which my group has, in fact, been able to confirm experimentally.

First, note the presence of a narrow ridge defined by genotypes with a combination of early flowering and fairly low leaf production. Second, there is a large, diffuse area occupied by genotypes characterized by late flowering and a range of intermediate to high number of leaves. Third,

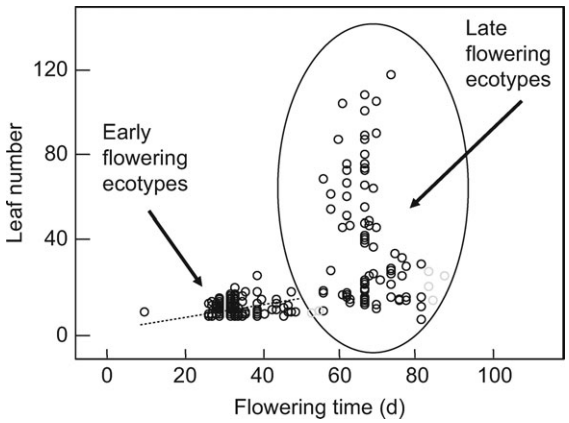

FIG. 1. Empirical definition of the flowering time/leaf number phenotypic space in natural populations of *Arabidopsis thaliana*. Data from http://www.arabidopsis.com. Note the sharp distinction between early and late flowering ecotypes, as well as the presence of large empty areas corresponding to the combinations early flowering/high leaf number (developmentally not achievable) and late flowering/low leaf number (strongly selected against).

there is a large empty space corresponding to the combination early flowering/high leaf number; lastly, a second empty space is found in the late flowering/low leaf number zone. As we shall see shortly, the entire pattern can be accounted for by a combination of different regimes of natural selection and distinct genetic/developmental constraints.

Our experimental approach to the study of the phenotypic space just outlined examined the within-population pattern of variation, and confirmed the existence of a strong genetic correlation between flowering time and leaf production in early flowering ecotypes (Mitchell-Olds, 1996; Camara and Pigliucci, 1999). But, as I argued above, a genetic correlation only hints at the presence of a constraint, it does not establish it. To test the hypothesis that the observed genetic correlation is in fact the result of a connection between the two traits at the level of genetic architecture, two experiments were conducted: first, Schlichting and I generated novel genetic variation by inducing mutations in a uniform genetic background (the Landsberg *erecta* genotype), and were able to observe the *de novo* appearance of the genetic correlation among the progeny of the mutated population (Pigliucci *et al.*, 1998). This provides strong evidence that the genetic architecture of the two traits is indeed at play here. Second, M. Camara and I (unpubl. res.) conducted an artificial selection experiment in which mutated and unmutated lines were selected for changes in both traits. The selection was conducted both along the genetic correlation (the 'line of least resistance': Schluter, 1996) and away from it (where significant resistance to change is expected if the correlation is a reflection of a true constraint). In accordance with the hypothesis, we did find a strong response to selection along the correlation line, but no response at all – despite strong selective pressure – away from it.

The combined results from these experiments argue for a real role of the genetic architecture in maintaining the

strong relationship between flowering time and leaf production. However, genetic correlations can be generated by selection, and may not simply reflect inevitable trade-offs. Work by Callahan and myself detected consistent (across sites and years) patterns of natural selection for early flowering and increased leaf production (Callahan and Pigliucci, 2002). When viewed together with the mutation and artificial selection experiments, these findings begin to give a reasonable explanation of the ridge on the lower left side of the phenotypic space in Figure 1: evidently, selection is pushing populations toward the upper left corner, where a short life cycle would be combined with a high production of photosynthetic tissue. However, this combination is impossible to achieve for obvious developmental and physiological reasons. On the other hand, selection clearly disfavours genotypes that occupy the late flowering/low leaf number zone. The upshot of this interplay of constraints and selection is the observed ridge that manifests itself as a tight genetic correlation between the two traits.

