PART VI

DOES EVOLUTIONARY DEVELOPMENTAL BIOLOGY OFFER A SIGNIFICANT CHALLENGE TO THE NEO-DARWINIAN PARADIGM?

Introduction

This book offers contemporary debates in philosophy of biology; thus, we have included papers pertaining to the new and burgeoning science of evolutionary developmental biology (evo-devo). Evo-devo's basic claim is broadly threefold:

(1) We have discovered that many genes produce—or can produce, when we manipulate them—a variety of different traits in different organisms, depending on which genes are being expressed or not (what is colloquially called the switching on and off of genes) in the very development of the organism. To take a simple and common example, genes in the larvae of fruit-flies can be moved around or replaced, and doing so causes eyes to grow where antennae should be, legs where heads should be, and other monstrosities in these flies. Also, such monstrosities are witnessed naturally without manipulation. Research on the genetic development of roundworms reveals similar phenotypic adjustments based upon genetic manipulation during development (e.g., Callaerts, Halder, & Gehring, 1997; Duncan, Burgess, & Duncan, 1998). This is the significant *developmental* part of evo-devo.

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(2) Further, for some time now, we have been mapping genes and manipulating the genetic toolkit that controls the phenotypic traits of animals like fruit-flies, mice, worms, and others. Through that work, we have discovered that all animals are built out of essentially the same genes. For example, the Pax6 gene seems to be responsible for eye development in both mice and fruit flies (Quiring, Walldorf, Kloter, & Gehring, 1994). Also, the melanocortin 1-receptor (MC1R) gene seems to be responsible for hair color in mice and humans, as well as plumage color in certain birds (Takeuchi, Suzuki, Yabuuchi, & Takahashi, 1996; Valverde, Healy, Jackson, Rees, & Thody, 1995). And there are many other examples (see Minelli, 2003).

(3) Here is the fascinating claim made by evo-devotees: evolution is not so much the result of wholesale genetic variation in terms of mutation as it is a matter of changing *when* and *where* genetic switches will be turned on and off in the development of an organism. Thus, according to many evo-devotees, the various species we see around us today actually are the result of genetic switches being turned on or off at various points in the development of an organism throughout evolutionary history. If true, this is a fascinating discovery—although, the development of organisms has been a significant area of research since Darwin's time; and Darwin (1859/1999) himself claims in the *Origin* that "characters derived from the embryo should be of equal importance with those derived from the adult, for a natural classification of course includes all ages" (p. 342).

There is now ongoing debate as to the extent to which evo-devo might challenge or complement various standard evolutionary principles since, according to Sean Carroll, Benjamin Prud'homme, and Nicolas Gompel in a fairly recent issue of *Scientific American*, "evolutionary changes to anatomy, particularly those involving pleiotropic genes, are more likely to happen via changes to gene enhancers than to the genes themselves" (p. 67). In the first paper included in this part, Manfred Laubichler wants to argue that, to a certain extent, evo-devo does offer a significant challenge to the neo-Darwinian paradigm that evolution is mostly the result of wholesale genetic variation in terms of mutation. Based upon recent evidence, Laubichler expands an important tenet of evo-devo that adaptive mutations which affect phenotypic characteristics are more likely to occur in the *cis*-regulatory regions of genes than in the regions where protein-coding occurs.

In his paper included as the second one in this part, although Alessandro Minelli wants to argue that evo-devo does not offer a significant challenge to the neo-Darwinian paradigm, he also maintains that "evo-devo is not simply developmental biology grafted onto evolutionary biology; rather, it deserves to be acknowledged as a research field of its own, with a specific agenda and a specific conceptual endowment." Through a discussion of the concept of *constraint* in evolutionary biology, complete with a few examples, Minelli ultimately claims that evo-devo "provides its unique contribution to understanding the evolutionary process by a description and analysis of *developmental* [emphasis ours] constraint and its elements."

Even as early as 1954, Gavin de Beer could maintain that it "has become increasingly clear from research in embryology that the processes whereby the structures are formed are as important as the structures themselves from the point of view of evolutionary morphology and homology" (p. 136). Research in evo-devo seems to be underscoring de Beer's point.

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THE LOCUS OF EVOLUTION: EVO DEVO AND THE GENETICS OF ADAPTATION

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An important tenet of evolutionary developmental biology ("evo devo") is that adaptive mutations affecting morphology are more likely to occur in the *cis*-regulatory regions than in the protein-coding regions of genes. This argument rests on two claims: (1) the modular nature of *cis*-regulatory elements largely frees them from deleterious pleiotropic effects, and (2) a growing body of empirical evidence appears to support the predominant role of gene regulatory change in adaptation, especially morphological adaptation. Here we discuss and critique these assertions. We first show that there is no theoretical or empirical basis for the evo devo contention that adaptations involving morphology evolve by genetic mechanisms different from those involving physiology and other traits. In addition, some forms of protein evolution can avoid the negative consequences of pleiotropy, most notably via gene duplication. In light of evo devo claims, we then examine the substantial data on the genetic basis of adaptation from both genome-wide surveys and single-locus studies. Genomic studies lend little support to the *cis*-regulatory theory: many of these have detected adaptation in protein-coding regions, including transcription factors, whereas few have examined regulatory regions. Turning to single-locus studies, we note that the most widely cited examples of adaptive *cis*-regulatory mutations focus on trait loss rather than gain, and none have yet pinpointed an evolved regulatory site. In contrast, there are many studies that have both identified structural mutations and functionally verified their contribution to adaptation and speciation. Neither the theoretical arguments nor the data from nature, then, support the claim for a predominance of *cis*-regulatory mutations in evolution. Although this claim may be true, it is at best premature. Adaptation and speciation probably proceed through a combination of *cis*-regulatory and structural mutations, with a substantial contribution of the latter.

KEY WORDS: ***Cis*-regulation, evolution of development, gene regulation, phenotypic evolution. structural gene.**

As new areas of research have been folded into the Modern Synthesis, each has claimed to offer unique and revolutionary insights into the evolutionary process. Punctuated equilibrium, for example, proposed novel and non-Darwinian explanations for a seemingly discontinuous fossil record. These included the fixation of nonadaptive macromutations by genetic drift in small populations, and the operation of "species selection," producing macroevolutionary trends via the differential splitting and extinction of entire taxa (Eldredge and Gould 1972; Gould and Eldredge 1977, 1993; Gould 1980).

Some advocates of "evo devo" (the new field that fuses developmental and evolutionary biology) also claim to have revolutionized the study of macro- and microevolution. Like advocates of punctuated equilibrium, adherents to evo devo extrapolate from

pattern to process. Their novel evolutionary theories include the notion that the new body plans (i.e., phyla) arise by mutations different from those distinguishing populations or species (Davidson and Erwin 2006); the idea that evolution involves the transformation of developmental "modules" that are relatively independent of each other genetically (Breuker et al. 2006); the view that evolution itself establishes traits that promote *future* evolution ("evolvability;" Kirshner and Gerhardt 1998); and the idea that most important evolution involves alterations in the regulation of genes rather than in their structure.

The emphasis on gene regulation is evo devo's most famous and widely accepted contribution to evolutionary theory. It began with the work of Jacob and Monod on bacterial operons (1961), and was formalized by Jacob (1977) in a now-famous paper suggesting that evolution acts as a "tinkerer," assembling new adaptations by puttering about with gene regulation. Around the same time, King and Wilson (1975), noting the similarity in protein and DNA sequence between humans and chimps, suggested that minor changes in gene regulation could yield major phenotypic change between taxa. Wilson and colleagues expanded this view in a series of papers (e.g., Wilson et al. 1974a,b; Wilson 1975). The emphasis on gene regulation was also a major theme of influential work by Britten and Davidson (1969, 1971), who suggested that morphological evolution resulted more from changes at "integrator" and "receptor" genes than from "producer" genes (categories that presumably correspond, respectively, to transcription factors, promoters, and structural genes).

As evo devo matured, the focus on gene regulation narrowed to a single one of its forms: that involving *cis*-regulatory elements (short, noncoding DNA sequences that control expression of a nearby gene). For various reasons, which we discuss below, *cis*-regulatory elements are now seen as not only the most likely target for the evolution of gene regulation, but also as the site of most important evolutionary change, at least for morphology.

Perhaps the first detailed argument for the importance of *cis*-regulation was made by Stern (2000). But the most vigorous advocate of this view has been Carroll, who, in a series of papers, scholarly books, and popular books (Carroll 2000; 2005a,b; Carroll et al. 2001, 2006), has repeatedly emphasized that the evolution of animal form and other macroevolutionary features resulted largely from changes at *cis*-regulatory sites:

> In the final chapter of this book [titled "From DNA to Diversity: The Primacy of Regulatory Evolution"], we consider why regulatory evolution is the creative force underlying morphological diversity across the evolutionary spectrum, from variation within species to body plans. The link involves evolution at the DNA level and phenotypic diversity involves the *cis*-regulatory elements acting as units of evolutionary change (Carroll 2001, p. 173).

> It has required several decades to obtain evidence that regulatory sequences are so often the basis for the evolution of form that, when considering the evolution of anatomy (including neural circuitry), regulatory sequence evolution should be the primary hypothesis considered (Carroll 2005a, p. 1165).

> This regulatory DNA [noncoding promoter regions] contains the instructions for building anatomy, and evolutionary changes within this regulatory DNA lead to the diversity of form (Carroll 2005b, p. 12).

These conclusions are delivered without caveats. The popular book *Endless Forms Most Beautiful* (Carroll 2005b), for example, begins with a quote from the Beatles' song "Revolution 1." In case the reader misses its significance, Carroll quickly explains (p. *x*):

> Over the past two decades, a new revolution has unfolded in biology. Advances in developmental biology (dubbed "evo devo") have revealed a great deal about the invisible genes and some simple rules that shape animal form and evolution. Much of what we have learned has been so stunning and unexpected that it has profoundly reshaped our picture of how evolution works.

Although Carroll's views have been by far the most influential in this area, other workers have also taken up the cudgels, showing the same unwavering confidence about the genetic basis of evolutionary change:

> The conclusion we draw from these inferences is that the evolution of plant form will be most readily accomplished by changes in the *cis*-regulatory regions of transcriptional regulators (Doebley and Lukens, 1998, p. 1081).

> For anyone interested in mechanism, there is in fact no other way to conceive of the basis of evolutionary change in bilaterian form than by change in the underlying developmental gene regulatory networks. This of course means change in the *cis*-regulatory DNA linkages that determine the functional architecture of all such networks" (Davidson 2001, p. 201).

> From what is already known, it is evident that the evolution of regulatory gene systems, rather than of structural alleles, has been chiefly responsible for the sorts of major morphological innovations revealed by the fossil record. . . Indeed, for the origin of bodyplans, involving the patterning of novel architectures, evolution of *cis*-regulatory elements appears to have been preeminent (Valentine 2004, pp. 77, 104).

(See also Wray et al. 2003).

But are these claims supportable? Considerable data now exist documenting the types of DNA changes underlying adaptive differences among species and higher taxa. Here we review these data. We will conclude that evo devo's enthusiasm for *cis*-regulatory changes is unfounded and premature. There is no evidence at present that *cis*-regulatory changes play a major role—much less a pre-eminent one—in adaptive evolution. We hasten to add, however, that future work may indeed show *cis*-regulatory change to be an important feature of evolution, and, as Carroll and

others suggest, one that should be studied carefully. At present, however, we can conclude only that changes in *both* the structure and regulation of genes have been important in adaptation, that their relative importance will not be known for a considerable time, and that the role of structural mutations in morphological evolution—and other adaptive change—is unlikely to be trivial.

The argument for the ubiquity of *cis*-regulatory evolution rests on two pillars. The first is a theoretical claim: the nature of gene regulation makes promoter elements perforce the most likely site of evolutionary change. Moreover, the involvement of promoters is said to have been far more important in the evolution of anatomical traits than of other sorts of traits. The second argument is empirical: *cis*-regulatory evolution has actually been the most important cause of adaptation. We will examine these arguments separately, but first we address two related questions: Do we expect a difference between the genetic basis of anatomical versus physiological evolution? And what is a regulatory change?

Form versus Function

It is a curious aspect of evo devo theory that *cis*-regulatory evolution is said to be enormously important for the evolution of body plans and anatomy, but not necessary for other types of adaptations. Thus, the "theory" of gene regulation largely ignores adaptations affecting behavior, biochemistry, metabolism, and physiology.

