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Time's arrow: heterochrony and the evolution of development

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ABSTRACT The concept of heterochrony, which denotes a change in the relative timing of developmental events and processes in evolution, has accompanied attempts to link evolution and development for well over a century. During this time the definition of heterochrony and the application of the concept have varied and by the late 1990's, many questioned the usefulness of the concept. However, in the past decade studies of heterochrony have been revitalized by a new focus on developmental sequence, an examination of heterochrony in explicit phylogenetic contexts and increasing tendencies to examine the heterochrony of many kinds of events, including cellular, molecular and genetic events. Examples of such studies are reviewed in this paper and it is argued that this new application of heterochrony provides an extraordinarily rich opportunity for understanding the developmental basis of evolutionary change.

KEY WORDS: *heterochrony, evolution, development*

Introduction

During development a series of events take place in a precisely regulated spatial and temporal context. For the most part there is a clear directionality to these events, so that most events occur at specific points in a stereotyped sequence of events. Commonly later events are contingent on the proper completion of certain prior events. At least in animals, there is rarely significant reversibility of the process (with a few exceptions such as regeneration and some processes that occur during metamorphosis). In multicellular organisms, development proceeds from large scale patterning of the whole organism to events that are increasingly smaller in scale, and more modular and localized as individual parts differentiate and become more specialized. The study of the mechanisms by which these intricate processes are controlled in space and time forms the field of developmental biology.

The burgeoning field of evolutionary developmental biology examines how modifications of this process lead to the evolutionary diversity present in nature. Since the first attempts to explicitly link development and evolution it has been recognized that changes in timing of various developmental processes – heterochrony – may account for many of the evolutionary changes we observe. Heterochrony in its simplest is a change in the time of onset or end, or the rate of a developmental process. The term heterochrony refers to changes in the relative time of developmental processes between ancestors and descendents, but in practice heterochrony is applied in a comparative sense to changes among taxa that are related at some level.

The concept of heterochrony has accompanied attempts to link evolution and development for well over a century, although through this period the specific application of the term has varied. The term was first defined by Haeckel to describe exceptions to his Biogenetic Law. As is well known, the now discredited Biogenetic Law asserts that ontogeny is the recapitulation of phylogeny. More specifically, Haeckel states that "The organic individual …. repeats during the rapid and short course of its individual development the most important of the form changes which its ancestors traversed during the long and slow course of their paleontological evolution according to the laws of heredity and adaptation" (as quoted from Haeckel in Russell, 1916, p. 253). Haeckel recognized two major types of departure from strict recapitulation. The first was heterotopy, in which an organ develops in a position or germ layer other than that in which it originally arose in phylogeny. The second, heterochrony, consists of processes that "arise through the dislocation of the proper phylogenetic order of succession… Heterochrony shows itself as a rule either as an acceleration or retardation of developmental events, as compared with their relative time of occurrence during phylogeny." (Russell, 1916, p. 259). Heterochrony thus originally denoted a shift where an element appeared at a different time (sequence) in an organism's development relative to the sequence of appearance in that organism's phylogeny. It was not, therefore a comparison of different ontogenies (see Richardson and Keuck, 2002 for an excellent review of Haeckel's work and its modern relevance).

In the middle part of the 20th century, de Beer (1930, 1940, 1951, 1958) wrote a series of books that aimed to synthesize

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developmental biology, evolutionary biology and genetics. One major focus of these books was the attempt to finally refute the concept of recapitulation (Ridley, 1985). De Beer discussed the concept of heterochrony in great detail and demonstrated how changes in the relative timing of events and rates of processes could generate differences among organisms. Most importantly he argued that heterochronic processes had little necessary relation to recapitulation. Additionally, whereas Haeckel used the concept of heterochrony to describe cases when the ontogenetic and phylogenetic sequence differed, de Beer used the concept to describe differences in the ontogenies of related taxa (Gould, 1977). This comparative definition is the dominant one in use today.

Although de Beer's work is remembered as an important early attempt to link evolution and development, it was the work of Gould (1977) that brought the concept of heterochrony to the general attention of evolutionary biology. Gould's work, which was extended by Alberch *et al.*, (1979) defined the scope of heterochrony for the next two decades. The view of heterochrony presented by these authors, and generally adopted by evolutionary biologists, was characterized by several distinctive features (Smith 2001b).