Has the coupling of flowering time and leaf production been in place for a long time during the evolutionary history of *Arabidopsis*? It would seem so. Work in my laboratory (Pigliucci *et al.*, 2003) has used a molecular-based reticulate phylogeny of several populations of *A. thaliana*, and then mapped the values of both flowering time and leaf number to see if they had a tendency to co-evolve. They did, with every increase or decrease in flowering time from one node of the phylogeny to another matched by a respective increase or decrease in leaf number (Fig. 2). We can make sense of this coupling at a molecular level, taking advantage of the wealth of information on the molecular biology of this model system. Simpson and Dean (2002) have pointed out that the historical transition always seems to be from a late flowering to an early flowering ecotype, which accords with the evolutionary data indicating that a winter annual life history is ancestral in *Arabidopsis* (Koch *et al.*, 2000). Moreover, the transition is often associated with a mutation at the *Frigida* locus, which in winter annual *A. thaliana* stimulates the *Flowering Locus C*, which in turns delays flowering until either the autonomous pathway or vernalization kick in and jump start the transition from the vegetative to the flowering phase. Why has this transition between life histories occurred repeatedly in *A. thaliana*? Presumably (though direct evidence from field studies of natural selection is still sparse) because the winter annual habit (Napp-Zinn, 1985; Donohue *et al.*, 2005) is advantageous where winters are mild (it leads to larger plants, better able to compete when the flowering season starts), while the spring annual habit is favoured where winter conditions are severe (but at the cost of decreased competitive ability in the spring).

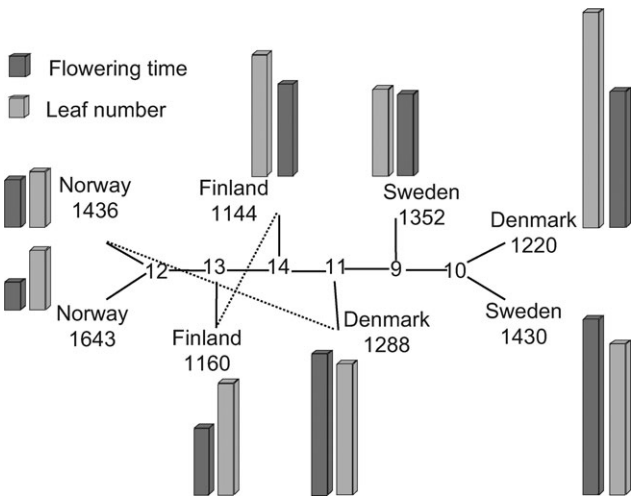

FIG. 2. Recent evolution of the coupling between flowering time and leaf number in phylogenetically related populations from Scandinavia (close to the northernmost area of distribution of the species). Numbers inside the reticulate phylogeny are mutational steps separating the branches. Note how whenever one trait increases or decreases in value the other one does too. Also note the similarity in phenotype between closely related populations.

We therefore now have an almost complete, and intellectually satisfying, account of why the flowering time/leaf production phenotypic space in *A. thaliana* is shaped the way it is. We started out with the depiction of the pattern and proceeded via several experimental lines to disentangle the roles of constraints and selective processes, and we have a good understanding of the molecular basis of the observed phenotypes and of the transitions between them. Of course, even the phenotype of a relatively simple plant such as *A. thaliana* is much more complex than the two-dimensional subset we have focused on, but there is no conceptual reason why similar approaches cannot be extended to a fuller characterization of the phenotype of this and other species. Indeed, some of the interesting follow-ups could include the study of the same pheno-space in phylogenetically close relatives of *A. thaliana* (often characterized by either an annual or a biennial life history), or traits such as leaf size (as opposed to number) and branching architecture, which are known to play major roles in the evolutionary ecology of this taxon.

TOWARD A GENERAL CONCEPTUAL FRAMEWORK FOR THE STUDY OF PHENOTYPIC SPACES

As I have argued at the beginning of this paper, the idea of a phenotypic space shaped by the interaction of various kinds of constraints and selective pressures provides a useful quantitative conceptual framework for the study of phenotypic evolution. On the other hand, recent attempts to use the tools of evolutionary quantitative genetics, such as **G** matrices, to quantify parameters describing the interplay between constraints and selection are of much more limited use.