It is not clear why this is so. Although advocates of evo devo certainly make a sharp distinction between the evolution of anatomy on one hand and the evolution of all other traits on the other, which they lump together as "physiological" (e.g., Britten and Davidson 2001; Carroll 2005b), they have offered no biological justification for this distinction. Certainly it cannot be because nonanatomical changes are unimportant in evolution. It must be the case that many major evolutionary innovations and transitions involved changes that were not reflected in body form. Think, for example, of the transition from water to land, which involved innovations in respiration, behavior, and reproduction. The evolution of new phyla certainly involved more than just the changes in body plan documented in the fossil record, as we can see from examining adaptations of living phyla.

We suspect that there are two reasons for omitting nonanatomical traits from evo devo theory. First, many practitioners are interested in macroevolutionary changes that *can* be studied in the fossil record, and these of course are limited to changes in form. This appears to have promoted the view that changes in form are the most important of all adaptations. As Carroll (2005b) notes:

> The evolution of form is the main drama of life's story, both as found in the fossil record and in the diversity of living species (p. 294).

> We do not address other forms of innovation, though they are fascinating in their own right, such as the evolution of physiological adaptations through protein evolution (for example, antifreeze proteins, lens crystallins, keratins, lactose synthesis, immune systems), because they do not concern morphological evolution per se (p. 160).

But the omission of "physiological" traits from the theory fails to acknowledge the tremendous amount of already-existing data showing that the adaptive evolution of such traits usually involves changes in structural regions. This, in fact, is acknowledged by evo-devotees. For example:

> There is ample evidence from studies of the evolution of proteins directly involved in animal vision, respiration, digestive metabolism, and host defense, that the evolution of coding sequences plays a key role in some (but not all) important physiological differences between species. In contrast, the relative contribution of coding or regulatory sequence evolution to the evolution of anatomy stands as the more open question, and will be my primary focus (Carroll 2005a, p. 245; references given in text omitted).

But why *should* there be a difference between the types of changes involved in the evolution of form versus function? Is there really an important evolutionary difference between making a bone long and making it strong? After all, physiological and biochemical changes are tissue- and organ-specific in exactly the same way as are anatomical changes, and both types of change occur within developmental networks. Indeed, the same impediments to protein evolution that are said to lead to *cis*-regulatory-based change of anatomy—the deleterious pleiotropic effects of protein-coding mutations—would seem to be at least as strong for physiological and biochemical innovations as for anatomical innovations.

We can find only one explicit biological rationale for distinguishing between the evolution of anatomy versus physiology:

> One absolutely crucial difference, then, between proteins involved in physiology and those involved in body-building, concerns the consequences of mutations that alter these proteins. A mutation that alters an opsin protein may affect the spectrum of light detected in either rods or cones in the eye. However, a mutation in a tool-kit protein [a transcription factor] may abolish the eye altogether, as well as affect other body parts. For this reason, mutations that alter tool-kit proteins are often catastrophic and have no chance of being passed on. The important consequence is that the evolution of form occurs more often by changing how tool-kit proteins are used, rather than by changing the tool-kit proteins themselves (Carroll 2006, p. 204).

But this argument is flawed on two grounds. First, taken at face value, it explains only *why transcription factors may evolve more slowly than other types of proteins*. It does not explain why physiology should evolve by changes in protein structure and anatomy by changes in *cis*-regulatory elements. After all, the

expression of both "physiology" and "anatomy" genes involves transcription factors and promoters, and so should be equally constrained. And there is no evidence that these two classes of genes are regulated in different ways. The study of comparative gene regulation is in its infancy, and although there are hints that different classes of genes may have different types of promoters (e.g., McNutt et al. 2005; Yang et al. 2006), these partitions neither include form versus function, nor say anything about the evolutionary *potential* of different classes of genes. The second problem with this argument is there is no necessary relation between the potential effects of mutations at a locus and the rate of adaptive evolution at that locus. We do not expect, a priori, that loci which can mutate to more lethal alleles (e.g., transcription factors) will evolve more slowly than loci whose extreme effects are more benign (e.g., genes producing structural proteins).

The artificiality of separating form and physiology becomes most evident when considering the evolution of pigmentation, which, although clearly involving physiological and metabolic processes, is nevertheless seen as an aspect of form:

> Changing the size, shape, number, or color patterns of physical traits is fundamentally different from changing the chemistry of physiological processes (Carroll 2005a, p. 1159).

The reason, then, why the evolution of anatomy is a "more open" question than that of physiology is not because there is some fundamental biological difference between the two classes of traits. It is *only because we have less evidence about the nature of change affecting form*, and therefore are less constrained by facts in speculating about its genetic basis. Because there is no clear theoretical reason for expecting different types of evolutionary changes for form than for physiology, we will, when dealing with the data, lump together both types of adaptations.

What is a Regulatory Change?

Historically, the literature on evo devo has conflated two concepts: regulatory genes and regulatory mutations. We will show that while trying to define a regulatory *gene* leads one into a tangled semantic thicket, one can define regulatory *mutations* (i.e., *cis*-regulatory changes) in a consistent way that allows us to address and evaluate the claims of evo devo.

On some level it can be argued that most genes regulate something, whether that something be a protein, a pathway or a biochemical product. True, the primary function of some genes is clearly regulatory. The main role of transcription factors, for example, is to bind to DNA elsewhere in the genome and thereby regulate the spatiotemporal expression of genes. Likewise, some genes have a distinct structural function. They may, for example, contribute to the physical structure of chromosomes and cells. One example is keratin, an insoluble fibrous protein found in hair, feathers, and scales.

There are, however, many cases in which it is hard to draw a simple dichotomy between "structural" and "regulatory" function of genes. Histones, for example, form nucleosomes, which act as spools around which DNA is coiled, maintaining its helical structure and forming chromatin. Although histones were once thought to have a purely structural function, their posttranscriptional modification also allows them to act in more diverse biological processes, including gene regulation (Strahl and Allis 2000). Similarly, the protein beta-catenin has dual regulatory and structural functions (Perez-Moreno and Fuchs 2006). As a structural protein, it is an essential component of cellular adhesion in the cytoskeleton. As a regulatory protein, it acts as a transcriptional coactivator in the *Wnt* signaling cascade. Because of the domain structure of the beta-catenin protein, these two functions can be separated; that is, mutations can alter beta catenin's regulatory function while maintaining its structural role, and vice versa (Bremback et al. 2006). Finally, other proteins have structural and regulatory functions that are inseparable. *SATB1* organizes chromosomes into distinct loop domains, and thus acts as a traditional structural gene. But this structural aspect has a regulatory end: *SATB1* orchestrates gene expression by remodeling chromatin at specific genomic locations, allowing enzymes access to target DNA for regulating DNA transcription (Yasui et al. 2002).

We have not singled out histones, beta-catenin and *SATB1* because they are among only a few genes having both structural and regulatory properties. We could give many similar examples. And when we understand development more fully, it seems likely that many "structural" proteins will act together with transcription factors to regulate gene expression.

A related issue is whether mutations *within* a gene should be classified as regulatory or structural. This question, too, is not straightforward. For example, amino-acid ("structural") substitutions in transcription-factor proteins may be more common than previously appreciated, and these can alter gene regulation. Like *cis*-regulatory elements, many transcription factors are modular in structure (having several functional elements that can act independently of one another), and there is increasing evidence that their coding changes can alter expression of a subset of downstream target genes without completely disrupting downstream pathways (Hsia and McGinnis 2003). In fact, Levine and Tjian (2003) suggest that the diversification of activation sites of transcription factors—whose DNA binding domains nevertheless remain conserved—also contribute to organismal diversity.

One example involves homeobox (Hox) genes, the most famous class of transcription factors, which help specify segmentation patterns along the anterior–posterior body axis of animals. Although the DNA sequences of Hox genes are largely conserved among major animal groups, some coding changes in the Hox gene *ultrabithorax* (*Ubx*) affect *its* ability to regulate downstream transcription levels and ultimately its ability to repress limb formation.

(In vitro studies implicate the loss of serine phosphorylation sites.) Thus, coding mutations in a transcription factor might be involved in a "macroevolutionary" change in animal body plan (Ronshaugen et al. 2002).

Likewise, in different groups of insects, evolution has exchanged binding motifs in the coding region of the Hox gene *ftz*. This swap has changed *ftz*'s downstream binding targets and hence its regulatory role. These swapped motifs may be associated with the diversity of body segments (Lohr et al. 2005). Should we consider such mutations regulatory—because they alter the expression levels of downstream genes—or structural—because they alter the structure of the transcription factor? And should we classify as structural or regulatory those amino acid changes in a protein that affect *its own* regulation (e.g., mutations in G-protein coupled receptors that downregulate the receptor [Benya et al. 2000; Rathz et al. 2002])? What about coding-region mutations that affect mRNA folding or stability (e.g., Wisdom and Lee 1991; Schiavi et al. 1994; Shen et al. 1999), protein level (Carlini and Stephan 2003) or tissue-specific expression pattern (e.g., Nakayama and Setoguchi 1992)? Or silent mutations in the coding regions that affect translation rate and protein function (Kimchi-Sarfaty et al. 2007)?

To escape this semantic tangle, we take two approaches. First, we refrain from classifying *genes* as either structural or regulatory, although some bits of DNA, like promoters, are clearly regulatory. Second, we classify *mutations* based on their physical location. Mutations must lie either inside or outside the coding region of a gene (either DNA that is transcribed into mRNA and translated into a protein, or "functional" RNA molecules such as ribosomal RNA, ribozymes, or antiviral RNA). If a mutation affecting a phenotype lies within the coding region, we consider it a structural mutation. Conversely, mutations that lie outside the coding region (including mutations in introns) are considered regulatory. Although regulatory elements are often poorly delineated, we can infer that if a noncoding mutation causes a change in phenotype, it usually occurs in a functionally important *cis*-regulatory element (e.g., enhancer, promoter core element, or other transcriptionally relevant element).

When considering a "causal locus" affecting a phenotypic difference, our distinction between regulatory and structural mutations covers all possible changes, and we no longer need to distinguish between *cis*- and *trans*-regulation. For example, if a *cis*-regulatory change alters the expression of gene A, which then has downstream effects on unlinked gene B, and the effects of gene B alter the phenotype, then the *causal* change is *cis*-acting for A, *trans*-acting for B, but is still a regulatory mutation in our classification. Finally, we will not distinguish here between the various types of regulatory elements (for a description see Alonso and Wilkins 2005), as this is irrelevant to our discussion.

Our distinction between structural and regulatory mutations comports with much current usage in evo devo. Of course, while it is easy to construct such a dichotomy, it is much harder to *identify* the mutation or mutations associated with a gene that causes an important phenotypic change.

Theory

Carroll (2005a,b; 2006) outlines what we call the "theoretical imperative" for *cis*-regulatory evolution. This derives from what we know about the nature of gene regulation (see Levine and Tjian 2003; Wray et al. 2003), and so a brief review is in order.

Eukaryotic genes are under the control of noncoding DNA sequences (e.g., *cis*-regulatory elements), including promoters usually located "upstream" (in the 5′ direction) from the start codon of a gene. Core promoter sequences are the sites where transcription is initiated. A gene can be controlled by several independent promoters (indicating different transcriptional start sites), which may or may not be close to each other. The default state of a gene is "off" (no expression or low basal expression), and mRNA transcription begins when RNA polymerase binds to the gene's promoter region. The binding of RNA polymerase is mediated by transcription factors, regulatory proteins that may themselves require other transcription factors or organic molecules for accurate binding.

By and large, transcription factors are evolutionarily conserved in both structure and function; the classic example is Hox genes, which are conserved in their amino acid homeodomains, genomic organization, and expression patterns among animals (Hill et al. 1989; Doboule and Dolle 1989). In addition, promoter regions often work together with other *cis*-regulatory elements (e.g., enhancers, silencers, insulators, etc.) to control the expression of the gene in a specific tissue or at a specific time. For example, enhancers (sequences a few hundred base pairs long) usually bind sequence-specific transcription factors to mediate expression within a specific tissue or cell type. Silencers, on the other hand, bind transcription factors that block or reduce transcription levels by impeding RNA polymerase binding. Both enhancers and silencers can be up to 100 kilobases away from their core promoter, making them difficult to identify. Taken together, these *cis*-regulatory elements are modular: that is, different *cis*-regulatory elements can independently affect the expression of a transcript at different times and places. Consequently, diversity in gene regulation can be achieved by different combinations of *cis*-regulatory elements working independently of one another to direct composite patterns of gene expression.