First, Gould again links heterochronic change to recapitulatory patterns, and only considered a shift in the timing of an event to be heterochrony if it produced a parallel between ontogeny and phylogeny. He limits heterochrony to two cases: 1) recapitulation, when the sequence of events in ontogeny is directly parallel to the sequence of characters in phylogeny, and 2) reverse recapitulation, where the ontogenetic sequence is the reverse of phylogeny. Gould excludes cases of timing shifts that do not produce parallels between ontogeny and phylogeny from his definition of heterochrony.

The second major change in the concept of heterochrony arising from Gould's treatment concerns the kinds of timing shifts considered. Haeckel defined heterochrony largely in terms of the sequence of developmental events. De Beer likewise discussed the order of structural changes or events. Gould however, focused almost entirely on rates of relative growth. He defines allometric growth not as growth but as differentiation, and goes on to treat heterochrony as the dissociation between (non-allometric) growth, "differentiation" (= allometric growth) and sexual maturation.

Gould therefore shifted the focus on heterochrony from the relative timing of developmental events to changes in the relation between size and shape. The almost exclusive focus on size and shape changes as the important heterochronic phenomenon was a significant redirection of the concept by Gould. During the explosion of attention to heterochrony in the 1980's and early 1990's this view was almost universally accepted. As a result, the concept of heterochrony became virtually synonymous with allometry. (For recent reviews see McKinney, 1988, 1999; Raff & Wray, 1989; Hall, 1992, 1999; McNamara, 1995, 1997; Raff, 1996; Zelditch & Fink, 1996; Reilly *et al.*, 1997; Klingenberg, 1998; Li and Johnston, 2000; Smith, 2001b, 2002; Zelditch 2001).

By the late 1990's, although many people continued to use the approach as defined by Gould, others began to question this approach. At the most basic level the fact that the concept of heterochrony was used at times in a non-specific manner received increasing criticism. In some cases a change in the relative proportion of any structure in two related organism was attributed to "evolution via heterochrony". While technically true (a change in relative proportions did require a change in rate, onset or offset of some process), such applications of the concept were sufficiently imprecise so that little was explained. Further, the concept of heterochrony acquired tremendous terminological complexity and discussion at times focused on defining the type of heterochrony rather than on establishing the specific processes that were modified.

More specific criticisms also arose. Because focus was on size and shape, size was often used as a substitute for time so that in many cases, the study of "heterochrony" was not a comparison of shifts in relative timing of developmental events but in growth curves. In some cases size is an appropriate surrogate for age, but in others this substitution is problematic because size, rate of development, and shape may evolve independently (e.g., Snow *et al.*, 1981; Roth, 1984; Emerson, 1986; Blackstone, 1987a&b; Klingenberg & Spence, 1993; Godfrey & Sutherland, 1995a&b; Klingenberg, 1998). In addition, the emphasis on size and shape limited the focus to global (whole body) events and relatively late processes (Raff & Wray 1989; Hall, 1992, 1999). Although many changes between closely related species arise through patterns of relative growth, some of the most critical events in development such as the appearance of segmental and regional identity, patterns of regulatory gene expression, induction and signaling cascades, cell and tissue specification and differentiation, and the differentiation of organs and organ systems cannot be studied by these means. Changes in the relative timing of such events are likely to be critical in producing evolutionary change (e.g., Hall, 1984; Langille & Hall, 1989; Wray & McClay, 1989; Swalla and Jeffery, 1990; Jeffery & Swalla, 1992; Collazo, 1994; Evans *et al.*, 1994; Swalla, *et al.*, 1994; Kai, *et al.*, 1995; Richardson, 1995; Smith, M., 1995; Wray, 1995; Cubbage & Mabee, 1996; Hanken *et al.*, 1997; Slack & Ruvkun, 1997; Velhagen, 1997; Hodin and Riddiford, 1998; Félix *et al.*, 1999; Irvine *et al.*, 1999; Cubo 2000; Mabee *et al.*, 2000; Kanki and Wakahara, 2001; Macdonald and Hall, 2001; Smith, K., 2001a,c; Forbis *et al.*, 2002; Vaglia and Smith, 2003).