These considerations are related to a fundamental aspect of the study of phenotypic spaces that provides researchers with serious empirical as well as theoretical challenges: the necessity to go back and forth between individual and population levels of analysis. Genetic correlations are population-level measures that are supposed to tell us something about individual-level phenomena such as pleiotropy, epistasis and physiological trade-offs. Analogously, the

covariance between phenotypic traits and estimates of viability or reproductive fitness are the way we measure natural selection as a population average, even though natural selection is in fact the result of a series of individual-level physical interactions between organisms and their environment (both biotic and abiotic).

Moving from the individual to the population levels of analysis is difficult enough, but the converse – which is what most analytical methods and empirical approaches in the evolutionary biology of phenotypic evolution attempt to do – is fraught with even more difficulties. Cheverud and Routman (1995) convincingly showed that one simply cannot use (quantitative genetic) statistical estimates of 'epistasis' to infer the existence or type of true physiological epistasis. On the other hand, if one knows sufficient details about the physiological level, one can in fact make reasonable predictions about what its 'statistical shadow' should look like. This is the shadow-theatre problem pointed out by Shipley (2000), and – I think – not currently taken seriously enough. Along similar lines, Kaplan and I have provided a detailed discussion of the conceptual and empirical issues surrounding the study of the interplay between constraints and selection, and of the additional difficulties that occur when one throws in the further complication of drift (Pigliucci and Kaplan, 2006). We concluded that the common view of evolution as driven by 'forces' conceptually analogous to those considered by physicists, including a 'zero-force' law represented by the Hardy–Weinberg principle (Sober, 1984), is problematic to say the least, and should probably be abandoned for more fruitful metaphors and approaches.

Consider a classic problem in evolutionary genetics: distinguishing between the effects of selection and drift. An elegant study by Phillips *et al.* (2001) attempted to do just that in the case of a set of traits in *Drosophila melanogaster*, summarized by a **G** matrix. Phillips and collaborators measured **G** in a large base population of *D. melanogaster* and then established a series of small subpopulations that underwent inbreeding and drift but no selection. The idea was that an examination of the descendant **G** matrices would be consistent with theoretical expectations and show that drift, but not selection, had occurred. If that were true, one could then use similar analytical techniques on naturally collected populations to pursue similar questions (with the difference that in the natural case the actual history of the population was the unknown objective to be inferred). It did not work, and for interesting reasons. While the *average* **G**, calculated across all descendant subpopulations, did indeed conform to theoretical expectations, individual **G** matrices varied in haphazard ways that would have been consistent with an interpretation calling for the action of diversifying natural selection – had the researchers not known already that there was no such history of selection. It is Shipley's point once again: we can accurately project the shadow forward, if we know enough details, but we cannot use it to infer directly the shapes behind the screen.

Consider, however, a positive example of the same problem: Roff (2002) re-examined data on four populations of the freshwater isopod crustacean *Gammarus minus*, two from cave habitats and two from surrounding springs. Roff found significant differences in the **G** matrices of each cave–spring pair, but no differences in cave–cave or spring–spring comparisons. He attributes these results to natural selection in the cave habitats. I think he is correct, but notice the following features of that study: (1) the cave populations are known to have been independently derived from the two nearby spring populations; and (2) there are known selective pressures on cave animals to reduce visual sensory organs and increase olfactory ones – exactly the correlations Roff found altered. In other words, Roff's conclusions are warranted because in addition to measurable differences in **G** he also has information about the phylogeny and likely selective history of his populations: the reason he is able to pinpoint the shape of the figures behind the screen is that he has access to much more than just their two-dimensional shadows!

What I mean to present here is no council for despair, only a call for caution. The central section of this paper, based on several years of work in my laboratory, has shown that it is indeed possible to make significant progress in understanding the causal mechanisms shaping phenotypic spaces, and the example just discussed of Roff's re-analysis of the *Gammarus* data is another case in point. But there is no direct inferential statistical shortcut available. Instead, one needs to start with statistical summaries of complex situations, use one's previous knowledge both of general biological principles and of specific aspects of the model system at hand, formulate causal hypotheses, and go about testing them by using the appropriate combination of experimental and observational methods. It is painstaking detective work, and as in the case of criminal investigations, it sometimes pays off and sometimes it does not, but keeping as varied a toolbox at hand as possible will surely help.

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