The fact that each gene is controlled by a set of modular *cis*-regulatory elements leads to the most important consequence for evo devo theory. Whereas a change in a protein sequence may have deleterious pleiotropic effects (proteins interact with other

proteins through the ramifying network of development, and a sequence change could affect every such interaction), a change in a *cis*-regulatory element may affect only the specific temporal or spatial expression of its single attendant gene. *Cis*-regulatory changes are therefore thought to be relatively free of negative pleiotropic effects on fitness. The problem with protein-sequence change seems even worse if the protein is a transcription factor, because every gene regulated by such a factor might show altered expression.

All other things being equal, then, a change in a *cis*-regulatory region is supposed to have a higher probability of being adaptive than is a random change in a structural gene or transcription factor. Moreover, if a mutation in a *cis*-regulatory element brings a gene under the control of a new transcription factor, a radical co-option of function can take place. Such co-option is said to underlie evolutionary innovations such as body segmentation and diversification of those segments (Carroll et al. 2001).

The final factor said to promote regulatory evolution is "the combinatorial action of the transcription factor repertoire in cells" (Carroll et al. 2001, p. 190). As Carroll et al. explain (p. 190), "The transcription factor repertoire is sufficiently diverse and the stringency of DNA binding [to the promoter region] sufficiently relaxed such that sites for most transcription factors can evolve at significant frequency in animal genomes." This idea—effectively that promoters have a higher rate of adaptive nucleotide substitution—could produce the differences in timing or tissue expression said to be involved in most evolutionary innovations.

Taken together, these facts about gene regulation underlie the theoretical imperative for *cis*-regulation:

> It [the nature of *cis*-regulatory regions] constitutes pervasive evidence that the diversification of regulatory DNA, while preserving coding function, is the most available and most frequently exploited mode of genetic diversification in animal evolution" (Carroll 2005b, p. 231).

However, there are several other ways to obviate the negative consequences of pleiotropy besides changing *cis*-regulatory elements. The most obvious is gene duplication followed by divergence of the duplicated copies (termed "paralogs"). This process allows a protein to retain an ancestral function while its paralog or paralogs evolve to new functions. (Gene duplication, of course, can also create new *cis*-regulatory regions that may likewise diverge adaptively.) In addition, a gene can mutate to new forms by creating alternative splicing sites or recruiting new coding domains while still allowing production of the ancestral protein; these two processes are relatively rare. The evolutionary fixation of duplications, however, appears to be fairly common. Nevertheless, Carroll (2005a) argues that duplications are established too rarely to play an important role in micro- and macroevolution, citing Lynch and Conery's (2000) calculation of one duplication fixed (or nearly fixed) per gene per 100 million years.

The theoretical argument for the importance of *cis*-regulation thus rests on eliminating evolutionary alternatives: changes in structural genes affecting anatomy must either be deleterious themselves or accompanied by deleterious pleiotropic effects, and recruitment of coding domains, alternative splicing, and gene duplication are rare. We are then left with *cis*-regulatory regions as the most likely site of adaptive change.

This logic, however, is not convincing in light of what we know about the population genetics of new mutations. The rate of fixation of *cis*-regulatory versus structural mutations depends on three factors: (1) their relative mutation rates, (2) their relative chances of being adaptive (positively selected; Fisher 1930), and (3) the relative sizes of the selection coefficients (Kimura 1983; Orr 2003). Even if a *cis*-regulatory mutation is less likely to have deleterious pleiotropic effects, this does not necessarily mean it is more likely to be fixed, because such mutations may be less likely to occur or their net selection coefficients may make them less likely to be fixed. For example, *cis*-regulatory sites at a given gene may be less numerous than protein-coding sites, and their mutation rate correspondingly lower. Moreover, it is easy to imagine that expressing a protein at a new time or place could have effects just as deleterious as—or more deleterious than—changes in protein sequence. Is it so clear that activating a gene in a new part of the body, or making twice as much of an enzyme, is more likely to be adaptive than, say, a single substitution of valine for leucine in an enzyme?

What about gene duplications? Are they, as Carroll maintains, too infrequent to explain much adaptation? This seems unlikely. It is curious that the paper cited by Carroll supporting the *infrequency* of adaptive change by gene duplication—that of Lynch and Conery (2000)—actually claims that duplications are not only frequent, but make important contributions to speciation and species-level differences. One duplication per gene per 100 million years is a low *per-gene* mutation rate, but not necessarily a low *per-genome* mutation rate; and it is the latter that is important for adaptation and speciation. As Lynch and Conery (2000 p. 1154) note, "With rates of establishment of 0.002 to 0.02 duplicates per gene per million years and a moderate genome size of 15,000 genes, we can expect on the order of 60–600 duplicate genes to arise in a pair of sister taxa per million years . . . " Moreover, gene copy number polymorphisms within species are well documented in the one species that has been extensively studied—humans (Sebat et al. 2004)—and are likely to be found in other groups.

One need only peruse Ohno's (1970) book to see the pervasiveness and potential importance of gene duplication, one of the few ways that new genes can actually arise in evolution. After all, nearly every gene can be considered a duplicate or chimera of earlier genes, and the origin of new genes must therefore have been important in adaptation. It is almost superfluous to list the gene families and adaptations deriving from duplications: they include

globins, the immune system, olfaction, opsins and, indeed, transcription factors themselves.

The multiply-and-diversify model of evolution does not depend solely on the duplication of single genes: the evolution of tetrapods probably involved at least two bouts of *whole-genome duplication* (Dehal and Boore 2005). Moreover, it is estimated that between 47% and 70% of angiosperms are polyploids (Ramsey and Schemske 1998), and thus harbor duplicated genes. Otto and Whitten (2000) calculated that ploidy changes represent between 2% and 4% of speciation events in flowering plants, although polyploidy is far rarer in animals. In view of these facts, it seems unwise to deny a priori that structural genes could play a major role in the evolution of plants and animals.

Moreover, there are other ways besides gene duplications that novel and useful structural genes can arise. These include gene fusion and fission (e.g., mammalian fatty acid synthase), recruitment of old genes to new functions (e.g., the antifreeze proteins permitting fish to live in frigid waters), exon shuffling (e.g., involved in the evolution of blood clotting), and the addition of transposons to coding sequences. In a review on the origin of new genes, Long et al. (2003) describes these and many other processes. Given the diverse ways that useful new genes can arise, one should be cautious about making sweeping evolutionary statements about likelihood. Before concluding, for example, that the difference between a man and a mouse rests largely on the nature of their promoters, one should realize that 21% of human protein-coding genes have *no known homologs* (gene copies related by descent) in mice (available from http://eugenes.org:7072/all/homologies/hgsummary-2005.html; Don Gilbert, Genome Informatics Laboratory, Indiana University).

Given the contrast between evo devo theory and the evidence that there has indeed been dramatic change in structural genes during evolution, it is no surprise that some have taken a position completely antithetical to the *cis*-regulatory imperative, viz. Li's statement (1997, p. 269) that "there is now ample evidence that gene duplication is the most important mechanism for generating new genes and new biochemical processes that have facilitated the evolution of complex organisms from primitive ones."

In the end, such back-and-forth assertions seem Talmudically irresolvable, at least on the basis of a priori considerations. The real way to settle the issue of the importance of *cis*-regulatory evolution is to look at the data. How often have new adaptations involved evolutionary changes of promoters versus changes in coding sequences? We now turn to the empirical evidence on the molecular basis of adaptation.

The Facts

GENOMIC STUDIES OF *cis* AND *trans* MUTATIONS

It is appropriate to begin our discussion with recent genomic data, because much of the inspiration for "regulatory gene" theories arose from early genomic data on the similarity of protein sequences between humans and chimps.

The recent production of complete genome sequences from many species has allowed far more refined analysis of adaptation using genome-wide patterns. Several studies are relevant to the question of *cis*-regulatory evolution. Andolfatto (2005), for example, showed that patterns of nucleotide variation in untranslated regions (UTRs) of the *Drosophila* genome are consistent with the view that changes in these regions affect fitness. He estimates that changes in UTRs probably contribute at least equally, if not more, to adaptation than do changes in coding regions. However, a major drawback of this approach, inherent in most genomic studies (discussed below), is that genome-wide surveys are conducted in the absence of phenotypic information, limiting our ability to identify the specific DNA mutations affect phenotype and are truly adaptive.

In the spirit of King and Wilson (1975), most of these genomic studies have focused on identifying genetic regions showing rapid evolution in the human lineage; the implicit goal is to discover mutations contributing to "human-ness." Early and highly publicized estimates that the DNA of chimps and humans is 99% identical led King and Wilson (1975, p. 115) to conclude that "a relatively small number of genetic changes in systems controlling the expression of genes may account for the major organismal differences between humans and chimpanzees." However, a 99% identity of DNA sequence still translates into a considerable difference in protein sequence, a conclusion confirmed by the data of Glazko et al. (2005) showing that 80% of the proteins of humans and chimps differ by at least one amino acid. Regulatory change, then, may not be necessary to explain the phenotypic differences between these species.

Several other studies have identified rapidly evolving proteins (and hence, structural mutations) that may have been involved in adaptive evolution in primates. For example, hundreds of genes show evidence of positive selection in the hominid lineage (Clark et al. 2003). Two more recent studies also showed evidence for rapid evolution of amino acid sequences (ca. 5–9% of genes under analysis), including genes involved in sensory perception and immune defenses (Dorus et al. 2004; Nielsen et al. 2005). In fact, one study (Bustamante et al. 2005) identified transcription factors as a particularly rapidly evolving class of proteins, contradicting the evo devo assertion that antagonistic pleiotropy precludes changes in the amino acid sequence of transcription factors. Indeed, the results of Bustamante and colleagues suggest that even if differences in gene expression played a prominent role in the divergence of humans from chimps, the ultimate cause may often involve structural mutations.

Only a few studies, however, have simultaneously compared regulatory with structural evolution. A recent one identified DNA elements in both coding and noncoding regions that showed rapid

divergence along the human lineage, elements termed "human accelerated regions" (HARs; Pollard et al. 2006a,b). The authors conclude that the majority of HARs are: (1) in noncoding regions, (2) contiguous to coding regions, and (3) if within coding regions, often in transcription factors. Together these results raise the possibility that *cis*-regulatory changes contribute disproportionately to human-specific traits. Unfortunately, because these genomic studies are conducted without reference to the phenotype, it is impossible without further work to determine which mutations in HARs contributed to adaptive evolution.

Recent technological advances allow us to gauge the relative contributions of *cis* versus *trans* mutations to interspecific changes in gene expression at many loci. In an elegant study, Wittkopp et al. (2004) examined the contributions of *cis*- and *trans*-acting factors to species-level divergence of gene expression in F_1 hybrids of *Drosophila melanogaster* and *D. simulans*. (In this study, the distinction between *cis*- and *trans*- mutations is not identical to our own distinction between regulatory and structural mutations). Wittkopp and colleagues clearly show that *cis*-acting factors are an important part of interspecific divergence in gene expression in *Drosophila*. Again, however, we do not know the phenotypic effects of any of the 29 genes analyzed. It is thus unclear whether any of the species differences in gene expression have an adaptive (or even phenotypic) effect, and, if so, which proportion of such adaptive changes were are caused by structural versus regulatory mutations.

Despite these problems, the studies discussed here raise the possibility that future genomic studies could address the relative contribution of *cis*-regulatory and structural mutations to biological diversity. At present, however, the genomic data are ambiguous. We turn now to the data from individual loci—data that constitute main bulwark of *cis*-regulatory theory.

SINGLE-LOCUS DATA

We begin by discussing the criteria for deciding whether a change in phenotype is caused by changes in *cis*-regulatory elements, protein structure, or both. We then describe the experiments necessary to demonstrate the relative contributions of *cis*-regulatory versus structural changes to adaptive variation at single loci.

A common method for determining the role of gene regulation in evolutionary change—a method that is the foundation of the evo devo approach—involves simultaneously comparing differences in a phenotype among species (often distantly related ones) with the pattern of expression of a single gene thought to influence that phenotype. Although this approach has successfully identified important pathways involved in phenotypic change (e.g., the calmodium pathway involved in beak-size evolution of Darwin's finches [Abzhanov et al. 2004, 2006] and *Notch*/*Distal-less* in the formation of butterfly wingspots [Beldade et al. 2002; Reed and Serfas 2004]), it does not give us the complete story because the source of phenotypic differences are not pinpointed:

> In many cases a gene required for the development of a trait in one species shows a difference in expression in other species that correlates with a difference in that trait . . . A causal relationship is plausible but not proven in these cases, because comparisons of gene expression cannot by themselves demonstrate that a change in transcriptional regulation is the genetic basis for a phenotypic difference (Wray et al. 2003, p. 1378).