In recent years the concept of heterochrony has been revitalized by two different trends. First, more and more the concept of heterochrony has been applied to shifts in the relative timing of developmental events rather than relative growth. In addition, the focus is less on identifying the types of heterochrony exhibited, but instead, on isolating the specific elements, and in some cases the underlying developmental mechanisms that have produced the observed changes. Further, a number of new analytical tools have provided means to study many events in many taxa, and to test hypotheses in an explicit phylogenetic context.

Finally, the kinds of processes being examined increasingly include study of shifts in the timing at molecular and genetic levels. These studies are particularly exciting as they have the potential of producing a fusion between modern studies of developmental biology and classic problems in evolutionary biology. Rather than simply approaching changes in size and shape these new studies of heterochrony are examining the basis for change in a variety of mechanisms and kinds of phenotypic change. The phenomena studied include patterning mechanisms, shifts in life history phases, the timing of appearance of various organs and structures, and overall morphological changes, while the processes include shifts in critical periods, inductive events, and relative timing of gene expression.

Sequence heterochrony

As workers began to focus on changes in the relative timing of developmental events, it was recognized that there was need for new analytical techniques, particularly in cases when a broad range of taxa were examined. One approach, taken by a number of workers was to focus on heterochronies in the sequence of developmental events (e.g., Wake & Hanken, 1982; Hanken & Hall, 1984; O'Grady, 1985; Irish, 1989; Strauss, 1990; Hufford, 1995; Richardson, 1995; Velhagen, 1995, 1997; Dunlap & Sanchiz, 1996; Mabee & Trendler, 1996; Smith, 1996, 1997, 2001c; Larsson, 1998; Nunn and Smith, 1998; Chipman *et al.*, 2000; King *et al.*, 2001; Schlosser, 2001, 2003; Sánchez-Villagra, 2002).

A focus on developmental sequence has a number of significant advantages. First, a sequence provides a ready means to compare the timing of events across taxa. A number of criteria have been proposed as a means to standardize development, including size, discrete landmarks, developmental stages, or chronological age. All of these criteria present significant theoretical and practical difficulties as measures for interspecific comparisons (e.g., Roth, 1984; Blackstone, 1987a&b; Raff & Wray, 1989; Reiss, 1989; Hall & Miyake, 1995, and references therein). A sequence, however, is independent of any variation in rate of development, whether the rate differences are intraspecific responses to environmental variation (e.g., temperature), or inter-specific adaptations. Further, it provides for a means of comparison at any stage of development – including early developmental phases when there may be little change in size for significant periods.

Focus on events means that any part of the developmental trajectory and changes in the timing of any kind of process or event can be included in a single study. Examples of the kinds of events that can be analyzed include the onset of expression of specific genes at specific sites, the differentiation of specific tissue types, the establishment of specific connections or interactions, the appearance of distinct morphological elements, numerical or quantitative landmarks, or the attainment of specific stages of morphological differentiation. Furthermore, multiple kinds of events may be incorporated and integrated in the single analysis.

A number of different kinds of approaches to analyze sequence heterochrony have appeared in recent years. For example, Larsson (1998) compares the relation between the sequence of character evolution in phylogeny with the sequence of appearance in ontogeny in crocodilians. He first uses fossil specimens to derive the phylogenetic sequence of the appearance of taxon-specific characters. He compares this temporal sequence with the sequence in which the same characters appear during the ontogeny of modern crocodiles by using bivariate plots and Spearman rank coefficients (see Larsson, 1998 for details). If the sequences in ontogeny and phylogeny are conserved, the ranks of the specific characters in the two sequences will be highly correlated. Larsson argues that characters that are highly correlated in development and evolution may be particularly integrated either functionally or developmentally and that this method provides a means to test hypotheses of developmental integration and dissociation.

A different approach to the question of integration and dissociation of characters was presented by Schlosser (2001) in a study of heterochronies in the direct-developing frog, *Eleutherodactylus*. Schlosser develops graphical methods with which the relative timing (sequence) of a series of events in two taxa can be compared. The sequence of events of one species is plotted on the x-axis, and the other on the y-axis. If the sequence is conserved, then the relative timing in the two events will appear as a straight line, with a slope of 45 degrees. Departures from this slope or movement of single events away from this line will indicate either overall rate change or heterochrony in specific events, respectively. He uses this technique to compare the developmental sequence of a wide variety of events in *Eleutherodactylus* and *Discoglossus*, a frog which undergoes a normal larval stage and metamorphosis. This analysis reveals a number of character complexes that exhibit particular heterochronies in response to this life history shift. Schlosser goes on to perform similar comparisons with a variety of levels of outgroups to determine the evolutionary polarity of these shifts. Other kinds of graphical depictions of sequence analysis were presented, for example by Alberch *et al.* (1979), Richardson (1995), and Hanken and Hall (1984).