The common methods of observing spatiotemporal patterns of gene expression (e.g., in situ hybridization, quantitative PCR) and experimentally manipulating protein levels (e.g., ectopic or misexpression studies) can do no more than show an association of gene expression with phenotype and perhaps implicate the developmental pathway in which the causal mutation lies. Although the causal mutation may be located in the *cis*-regulatory region of the protein of interest, it is equally likely, if not more likely, to lie somewhere upstream of the gene of interest, somewhere in the panoply of *trans*-regulatory factors or cofactors that affect regulation of the gene. Although these correlational studies often proclaim that change in *gene regulation* contributes to phenotypic diversity, this result is neither novel nor surprising. In fact, it would be surprising if a mutation did *not* affect gene regulation, for most mutations (either structural or *cis*-regulatory) have effects on the regulation of gene products downstream in their respective pathways.

Likewise, structural mutations can also explain divergence in gene expression among species. Differences in the amount of mRNA or protein may reflect structural rather than regulatory changes if they result from differential stability of the gene product. For example, in *D. melanogaster* the replacement of certain synonymous codons in the coding sequence of *alcohol dehydrogenase* causes a significant decrease of enzyme production (Carlini and Stephan 2003). Others have noted that experiments documenting changes in gene expression do not necessarily implicate regulatory changes:

> Many comparative studies that use in situ hybridization interpret different probe patterns as an indication of transcriptional changes in enhancer [in the *c*is-regulatory region], openly ignoring the possibility of post-transcriptional events that alter mRNA stability or changes in splicing profiles that affect the sequences detected by (often) a single probe (Alonso and Wilkins 2005, p. 713).

While studies of gene expression at either individual candidate loci or many loci simultaneously (e.g., microarrays) can test developmental pathways involved in phenotypic variation, determining whether variation in gene expression among species involves structural versus regulatory changes usually requires genetic analyses. Genetic crosses (e.g., quantitative trait locus

mapping) and genetic complementation approaches (e.g., deletion mapping) can initially be used to localize genomic regions, often containing hundreds of genes, of which one or more contain mutations that contribute to the phenotypic difference of interest.

For candidate loci, the challenge is then to determine whether causal mutations occur in the structural or in the *cis*-regulatory regions. This requires identifying mutations that are functionally important as well as excluding mutations that are not. For amino acid changes, functional assays (e.g., cell-culture based or enzyme assays) can provide evidence for the role of particular mutations on protein behavior.

For regulatory changes, *cis*-regulatory elements can be tested for their ability to drive the expression of reporter constructs (e.g., green fluorescent protein; GFP) to determine the association between particular *cis*-regulatory regions and the spatial pattern of expression. These experiments, however, are still one step removed from the phenotype. For both structural and regulatory changes, the ultimate test of mutational effect is the use of transgenics together with a thorough examination of the phenotype of interest. In such tests, a construct containing the mutation(s) of interest is expressed in the appropriate genetic background to determine if it affects the phenotype of interest—and not just the protein activity or gene expression pattern. Of course, such experiments are not always technically feasible.

Excluding structural or regulatory mutations that do not play a role in the phenotypic difference can be even more difficult than identifying the causal mutations themselves. If there are no nucleotide differences in the entire coding region between individuals that differ phenotypically, then one can rule out structural mutations at that gene. Unfortunately, interspecific comparisons, (which predominate in evo devo) usually show some nucleotide differences, making it difficult to pinpoint the causal substitution(s) in a sea of irrelevant substitutions.

Determining the relative contribution of structural and *cis*-regulatory changes at any genetic locus is challenging, and most studies have not examined both types of change. Nonetheless, Table 1 lists and describes mutations implicated in adaptive change between closely related taxa. Each mutation is accompanied by a description of the adaptive nature of the change, its effect on protein function, the evidence supporting the causal link between mutation and phenotype, and the information still needed to fully characterize the mutation's effects.

We chose these examples because each demonstrates fairly rigorously both the adaptive nature of the genetic change as well as whether that change is *cis*-regulatory, structural, or both. We have omitted examples of structural gene changes between distantly related groups that are undoubtedly adaptive (e.g., α vs. β vs. fetal hemoglobin). Including such cases would strengthen the evidence for structural versus *cis*-regulatory evolution, for while the adaptive significance of amino acid substitutions in many of these cases is fairly clear, we know little about changes in *cis*-regulation.

Although there have been many arguments (some verging on the philosophical) about how to define and recognize an "adaptation," in Table 1 we have used a fairly loose criterion: if a trait is generally recognized to increase fitness or is maintained by selection, we regard it as an adaptation. So, for example, Table 1 includes features like cryptic coloration, antifreeze proteins in ectotherms, polymorphisms apparently maintained by balancing selection, and clinally varying traits. Some traits have shown the expected fitness effects in laboratory or field tests, while others have not been rigorously tested. Indeed, for one trait—a species difference in larval bristle pattern in *Drosophila*—we have no idea of its adaptive significance (see below); we include this trait because it is an oft-cited example of *cis*-regulatory change in evolution.

We do not claim that this table shows the relative importance of structural versus *cis*-regulatory change in evolution. There is almost certainly an ascertainment bias in favor of structural changes, because these are far easier to detect than changes in promoter regions. Differences in protein structure, for example, can be identified by simply comparing nucleotide or cDNA sequences. In contrast, most regulatory elements are small, not strictly conserved, and often far removed from the gene, making them difficult to identify and to pinpoint their functionally relevant sites. Also, although we know something about mutational effects in protein-coding regions based on the type of DNA or amino acid change (e.g., nonsynonymous vs. synonymous, conservative vs. radical, hydrophobic vs. hydrophilic) and its location (e.g., conserved motifs, active sites), the functional effects of mutations in regulatory elements remain largely unknown. Of course, identifying *cis*-regulatory changes would be facilitated by a better understanding of gene regulation. Finally, we hasten to add that the examples given in Table 1 are not exhaustive: we have inevitably missed relevant studies. Nevertheless, the list gives an idea of what we know at present about the molecular genetics of adaptation, and how much empirical evidence supports a claim for the importance of *cis*-regulatory variation.

Table 1 clearly shows that we have far more evidence for structural than for *cis*-regulatory changes. While the most well-supported examples of *cis*-regulatory based adaptation (the first three entries of Table 1) have not yet identified precise causal mutations, there are, in contrast, many examples of individual structural mutations contributing to adaptation. Moreover, most of the *cis*-regulatory examples involve loss of an ancestral trait (usually via loss-of-function alleles), whereas the structural mutations involve both gains and losses of traits. We discuss the two types of mutations, cis-regulatory and structural, below.

Empirical evidence for cis-regulatory adaptation

The claim that adaptive change is predominantly driven by *cis*-regulatory mutations rests on a handful of elegant but still incomplete studies. The three most relevant analyses, which focus respectively on skeletal armor in threespine sticklebacks (Shapiro et al. 2004), pigmentation on *Drosophila* wings (Gompel et al. 2005; Prud'homme et al. 2006), and dorsal bristle (trichome) density on *Drosophila* larvae (Sucena and Stern 2000), have been repeatedly cited as exemplars of *cis*-regulatory evolution. We will show, however, that in each case additional data are needed to identify the molecular basis of phenotypic change. It is also important to note that these three studies focus primarily on the genetic dissection of trait *loss*, so from the outset these data may be biased by a specific type of phenotypic change, and thus quite possibly by a specific type of mutational change. (It may, for example, be much easier for a *cis*-regulatory change to eliminate a trait than to create a new one.)

Perhaps the most comprehensive study of "*cis*-regulatory" adaptation comes from comparing pelvic spine morphology in marine versus benthic sticklebacks (*Gasterosteus aculeastus*; Shapiro et al. 2004). This work involved genetic analysis of phenotypic differences between an ancestral marine form, clad with armor plating and rigid spines that protect against predators, and a derived benthic form having reduced pelvic spines and very little armor. The use of genome-wide molecular markers allowed Shapiro and colleagues to map the difference in pelvic morphology to several chromosomal regions, one of which contains the candidate gene *Pitx1*. Because there is no difference in amino acid sequence between the *Pitx1* proteins of the two phenotypes, we can rule out the possibility that amino acid change in the *Pitx1* transcript caused the loss of pelvic spines. However, the absence of amino acid variation does not by default *prove* that the causal mutation(s) is located in an upstream *cis*-regulatory element, as there are alternative hypotheses (e.g., mutations in closely linked loci).

To examine divergence in *Pitx1* gene expression, Shapiro et al. (2004, 2006) used in situ hybridizations to compare *Pitx1* transcript levels between marine and benthic fish. This experiment yielded two important results. First, in some structures, like the mouth and jaw, the spatial expression pattern of *Pitx1* is conserved between the marine and benthic phenotypes. Second, in the pelvis, the *Pitx1* transcript is undetectable in the less-armored benthic form, and thus its absence is correlated with the absence of pelvic spines. These results suggest that there has been tissue-specific divergence in the regulation of *Pitx1*. Based on these two patterns of *Pitx1* expression, it is possible that benthic fish have undergone an inactivating mutation in a *cis*-regulatory element specific responsible for pelvic expression. However, additional data, including identifying the precise mutation(s), are necessary to prove that a *cis*-regulatory mutation(s) contributes to the adaptive pelvic reduction. The crucial experiments (undoubtedly underway) include the following:

(1) fine-scale mapping to exclude the contribution of neighboring genes (e.g., transcription factors, miRNAs) to protein expression and ultimately to morphological variation.
(2) identifying and verifying through functional analysis (e.g., transgenic experiments) the causal mutations in the cis-regulatory region.

Moreover, to support the ancillary hypothesis that modularity in the *cis*-regulatory region promotes evolutionary change, it will be necessary to identify multiple *cis*-regulatory elements and demonstrate that each element, by binding distinct transcription factors, independently controls tissue-specific expression. (A second locus, *ectodysplasin* (*Eda*), has been implicated in the loss of lateral plates in freshwater populations of sticklebacks [Colossimo et al. 2005]. However, nothing is yet known about the relative role of structural versus regulatory mutations at this locus.)

Two other examples of *cis*-regulatory evolution come from *Drosophila,* one on larval trichome loss and the other on pigmentation. These studies both compared divergent *Drosophila* species, one or more of which experienced the loss of a trait during their evolutionary history. The first pair of studies examined the role of species-specific differences in the expression of the *yellow* protein in the formation of male wing spots that may play a role in courtship behavior (Gompel et al. 2005; Prud'homme et al. 2006). Most notably, this work used transgenic methods to test individual sub-regions of the 5′ *yellow* promoter and to determine which regions drove reporter expression in the developing wing. Together with sequence data, these experiments show that the gain and loss of binding sites in the *cis*-regulatory region affect the expression of *yellow* protein among species. Although in this case the promoter region clearly contains regulatory modules controlling the spatial expression of *yellow*, the direct link between genotype and phenotype is not complete. This is because changes in the *cis*-regulatory elements of *yellow* alone are not sufficient to produce the phenotype of interest—the pigmented wing spot (Gompel et al. 2005). Additional loci must therefore be involved. Although it is not surprising that different *cis*-regulatory elements in the *yellow* promoter affect *yellow* expression, a critical piece of evidence is still missing: the demonstration that species-specific *cis*-regulatory elements produce the species-specific difference in the wing spot.

A third study, that of Sucena and Stern (2000), used genetic mapping (genetic crosses with visible markers, deletion mapping, and single-gene complementation) to pinpoint the gene *ovo*/*shavenbaby* (*svb*) as the cause of differences in trichome pattern between species. While larvae from most species in the *Drosophila melanogaster* subgroup have robust denticles and a

lawn of fine hairs on their abdominal segments, *Drosophila sechellia* (and four other species) maintain the rows of robust denticles but have lost the fine hairs, thus acquiring a naked cuticle. The adaptive significance of the interspecific difference in trichome pattern, if any, is unknown. There are several lines of evidence that the causal mutation(s) is regulatory. First, expression of *svb* mRNA is correlated with phenotypic variation in trichome pattern. That is, in *D. melanogaster* the *svb* transcript is abundant in cells that form robust denticles and less abundant (but still present) in cells that produce fine hairs. In *D. sechellia*, the *svb* transcript is similarly abundant in cells that produce denticles but absent in cells producing fine hairs. Second, transgenic assays show that a 60 kb region upstream of the *svb* coding region contains several sites influencing the species difference in trichome pattern (D. Stern, pers. comm.). Third, the *sechellia* naked-cuticle phenotype is not consistent with a null mutant at the *svb* locus itself, because such mutants completely lack trichomes (both the robust denticles and the fine hairs). Finally, recombination studies show that the coding region of *svb* is not responsible for the phenotypic difference (D. Stern, pers. comm.). Nevertheless, the precise locations of the DNA changes that produce the interspecific difference in trichome pattern remain elusive.

The three sets of studies described above are the strongest (and most widely cited) cases used to show the evolutionary importance of *cis*-regulatory mutation. None of them has yet identified an individual mutation in a *cis*-regulatory element, or functionally verified via transgenics that that mutation contributes to the phenotypic difference of interest. By raising these issues, we do not mean to criticize these studies, for the conclusive experiments are almost certainly in progress and may well show that *cis*-regulatory evolution is involved. Indeed, this seems likely for the cases of *Pitx1* in sticklebacks and *ovo*/*shavenbaby* in *Drosophila*. We claim only that, at present, these studies cannot serve as formal demonstrations of *cis*-regulatory change in evolution. Moreover, even if all of these cases do prove to involve *cis*-regulatory change, we are still left with only a handful of such examples compared to the much larger amount of data implicating structural changes. Finally, we must recall that these three studies focus primarily on the *loss* of traits (pelvic spines, wing spots, and trichomes). Supporting the evo devo claim that *cis*-regulatory changes are responsible for morphological innovations requires showing that promoters are important in the evolution of *new* traits, not just the losses of old ones.

Empirical evidence for structural adaptation

In contrast to the dearth of evidence for *cis*-regulatory changes are the many cases in which an adaptation has involved changes in a structural region (see Table 1). Except for insecticide resistance, all of these are "natural" adaptations that do not involve human intervention or selection. (Although we did not use examples from animal or plant breeding, we included genes involved in insecticide resistance because such adaptations still take place in a semi-natural environment with all of its constraints, and because evolutionary responses to insecticides highlight the diverse ways that the genome handles the adaptive challenge of toxicity).

Inspecting these data yields several conclusions. The first is obvious: there are many examples of simple changes in amino acid sequence contributing to adaptive evolution. Some cases involve changes in morphological traits (e.g., *Mc1r* in pigmentation), while most involve physiological traits (e.g., lysozymes in digestion). It is important to add that not all of these structural mutations have yet been tested using functional assays.

As we emphasized above, the larger number of documented structural changes may partly result from a bias in our understanding of underlying molecular pathways. While most of us have seen the detailed physiological pathways illustrated in textbooks (e.g., the Krebs cycle), there is little similar information about the genetic network for morphology. Therefore, candidate loci for physiological traits are more readily identified and their coding regions more readily sequenced. In contrast, traditional evo devo studies are motivated by understanding differences in morphology (body plan) and use comparisons of gene expression pattern as their primary tool.

Several examples of amino acid substitutions are clearly involved in species adopting new ways of life, that is, occupying new "adaptive niches." These include changes in hemoglobin structure that allow birds to migrate over high mountain ranges, in "antifreeze" proteins of fish that permit them to inhabit frigid waters, and in pancreatic RNAase in monkeys associated with increased herbivory. Finally, virtually every change in the color of animals and plants analyzed so far appears to involve changes in the coding regions of genes (see Hoekstra 2006), even though pigmentation is often considered to be a "form" trait, and thus hypothesized to evolve by changes in *cis*-regulation.

"Speciation" genes

Evo devo research is often explicitly motivated by a desire to explain the generation of biological diversity. While adaptation within lineages (anagenesis) represents part of the story, speciation and the generation of new lineages (cladogenesis) is the other part, without which morphological diversity would not be preserved. Here we briefly discuss what is known about the contribution of *cis*-regulatory and structural mutations to reproductive isolation.

The study of "speciation genes" (the name we use for any gene causing reproductive isolation between related taxa, even though some of these must have evolved after rather than during speciation [Coyne and Orr 2004]) is in its infancy, and hence only a handful of genes contributing to reproductive isolation have been identified. However, several patterns are already emerging (reviewed in Orr et al. 2004; Orr 2005; Noor and Feder 2006). One

is that all known speciation genes whose divergence in DNA sequence causes hybrid sterility or inviability (e.g., *OdsH*, *Hmr*, *Lhr*, *Nup96*) show evidence of rapid evolution and the signature of positive selection on mutations in coding regions (see Orr et al. 2004; Brideau et al. 2006). In addition, it is clear that *cis*-regulatory changes alone cannot be the cause of postzygotic isolation: for example, complementation tests show that *Nup96*'s effect on hybrid inviability is probably due to divergence in the protein itself (Presgraves et al. 2003). Indeed, *none* of these cases have found a contribution of *cis*-regulatory changes to reproductive isolation. And, unlike studies of genes involved in adaptation, the methods for identifying speciation genes do not suffer from the same ascertainment bias: there is no a priori expectation that genes causing inviability of hybrids, for example, should be "physiological" rather than "anatomical."

Conclusions

While the study of *cis*-regulatory evolution is an important endeavor, justifiably championed by Carroll and others, our survey of the theory and empirical data shows that the widespread enthusiasm for the importance of *cis*-regulatory change in evolution is at best premature. Analyzing the verbal theory, one finds no compelling reason to draw a distinction between the genetic basis of anatomical versus physiological evolution. Nor is there good reason to accept the a priori argument that—for either anatomy or physiology—changes in *cis*-regulatory genes are more likely to be fixed in evolution than are changes in the coding region of genes.

The data, though they may suffer from ascertainment bias, also show no strong evidence for important *cis*-regulatory change in evolution. In contrast to the many known adaptive changes in protein structure (some of which may have opened new ways of life for animals), there are only a handful of examples that are probable cases of adaptive *cis*-regulatory evolution. And, in contrast to the evidence for structural change, all three of the most widely cited cases have not yet produced definitive evidence that *cis*-regulation is involved. Moreover, these three cases focus on losses of traits rather than the origin of new traits, and in only one of the three (loss of pelvic structures in stickleback fish) is there a clear adaptive explanation for the trait loss. Obviously, we still cannot make sound generalizations about the molecular basis of adaptation. What we *can* say is that adaptations of both form and physiology are likely to involve a mixture of structural and *cis*-regulatory changes, and that structural changes are unlikely to be negligible.

At present, then, we should neither draw conclusions stronger than this nor represent to the general public that we fully understand the genetic basis of adaptation. Those who feel otherwise would do well to remember Carl Sagan's (1987, p. 45) testy remark when pressed to give an opinion about the probability of extraterrestrial intelligence: "Really, it's okay to reserve judgment until the evidence is in."

NOTE ADDED IN PROOF

Since this paper was accepted, four additional relevant studies have been published. Contrary to our view, Wray (2007) concludes that there is ample empirical evidence to support the claim that *cis*-regulatory mutations are more important than structural mutations in phenotypic evolution. However, empirical studies continue to support the importance of structural mutations in adaptive evolution. Tang et al. (2007) describe a genome-wide survey of polymorphism in humans, estimating that 10–13% of amino acid substitutions between humans and chimpanzee may be adaptive. Demuth et al. (2006) show that in humans and chimpanzees at least 6% (1,418 of 22,000 genes) of the genes in one species has no known homologue in the other, suggesting that gene duplication and gene loss occur frequently and contribute to the genetic (and perhaps phenotypic) differences between even closely related species. Both of these genomic studies, then, point to a potentially important role of structural mutations in human evolution. Finally, one other study provides yet another example of structural mutations in phenotypic evolution: loss-of-function mutations in the structural region of *anothcyanin2* (*An2*) have evolved five times independently (through five different mutations causing premature stop codons or frame-shifts), leading to an adaptive shift in pollinator syndrome in Petunia.

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Appendix. Mutations in *cis*-regulatory or structural regions that contribute to adaptation.

Gene	Structural/ regulatory	Gain/ Loss†	Main result	Evidence	Missing evidence	Reference
MORPHOLOGY						
Pitx1 (transcription factor)	R	L	Loss of pelvic spines associated with novel habitat (predators) in threespine sticklebacks (*Gasterosteus aculeatus*).	QTL mapping; in situ hybridization; candidate gene information; amino acid sequence comparison.	Neighboring genes not ruled out; functional mutations not identified; transgenic assays needed.	Shapiro et al. 2004, 2006
Ovo/shavenbaby	R	L	Loss of trichomes in several species of *Drosophila* larvae. *D simulans/D. sechellia.*	Genetic mapping; deletion mapping; gene complementation.	Functional mutations not identified; adaptive significance unknown.	Sucena and Stern 2000; Stern (pers. comm.)
Yellow (pigment enzyme)	R	G/L	Male wing spots used in courtship displays in some *Drosophila* species.	Transgenic assays with different *cis*-regulatory elements associated with changes in *yellow* protein expression.	a.a. changes not ruled out; functional mutations not identified; association with pigmentation changes not shown.	Gompel et al. 2005; Prud'homme et al. 2006
Eda (transcription factor)	R/S?	L	Loss of lateral plates associated with novel habitat (predators) in threespine sticklebacks (*Gasterosteus aculeatus*).	QTL and LD mapping; candidate gene information; amino acid sequencing; transgenic results in recovery of some plating.	Functional mutation not identified (only haplotype); contribution of mutations in coding region not ruled out.	Colossimo et al. 2005
Ubx (Hox transcription factor)	S	G	Gain of limbs via loss of serine phosphorylation sites in C terminus results in loss of limb repression in *Artemia* crustaceans versus *Drosophila.*	In vitro assay using site directed mutagenesis shows loss of binding and pathway disruption.	In vivo transgenic assay.	Ronshaugen et al. 2003
Oca2 (ocular albinism enzyme)	S	L	Loss of pigment in three independent cave populations (*Astyanax fasciatus*).	Deletions in different exons in different populations; loss of expression using cell-based functional assay.	Mutation in *Oca2* not identified in 1 of the 3 populations; transgenics assays needed.	Protas et al. 2006
Tyrp1 (tyrosinase-related protein 1)	S	L	Single a.a. mutation associated with light-colored island sheep (*Ovis aires*) maintained as a balanced polymorphism.	One a.a. change is perfectly correlated with color in pedigree of 500 sheep; no change in expression level between color morphs.	Functional assay showing the effect of a.a. substitution on protein function and phenotype; selective agent unclear.	Gratten et al. 2006

Continued.

Appendix. Continued

Gene	Structural/ regulatory	Gain/ Loss†	Main result	Evidence	Missing evidence	Reference
Mc1r (melanocortin receptor)	S	L (partial)	Cryptic pigment pattern in beach mice (*Peromyscus polionotus*).	One derived a.a. in coding region associated with patterning in large cross; a.a. change shown to reduce receptor signaling and ligand binding in cell culture assays.	Neighboring genes not ruled out (but no changes in mRNA expression levels observed between color morphs).	Hoekstra et al. 2006
Mc1r (melanocortin receptor)	S	G	Melanism in lava-dwelling pocket mice (*Chaetodipus intermedius*).	Four linked a.a. changes in coding region perfectly associated with color.	Individual effects of a.a. mutations unknown; functional assay needed; regulatory regions not ruled out.	Nachman et al. 2003
Mc1r (melanocortin receptor)	S	G (additive)	Sexually selected plumage pattern in snow geese (*Anser caerulescens*) involved in assortative mating.	Two a.a. in coding region correlated with quantitative plumage variation.	Functional assay needed; regulatory regions not ruled out.	Mundy et al. 2004
Mc1r (melanocortin receptor)	S	G (additive)	Plumage pattern in and artic skua (*Stercorarius parasiticus*).	One a.a. in coding region correlated with quantitative plumage variation.	Functional assay needed; regulatory regions not ruled out.	Mundy et al. 2004
Mc1r (melanocortin receptor)	S	G	Melanism in bananaquits (*Coerebra flaveola*); clinal variation.	One a.a. in coding region perfectly correlated with melanism.	Functional assay needed; regulatory regions not ruled out; selective agent unknown.	Theron et al. 2001
Mc1r (melanocortin receptor)	S	L (additive)	Cryptic pigmentation in little striped whiptail (*Aspidocelis inornata*).	One a.a. change statistically correlated with blanched color.	Functional assay needed; regulatory regions and linked genes not ruled out.	Rosenblum et al. 2004
Mc1r (melanocortin receptor)	S	L (additive)	Cryptic pigmentation in eastern fence lizards (*Sceloporus undulatus*).	One a.a. change statistically correlated with blanched color.	Functional assay needed; regulatory regions and linked genes not ruled out.	Rosenblum et al. 2004
Mc1r (melanocortin receptor)	S	L (additive)	Cryptic pigmentation in lesser earless lizard (*Holbrookia maculata*).	One a.a. change statistically correlated with blanched color.	Need to control for population structure; functional assay needed; regulatory regions and linked genes not ruled out.	Rosenblum et al. 2004

Continued.

Appendix. Continued

Gene	Structural/ regulatory	Gain/ Loss†	Main result	Evidence	Missing evidence	Reference
Mc1r (melanocortin receptor)	S	G	Association with melanism in jaguars (*Panthera onca*).	Deletion in first transmembrane region perfectly associated with melanism; segregation in pedigree.	Functional assay needed; regulatory regions and linked genes not ruled out; selective. agent unclear	Eizirik et al. 2003
Mc1r (melanocortin receptor)	S	G (additive)	Association with melanism in jagurundis (*Herpailurus yaguarondi*).	Single a.a. change perfectly associated with melanism; segregation in pedigree.	Functional assay needed; regulatory regions and linked genes not ruled out; selective agent unclear.	Eizirik et al. 2003
Ipmyb1 (*myb* transcription factor)	S	L	Blue to white flower color change in morning glory (*Ipomoea purpura*), balanced polymorphism.	Transposition causing a frame-shift mutation.	Functional assay needed; regulatory regions and linked genes not ruled out.	Chang et al. 2005
An2, homologous to *Impmyb1* (transcription factor)	S	L (additive)	Purple to white flower color change in petunias (*Petunea axilaris*) associated with a change in pollination syndrome.	Transposition mediated deletion/frameshift and in an independently derived allele a nonsense mutation are both large contributors to phenotypic divergence.	Structural changes in enzymes may also contribute; functional assay needed; regulatory regions and linked genes not ruled out.	Quattrocchio et al. 1999
F3'H and *DFR* (anthocyanin enzymes)	S	L	Blue to red flower color change in morning glories (*Ipomoea quamoclit*) associated with change in pollination syndrome.	Knockout allele of *F3'H* enzyme and amino acid change in *DFR* enzyme (independent effects of each gene mutation are unknown).	Functional assays needed; regulatory regions and linked genes not ruled out (note that reduced *F3'H* expression can also lead to color change but casual mutation unknown).	Zufall and Rausher 2003, 2004
PLANT LIFE HISTORY						
Flm (flower timing transcription factor)	S	L	Early flowering time in isolates from nature (*Arabidopsis thaliana*).	QTL; microarrays; locus deletion; isogenic lines; quantitative transgenic complementation.	Looked in accessions; functional assay needed; regulatory regions and linked genes not ruled out.	Werner et al. 2005
Flc (flower timing transcription factor)	S	L	Clinal variation in leafy phenotype (*Arabidopsis thaliana*).	Nonsense a.a. mutation associated with early flower timing and alternative splicing disrupts function (null allele).	Looked in accessions; functional assay needed; regulatory regions and linked genes not ruled out.	Werner et al. 2005

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Appendix. Continued

Gene	Structural/ regulatory	Gain/ Loss[†]	Main result	Evidence	Missing evidence	Reference
Cry2	S	R	Clinal variation in flowering time (*Arabidopsis thaliana*).	QTL and positional cloning; protein polymorphism; single a.a. change induces light-induced downregulation of Cry2.	Looked in accessions; functional assay needed; regulatory regions and linked genes not ruled out.	El-Assal et al. 2001
Frigida (flower timing transcription factor)	S	L	Clinal variation in flowering time (*Arabidopsis thaliana*).	Deletion polymorphism disrupting open reading frame associated with early flowering times.	Looked in accessions; functional assay needed; regulatory regions and linked genes not ruled out.	Johanson et al. 2000
ALTITUDINAL PHYSIOLOGY						
Hb (hemoglobin tetramer)	S	G	Comparison high-altitude bar-headed goose (*Anser indicus*) and Andean goose (*Chloephaga melanoptera*) to low-altitude greylag goose (*Anser anser*).	Single a.a. change; increased O_2 affinity binding assays.	Functional assay needed; regulatory regions and linked genes not ruled out.	Perutz 1983; Jessen et al 1991
Hb (hemoglobin tetramer)	S	G	Comparison of high-altitude camelids [llama (*Lama glama*), alpaca (*L. pacos*), guanaco (*L. guanacoe*), vicuna (*L. vicugna*)] relative to low-land camelids (genus *Camelus*).	a.a. change; O_2 affinity binding assay.	Functional assay needed; regulatory regions and linked genes not ruled out.	Perutz 1983; Kleinschmidt et al. 1986; Piccinini et al. 1990
Hb (hemoglobin tetramer)	S	G	Analysis of high-altitude Andean frog (*Telmatobius peruvianus.)*	a.a. changes in alpha chains to reduce chloride binding; O_2 affinity binding assay.	Regulatory regions and linked genes not ruled out.	Weber et al. 2002
INSECTICIDE RESISTANCE						
Rdl (GABA receptor)	S	L (binding)	Resistance to insecticide dieldrin in *D. melanogaster* (& five other species of insects).	Single a.a. change in coding region (ala -> ser or gly). In *D. melanogaster*, RNA injected into frog oocytes renders them less sensitive to dieldrin. In five other species, exact same substitution associated with resistance.	Regulatory regions not ruled out.	ffrench-Constant 1994; ffrench-Constant et al. 1993, 2004.

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Appendix. Continued

Gene	Structural/ regulatory	Gain/ Loss†	Main result	Evidence	Missing evidence	Reference
kdr (knockdown resistance) sodium channel gene	S	L	Resistance to DDT in house flies (*Musca domestica* & six other species of insects).	1–2 a.a. subs. depending on allele. Correlation in species between substitution and resistance.	Functional assay needed; regulatory regions and linked genes not ruled out.	Williamson et al. 1996; Miyazaki et al. 1996; Soderlund and Knipple 2003
Ace-1 (acetyl cholinesterase enzyme)	S	L	Resistance to insecticide in mosquitoes (*Anopheles gambia, Culex pipiens*).	Single a. a. substitution, same in both species. Correlation between substitution and resistance.	Functional assay needed; regulatory regions and linked genes not ruled out. Small sample of strains.	Weill et al. 2003
Cy6g1 (cytochrome P450 enzyme)	R	G (overtranscription)	Resistance to DDT in *Drosophila melanogaster*	Insertion of *Accord* transposon at 5′ end outside of gene, causes overtranscription of gene; Perfect correlation between transposons and resistance; genetic manipulation by overtranscription; transgenic constructs containing the TE in flies.	None	Daborn et al. 2002; Chung et al. 2007
LcαE7 (esterase)	S	G (amplification)	Resistance to organophosphate insecticides in sheep blowfly (*Lucilia cuprina*).	Single a.a. replacement; recombinant mutant enzyme has increased organophosphatehydrolysis.	Show change in biochemistry associated with resistance but do not higher resistance of genetically engineered flies.	Newcomb et al. 1997
E4 (esterase)	S	G (amplification)	Mutant protein sequesters insecticide in peach potato aphid *(Myzus persicae*).	Either truncated protein (FE4) or amplified protein (E4). Genes are regulated in different ways, too. Correlation between resistance and no. of genes, as well as known mechanism of sequestration of insecticide by enzyme.	No experiments to show whether regulation difference is adaptive, although duplicated genes have different regulation.	Field et al. 1988, 1998, 1999; Devonshire et al. 1998

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Appendix. Continued

Gene	Structural/ regulatory	Gain/ Loss†	Main result	Evidence	Missing evidence	Reference
CKKov1 (choline kinase)	S	G (novel protein)	Resistance to pesticide AZM in *D. melanogaster.*	Allele makes a truncated peptide by insertion of TE in coding region followed by 7 a.a. changes in remaining peptide. Correlation of genotype with pesticide resistance.	Functional assay needed; regulatory regions and linked genes not ruled out.	Aminetzach et al. 2005
VISUAL PIGMENTS						
SWS1 opsin	S	G (novel protein)	UV sensitivity in bird vison.	Change of single a.a. makes violet pigment into UV pigment; determined by absorption spectrum of purified pigment.	Regulatory regions not ruled out.	Yokoyama et al. 2000
RH1 and RH2 opsins	S	G (novel protein)	Blue sensitivity of coelocanth vision (*Latimeria chalumnae*).	Change of two a.a.'s in each of two pigments changes sensitivity in expected direction; determined by absorption spectrum of purified pigment.	Regulatory regions not ruled out.	Yokoyama et al. 1999
OTHER						
AFPs (antifreeze proteins) and AFGPs (antifreeze glycoproteins)	S	G (novel protein—duplication & a.a. subs).	Resistance of cytoplasm to freezing in various fish, insects, and plants.	Changes in duplicated genes (often involving repeated a.a.'s), confers resistance to ice crystals as shown in functional studies of purified proteins.	Regulatory regions not ruled out: no information about concordant changes in regulation of antifreeze genes.	Cheng 1998; Duman 2001; Fletcher et al. 2001
pancreatic RNAse RNAASE1B	S	G (duplication & a.a. subs)	Ability to recycle nitrogen in the small intestine by increased RNAase activity in the guereza monkey (*Colobus guereza*) and douc langur (*Pygathrix nemaeus*).	pHs examined of recombinant purified proteins: new proteins operated better at lower pHs (as in small intestine).	Regulatory regions not ruled out.	Zhang 2006
lens crystallin	S	G [co-opted (and often altered) proteins & enzymes]	Recruitment and change of enzymes and proteins in vertebrates and invertebrates (many of them products of duplicate genes) into eye helps focus light.	Proteins function as an intraocular matrix for focusing light.	Regulatory regions not ruled out.	Wistow et al. 1987; Tomarev and Piatigorsky 1996; Fernald 2004

†Change in protein expression or function associated with mutations causing gain of function (e.g., increased or altered expression or activity) or loss of function (e.g., decreased or absence of expression, reduced activity).

Defending Evo-Devo: A Response to Hoekstra and Coyne*

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The study of evolutionary developmental biology ("evo-devo") has recently experienced a dramatic surge in popularity among researchers and theorists concerned with evolution. However, some biologists and philosophers remain skeptical of the claims of evo-devo. This paper discusses and responds to the recent high profile criticisms of evo-devo presented by biologists Hopi E. Hoekstra and Jerry A. Coyne. I argue that their objections are unconvincing. Indeed, empirical research supports the main tenets of evo-devo, including the claim that morphological evolution is the result of *cis*-regulatory change and the distinction that evo-devo draws between morphological and physiological traits.

1. Introduction. The study of evolution was transformed between the 1920s and 1950s. During this time, Mendelian genetics and Darwinian natural selection underwent a theoretical integration via the mathematical theory of population genetics, resulting in a new framework for the study of evolution that includes the fields of paleontology, botany, systematics, and zoology (Mayr and Provine 1980; Laubichler 2007). This so-called Modern Synthesis resulted in a fruitful population-based research framework that has dominated evolutionary biology since the mid-twentieth century. Many biologists (e.g., Gould 1982; Eldredge 1985), philosophers (e.g., Ghiselin 1980; Jablonka 2006) and historians (see discussions in Amundson 2005; Laubichler 2007), however, have argued that the theory developed during the Modern Synthesis is incomplete and, so, does not allow for a complete understanding of macroevolutionary phenomena.

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Indeed, many biologists, philosophers, and historians agree that we could better understand how species evolve, specifically the origin of morphological novelties and other macroevolutionary phenomena, by adding the studies of embryology and developmental biology to the research framework provided by the Modern Synthesis (e.g., Amundson 2005; Burian 2005; Carroll 2005; Carroll, Grenier, and Weatherbee 2005; Müller 2005). This general consensus can be traced back to controversy surrounding one of the major theoretical tenets of the Modern Synthesis, the tenet of extrapolation. According to this tenet, macroevolutionary phenomena are completely explained by microevolutionary processes, so, phenomena such as the origin of novel morphological varieties are thought to be explained by population genetics alone (Gilbert, Opitz, and Raff 1996, 358). Extrapolation from micro– to macroevolutionary phenomena has been criticized since the Modern Synthesis when geneticist Richard Goldschmidt (1940) and developmental biologist Conrad. H. Waddington (1940) questioned how the origin of new body structures could be explained by the accumulation of changes in gene frequency without embryological and developmental insights (Gilbert et al. 1996, 362).

In the 1970s and early 1980s, the tenet of extrapolation was further criticized by paleontologists Niles Eldredge and Stephen Jay Gould (Eldredge and Gould 1972; Gould and Eldredge 1977), along with other biologists (e.g., Stanley 1979; Ayala 1983), who argued that changes in gene frequencies in a population could not provide a complete explanation of the evolution of higher taxa and the origin of novel morphologies. Empirical studies in molecular evolution also support the claim that microevolutionary processes alone cannot explain macroevolutionary events (e.g., King and Wilson 1975). Today, many evolutionary theorists agree that because of the emphasis placed on microevolutionary processes and population genetics and the absence of developmental insights, the theoretical framework for the study of evolution provided by the Modern Synthesis precludes complete explanations of speciation and morphological variation across higher taxa (e.g., Gilbert et al. 1996; Amundson 2005; Burian 2005; Carroll 2005; Carroll et al. 2005; Müller 2005; Pigliucci 2007). These authors also agree that a closer look at the relationship between organismal development, genetics, and molecular evolution is needed for a complete explanation of macroevolutionary phenomena.

For molecular biologist Sean Carroll and like-minded developmental biologists, a revolution that integrates developmental biology with the framework provided by the Modern Synthesis is currently underway in the field of evolutionary developmental biology ("evo-devo"). Carroll claims that recent advances in evo-devo research have led to a new understanding of the role of developmental networks in the origin and evolution of organismal form (2005, x). It had long been assumed that the

great variation in body structure observed across species was due to different developmental genes acting in different species. This, however, is not the case. Work carried out in the late twentieth century has revealed that despite incredible morphological differences, all complex animals, from *Drosophila* to dinosaurs, share a common set of genes that dictate the development of body patterning and form (e.g., Duboule and Dollé 1989; De Robertis 1997; Panganiban et al. 1997; Hersh and Carroll 2005). This research shows that the same developmental genes are responsible for incredibly different body forms and that differences in form are the result of differences in the regulation of these genes, not differences in the genes themselves (e.g., Britten and Davidson 1969, 1971; Davidson 2001; Valentine 2004; Carroll et al. 2005).

It is apparent to evo-devo proponents that the study of individual organismal development and differential regulation of developmental genes does indeed have clear implications for our understanding of speciation and the origin and evolution of form (e.g., Davidson 2001; Valentine 2004; Carroll et al. 2005). The investigation of the developmental processes of individual organisms, starting with gene expression in the earliest embryological stages, has revealed that macroevolutionary phenomena such as speciation are intimately linked to the evolution of regulatory networks and developmental processes. Evo-devo researchers claim their work illustrates that there is much more to learn about evolution and promises to provide substantial insights into the intricacies of the origin and evolution of form, in particular, by investigating further the relationship between development and the evolution of regulatory networks in higher taxa (e.g., Carroll 2005).

Some biologists and philosophers interested in understanding evolution are skeptical of the conclusions of evo-devo research, and the call for the addition of embryological and developmental studies to the Modern Synthesis has been met with resistance (e.g., Mayr and Provine 1980; Hoekstra and Coyne 2007). Those skeptical of the importance of current evo-devo research to the study of evolution not only argue that evo-devo is theoretically problematic but that the so-called developmental revolution lacks empirical support as well. Recently, in a high profile examination of evo-devo, evolutionary biologists Hopi E. Hoekstra and Jerry A. Coyne (2007) present a skeptical analysis and critique of the main tenets of evo-devo. While they do not deny the relevance of embryology and developmental biology to the study of evolution, they do conclude that many of the claims of evo-devo lack empirical evidence and are at best premature. Because of this, Hoekstra and Coyne advocate a resistance to the developmental revolution championed by Carroll. In what follows, I discuss the relevant main tenets of evo-devo and Hoekstra and Coyne's arguments against them. I argue that Hoekstra and Coyne are mistaken. Indeed, my

view is that empirical evidence from recent genetic and developmental studies shows that the criticisms offered by Hoekstra and Coyne are flawed and should fail to dissuade one from joining Carroll's developmental revolution.

To show that the criticisms of Hoekstra and Coyne can be successfully dismantled, I first discuss the evo-devo claim that *cis*-regulatory elements are the most likely sites of adaptive change leading to the evolution of form. I next discuss Hoekstra and Coyne's claim that evo-devoers have unfairly ignored available alternative explanations of the evolution of form and that other genetic changes, specifically gene duplication and subsequent functional divergence of paralogs, could be responsible for adaptive morphological divergence. I respond by using recent biological literature to show that researchers do acknowledge the role of gene duplication in the evolution of form but that such duplication does not play the causal role that Hoekstra and Coyne suggest. I next discuss the importance of the mode of post-duplication paralog divergence found in *Hox* genes for evo-devo's claim that form evolves differently than other physiological traits. Finally, I reconstruct Carroll's call for a developmental revolution in evolution studies. I conclude that a complete understanding of evolution must include an understanding of the evolution of form and that the time to embrace evo-devo is now.

2. Evo-Devo: Claims and Criticisms. One of the most revolutionary claims made by evo-devo researchers is that the evolution of animal form, from the spots on butterfly wings to the number of fingers on a human hand, is primarily the result of changes in *cis*-regulatory regions at the DNA level. These regulatory regions interact with transcription factors, proteins that bind to *cis*-regulatory elements and control when corresponding genes are turned on, present in the cell nuclei to assess the regulatory state of the cell. The expression of genes that code for transcription factors is dependent on the information gleaned from the interpretation of the cell's regulatory state. So, *cis*-regulatory regions, via interaction with the regulatory state of the cell, play an important causal role in the spatial and temporal expression of genes that code for transcription factors.

Evo-devo's claim that the evolution of form is the result of changes in *cis*-regulatory regions rests on the notion of modularity. One gene may be controlled by several regulatory elements, and regulatory elements may affect gene expression in one part of a developing organism without affecting expression in another region. Because of the multiplicity of *cis*-regulatory elements and the fact that different *cis*-regulatory elements can independently affect the expression of a gene at different times and locations, there is an enormous variety of regulatory outcomes. In other words, because of their modularity, *cis*-regulatory elements come in a

myriad of combinations that lead to different patterns of gene expression. Different patterns of expression, caused by different combinations of modular regulatory elements, lead to differences in organismal patterning and form (Stern 2000; Davidson 2001; Carroll 2005).

According to Hoekstra and Coyne, modularity leads to "the most important" consequence for evo-devo: change in a modular *cis*-regulatory element avoids the negative pleiotropic effects often caused by change in a protein-coding region (2007, 999). This is because change in a *cis*-regulatory element may only affect when or where a single gene is expressed. Thus, changes in *cis*-regulatory regions, according to evo-devo, are more likely to be adaptive than changes in a structural gene. This conclusion leads to another, what Hoekstra and Coyne refer to as the "theoretical imperative for *cis*-regulation"—due to their modularity, *cis*-regulatory elements are the primary unit of evolutionary change responsible for variation in animal form (2007, 1000).

Hoekstra and Coyne are critical of these conclusions of evo-devo theory. They state that "there are several other ways to obviate the negative consequences of pleiotropy besides changing *cis*-regulatory elements. The most obvious is gene duplication followed by divergence of the duplicated copies" (2007, 1000). Their claim is that gene duplication is a more probable genetic mechanism for the evolution of form than *cis*-regulatory change. They site Susumu Ohno's (1970) book in order to argue that gene duplication is in fact quite pervasive and an important mechanism for the evolution of novel genes. In addition, Hoekstra and Coyne claim that Carroll, one of evo-devo's most prominent advocates, unfairly dismisses the available alternative genetic mechanism of gene duplication as a means for avoiding negative pleiotropic effects by claiming that such duplications are too rare to significantly contribute to evolutionary change. In general, Hoekstra and Coyne accuse Carroll of faulty logic, of excluding viable alternatives in the name of *cis*-regulatory change.

Hoekstra and Coyne's criticism of the *cis*-regulatory change hypothesis of evo-devo is unpersuasive, however. Recent biological studies make it clear that this criticism of evo-devo theory, that gene duplication and divergence is overlooked as an alternative to changes in *cis*-regulatory regions, is unconvincing. Indeed, researchers interested in the evolution of *Hox* genes, genes thought to play a significant role in the evolution of form and are shared by all complex animals, seem to agree that gene duplication has played an important role in the evolution of *Hox* genes. Evidence suggests that duplication events led to the four *Hox* clusters found in jawed vertebrates and that a separate duplication event took place approximately 400 million years ago after the divergence of ray-finned and lobe-finned fishes (McClintock et al. 2001). While biologists are still trying to pinpoint exactly when such duplication events took place,

there is a consensus amongst *Hox* researchers that multiple duplication events played an important causal role in the evolution of *Hox* genes (e.g., Crow et al. 2006; Lemons and McGinnis 2006; Lynch, Roth, and Wagner 2006). The biological literature shows that gene duplication and subsequent functional divergence have not been overlooked by those studying the evolution of form. Instead, this research demonstrates that gene duplication has been embraced as part of the evolutionary history of genes that code for particular transcription factors.

In fact, it is the mode of post-duplication divergence that has important consequences for evo-devo, not the claim that gene duplication plays a role in the evolution of form. In further support of the claim that *cis*-regulatory changes are primarily responsible for morphological variation, the biological literature suggests that changes in regulatory elements post-duplication are selected for, leading to functional divergence in duplicated genes (e.g., Chiu et al. 2001; Fares et al. 2003; Hersh and Carroll 2005; Wagner et al. 2005). Interestingly, Hoekstra and Coyne do not consider the results of this research, which contradict their claim that "evo devo's enthusiasm for *cis*-regulatory changes is unfounded and premature" (2007, 996). The biological literature provides an abundance of support for the claim that novel expression patterns of *Hox* genes are present post-duplication as a result of regulatory change and that these regulatory changes are the result of natural selection (e.g., McClintock et al. 2001; Hersh and Carroll 2005; Crow et al. 2006). Thus, evo-devo's emphasis on *cis*-regulatory change is not unfounded or premature and is empirically supported, contra Hoekstra and Coyne. In other words, research results support evo-devo's claim that adaptive *cis*-regulatory change does play an important causal role in the evolution of form.

Hoekstra and Coyne further criticize the thesis that *cis*-regulatory change is responsible for adaptive changes in morphology. They argue many studies that claim *cis*-regulatory change is the predominant means for adaptive morphological change arrive at this conclusion without reference to the resulting phenotype. Without phenotypic information, there is no way of knowing whether a genetic change of any type is truly adaptive. However, Hersh and Carroll (2005) avoid this objection. A wing-specific regulatory element was identified for the *knot* gene, which is expressed during the development of *Drosophila* wings but not during the development of the haltere, small, club-shaped balancing organs located behind the wings. Their study reports that a minimal regulatory element in the haltere is not evolutionarily conserved but a second element is conserved. Surprisingly, this second regulatory element is located more than 500 bp from the minimal region. This finding, in addition to evidence of natural selection on *knot* regulatory elements, leads to Hersh and Carroll's conclusion that "selection is acting on a larger region than the

minimally defined regulatory module" (2005, 1568). Here, we have empirical evidence of selection acting on *cis*-regulatory regions that are associated with a particular morphological phenotype, the *Drosophila* wing. Hence, Hersh and Carroll's work is evidence of *adaptive* regulatory change in reference to a particular phenotype, and Hoekstra and Coyne's objection does not apply to the results of Hersh and Carroll's study.

Of great importance for evo-devo theory is the sharp distinction between morphological and all other traits, which are classified together as "physiological" traits. Evo-devoers claim that morphological traits are different from physiological traits because they evolve differently—morphological traits evolve via regulatory changes while physiological traits evolve via structural or coding-region changes. Hoekstra and Coyne argue that this distinction is not adequately justified by advocates of evo-devo because there is no good reason to believe that the genetic changes responsible for the evolution of form are different from genetic changes responsible for physiological traits. In fact, they claim, "the omission of 'physiological' traits from [evo-devo] theory fails to acknowledge the tremendous amount of already-existing data showing that the adaptive evolution of such traits usually involves changes in structural regions" (2007, 997).

But there is a likely biological justification for the distinction between morphological and physiological traits. Researchers agree that gene duplication has led to multiple *Hox* clusters in complex animals, and evidence suggests that the mode of post-duplication functional divergence of paralogous *Hox* genes is unlike other mechanisms of divergence (e.g., Chiu et al. 2001; Wagner et al. 2005; Lynch et al. 2006). For example, Crow et al. (2006) claim that evidence for strong directional selection on both paralogs post-duplication is inconsistent with traditional models of functional divergence, such as the neofunctionalization and subfunctionalization models. Such results suggest that the mode of post-duplication divergence of *Hox* genes is novel and distinct from known divergence mechanisms. Similarly, Lynch et al. (2006) state that the mechanism for duplicated *Hox* gene divergence does not follow known mechanisms and remains unknown.

The biological literature suggests that after a duplication event, *Hox* genes may diverge in function via a unique mechanism that distinguishes *Hox* genes from other duplicated genes. This unique mechanism may involve a window of increased "evolvability" immediately following a duplication event, which is not seen in other genes (Wagner et al. 2005; Crow et al. 2006). This is evidence that suggests that the genes involved in the evolution of form do indeed diverge, and thus, evolve, differently than genes responsible for physiological traits. This means that the distinction between morphological traits and physiological traits is biolog-

ically justified. The possibility that the distinction may be justified by further exploration of the mechanism of *Hox* gene divergence warrants continued research.

3. Conclusion. Our understanding of how species change over time has dramatically progressed since the 1859 publication of Darwin's *On the Origin of Species*. In the past 150 years, biologists have made advances in the study of evolution from a multitude of different research perspectives. From genetics to animal behavior, from proteomics to psychology, evolutionary questions and explanations abound. However, we still know relatively little about how the developmental processes and morphologies characteristic of different species diverged over time. The recent developmental research discussed above clearly contributes to our understanding of the evolution of developmental genes and consequently the evolution of morphological traits in complex organisms. There is considerable evidence for evo-devo's claim that regulatory changes play an important role in the evolution of form. There is also good evidence that *Hox* genes evolve differently than other genes since they do not follow any of the traditional models of post-duplication divergence. This novelty, along with the role of regulatory change in the evolution of form, represents biological justification of the claim that morphological traits truly evolve differently than physiological traits. Nonetheless, further evo-devo research is needed for a more detailed understanding of how *Hox* genes and other developmental genes evolve.

As evo-devo research continues, it promises to answer many more of our questions about the evolution of form. In particular, embryological and developmental research promises to further explain how *Hox* genes causally contribute to the development of an impressive array of different organismal forms. This paradox of morphology, the question of how a family of similar genes can contribute to the development of drastically different forms, represents one of many gaps in our understanding of evolution. As Carroll correctly argues, the results coming out of evo-devo research programs provide hope that such developmental puzzles can and will be solved by paying more attention to developmental processes. Studying these processes and how they have changed over time will further our understanding of evolution in general.

The debate surrounding the importance of evo-devo and the integration of developmental studies with the theoretical framework provided by the Modern Synthesis threatens to stagnate progress in evolution studies. The threat of stagnation is real and exists because the synthetic theory of evolution developed during the Modern Synthesis is indeed unable to provide an adequate framework for the study of macroevolution. That this is true is made obvious by what we do not know about evolution.

Our understanding of the evolution of form is drastically limited, which suggests that evo-devo should be integrated with the framework of the Modern Synthesis in order to increase our understanding of how organisms evolved to have such remarkably different shapes and structures.

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The Problem with Darwinian Solutions:

Sean Carroll and Michael Ruse argue that "Evo Devo" Undermines Intelligent Design. ID Advocate William Dembski Begs to Differ.

William A. Dembski

Science & Theology News
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Original Article

Despite its early potential, evolutionary developmental biology — evo devo for short — has yet to make good on its promise.

In his review of *Endless Forms Most Beautiful* Sean Carroll's new book on evo devo, Michael Ruse faults intelligent design (ID) for harping on evolution's unsolved problems. Moreover, Carroll as well as Ruse suggest that evo devo has now resolved one of the major problems on which design theorists have been harping.

Wrong on both counts. Intelligent design does not have a problem with problems. It has a problem with bogus solutions that Darwinists like Ruse and Carroll dress up as real solutions to the problems of biological origins.

Evo devo is a case in point. This term, coined in the mid 1990s, attempts to merge two sub-disciplines of biology: evolutionary biology, which studies the mechanisms by which populations of organisms change over generations, and developmental biology, which studies the mechanisms by which individual organisms grow from conception to mature form.

Evo devo takes as its starting point that genetic mechanisms are the key to both evolutionary and developmental biology. The merger of evolutionary and developmental biology, therefore, looks to key genes that influence development and could in principle also influence changes in development and, thereby, lead to macroevolutionary change.

What if, for instance, a gene that controls development could somehow induce a change early in development? Even a small change early in development might have huge consequences for the organism's anatomy and physiology. Think of an arrow aimed accurately at a target. Left to fly unperturbed, the arrow will land in the target's bull's-eye. Yet the earlier in flight that the arrow is diverted from its trajectory, the wider it will be off the mark when it lands.

The promise of evo devo is that genetically induced changes early in development, though small and easily attainable in themselves, might nonetheless lead to macroevolutionary changes.

In other words, just as the arrow diverted early from its course will land wide of the mark, so development diverted early from its course might lead to significant evolutionary change. In this way evo devo seeks to do an end-run around the more traditional neo-Darwinian approach to macroevolution, with its steady accumulation of microevolutionary changes leading to macroevolution. Evo devo, by contrast, promises rapid evolutionary change at a small cost, namely, the cost of mutating a few key genes that control early development.

AMERICAN Scientist

BOOK REVIEW

Clever Tinkering

Lewis Wolpert

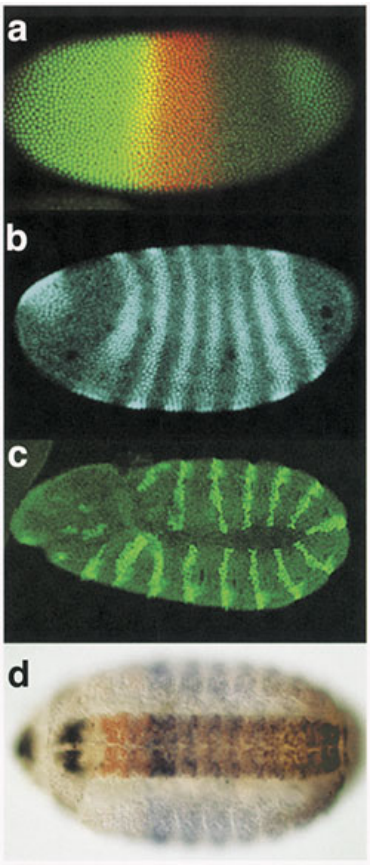
Endless Forms Most Beautiful: The New Science of Evo Devo and the Making of the Animal Kingdom. Sean B. Carroll. xiv + 350 pp. W. W. Norton, 2005. $25.95.

All plants and animals, including humans, are essentially societies of cells that vary in configuration and complexity. As Darwin's theory made clear, these multitudinous forms developed as a result of small changes in offspring and natural selection of those that were better adapted to their environment. Such variation is brought about by alterations in genes that control how cells in the developing embryo behave. Thus one cannot understand evolution without understanding its fundamental relation to development of the embryo. Yet "evo devo," as evolutionary developmental biology is affectionately called, is a relatively new and growing field.

Sean B. Carroll, as a leading expert both in how animals develop and in how they have evolved, is ideally placed to explain evo devo. His new book on the subject, *Endless Forms Most Beautiful: The New Science of Evo Devo and the Making of the Animal Kingdom* (the title borrows a phrase from Darwin's *On the Origin of Species)*, was written, he says, with several types of readers in mind—anyone interested in natural history, those in the physical sciences who are interested in the origins of complexity, students and educators (of course), and anyone who has wondered "Where did I come from?" Carroll has brilliantly achieved what he set out to do.

One of the most striking discoveries of the last half-century has been that, despite the fact that animals differ greatly in appearance, common principles control their development from a single fertilized egg. They even have in common many master genes—genes that control many aspects of development. One can almost imagine *Drosophila* fruit flies saying to one another that they are amazed at how similar humans are to them. Indeed, many of the genes that have been identified as controllers of vertebrate development were originally discovered in these flies.

It's a key point that when and where genes are expressed determines how animals develop. The control regions of the genes (switches that change an existing pattern of gene activity into a new pattern of gene activity) are crucial, as Carroll makes clear, and one gene can have many control regions. (For example, in the fruit fly, there is a group of genes—known as the pair-rule genes—that express proteins in seven stripes along the body axis of the embryo [see illustration on next page]; each of these genes has seven discrete control regions, and each region specifies one stripe.) It is thus unsurprising that 95 percent of the genes that code for proteins are similar in humans and mice. Evolution of control regions has made us human—and different from our primate ancestors.



Carroll explains the basic tool kit for development that all animals share, placing particular emphasis on *Drosophila.* He introduces both *Hox* genes, which are considered master genes, and widely used intercellular signaling molecules such as the proteins specified by *hedgehog* genes. It is striking how few signaling molecules animals use in development. This is because the same molecules can be employed again and again, as cells will respond differently according to their genetic constitution and developmental history.

Carroll doesn't give much attention to the fact that a cell has a positional identity (based on the position it occupied on the axis of the body of the early embryo) or to how that positional identity is acquired. Nor does he delve into how a cell senses its position and figures out how to act according to its genetic constitution and developmental history, thereby differentiating to give any imaginable pattern. Consider that a change in a single *Hox* master gene can convert the antenna of the fly into a leg. There is evidence that cells in the leg and those in the antenna have the same positional identity. It is somewhat embarrassing that we still do not know how the change in that particular *Hox* gene controls the response of all those unknown downstream genes to make a leg rather than an antenna. And this downstream target problem is present for all *Hox* genes.

Carroll emphasizes that individual animals are made up of similar parts, such as vertebrae, bones in fingers and spots on butterfly wings, and that modular construction played an important role in evolution. He is a supporter of Williston's law, which states that "in evolution . . . the parts in an organism tend toward reduction in number, with the fewer parts greatly specialized in function." I must confess to finding the idea of modules not that easy to appreciate. Is, for example, the leg/antenna basic structure a module?

The earliest complex animals, fossils of which were found in the Burgess Shale, appear to have arisen about 500 million years ago, over a period of some 15 million years. Evidence from evo devo shows that all the genes for building those complex animals existed long before that morphological explosion. The dominance of arthropods at the time of the explosion may have been due both to Williston's law and to the power of *Hox* genes to specify differences between the body segments that formed different appendages at specific positions along the body. But how, asks Carroll, did the number of distinct appendage types increase? His answer is that the relative shifting of *Hox* genes could have provided the mechanism. That still leaves a big problem—how did arthropod appendages such as limbs and wings evolve? An answer lies, he says, in the origin and modification of the ancestral biramous (forked) limb. But even if the origin of the limb can be explained, wings are even more difficult. One answer is that they evolved from the gills on the limbs of aquatic ancestors.

But this conclusion raises a key, and much neglected, problem that even Carroll does not properly explore. If evolution proceeds in small steps, what were the intermediate stages in the evolution of wings from gills, and what was the selective advantage of each of those forms? How could the intermediate structures have been an advantage before the animal could fly? One possibility is that they played a role in thermoregulation, but there is no good evidence for that hypothesis. This is a general problem in evo devo, and Darwin fully understood the difficulties it poses.

A related problem is how to explain the evolution of the autopod—the digits—from fins. One possibility is that

the autopod is merely a distal extension of the mechanism that gives rise to more proximal elements, such as the humerus, radius and ulna. A much more difficult problem is raised in the evolution of development itself. Gastrulation (during which an embryo forms its innermost, middle and outer layers) occurs in the early development of all animals and has evolved in a variety of ways related to later development; it is at present not possible to account for the intermediate forms or their advantage to the animal. Although evolution, as François Jacob pointed out, tinkers with what is there, rather than inventing something new, these problems remain unsolved.

A nice example of what could be considered clever tinkering is butterfly spots. Each spot appears to evolve its shape, color and size independently of other elements. Evolution has tinkered not only with the qualities of each spot, but with the making of the spot itself. Carroll's group discovered that at the center of each spot, the gene *Distal-less* (a key gene controlling the distal development of appendages such as insect limbs) is expressed and initiates spot development.

Even the evolution of humans can be thought of as tinkering with the genes of our primate ancestors. But this view is totally unacceptable to religious creationists. Carroll criticizes their views and emphasizes how important it is for evo devo to be taught in schools.

Evo devo is fundamental to understanding the biological world we live in, including ourselves. This is a beautiful and very important book.