A second kind of approach compares developmental sequences in a broader phylogenetic framework, and was independently developed by Mabee (Mabee & Trendler, 1996), Smith (Smith, 1996, 1997) and Velhagen (1995, 1997). The technique converts sequence data into characters, which are assigned various character states that represent changes in the relative timing of the two events. To convert relative timing data to characters, a series of "event-pairs" are constructed, in which the timing of every character in the sequence is compared with every other character in the sequence (See Smith, 1997 for more detail on the method). The relative timing of two events (e.g., A and B) in the pair is expressed as one of three character states: 1) A and B occur at the same time, 2) A occurs before B, or 3) A occurs after B. Each of these states is given a character state value, which may then be plotted on independently derived phylogenies to examine phylogenetic patterns of change in developmental timing.

Both of the above approaches to sequence analysis are cumbersome when many characters in many taxa are analyzed. The graphical approaches of Larsson and Schlosser become difficult when many different taxa are included, while the event-pair approaches are exceedingly cumbersome when many different events are used. Richardson, Jeffery and Bininda-Emonds (e.g., Bininda-Emonds *et al.*, 2002; Jeffery *et al.*, 2002) have developed methods that aim to streamline and semi-automate the event-pair approach. They term their technique "event-pair cracking" and use it to study patterns of sequence evolution across vertebrates. For example, Bininda-Emonds *et al.* (2002) use this approach to test, and they argue refute, the hypothesis that a conserved phylotypic stage exists in vertebrate development (e.g., Slack *et al.*, 1993; Duboule, 1994; Richardson *et al.*, 1997). The above papers mapped event-pairs on previously reconstructed phylogenetic trees. More recently Koenemann and Schram (2002) have examined the utility of such techniques in phylogenetic analysis and show that developmental sequence data contain a phylogenetic signal, and under some conditions can be useful in phylogeny reconstruction.

A final way to approach sequence analysis has been quantitative. Several authors (Mabee and Trendler, 1996; Strauss, 1990; Nunn and Smith, 1998) have used various rank coefficients to express the degree of conservation of an entire sequence. In addition, Nunn and Smith (1998) used ANOVA to compare changes in sequence position in a number of events in two major clades, each of which contains multiple taxa. This approach is a quantitative way to identify the specific events whose place in a developmental sequence is shifted

and is most useful when many events are compared in multiple taxa that are grouped into two or more major clades. It could also be applied to test differences in sets of individuals with any kind of group structure, such as different experimental treatments, litters, and so forth.

The analysis of sequence heterochrony has proved useful for a large number of different kinds of questions. However, it is important to emphasize that except for cases in which a clear causal relation exists among the events in the sequence (i.e., a set of events that are linked by a single set of developmental processes) the developmental sequence is usually just an analytical artifact (Alberch, 1985). This is particularly true when events from a wide variety of organ systems are examined. The relative order of events in a developmental sequence reflects changes in the rate, onset or offset of processes governing each of those individual events and the underlying processes of different events may have no relation to each other. There is, for example, no single mechanistic relationship governing the ossification sequence of cranial bones discussed by Clark and Smith (1993; Smith, 1996, 1997). The ossification of basicranial elements is mechanistically related to their cartilaginous precursors; ossification of other bones is related to processes tied with central nervous system maturation, and still others ossify in relation to processes occurring in the oral and facial region (Smith, 1996).

Nonetheless, sequence analysis, and the patterns of sequence heterochrony that can be identified, has provided an extremely useful tool to provide potential evidence for a wide variety of hypotheses. For example, sequence analysis may help identify which developmental events exhibit little change in relative timing across a wide range of taxa. Once such sets are identified, it is plausible to propose that the events may represent a developmental module and further studies may reveal mechanistic relations. Alternatively, sequence analysis may determine which parts of development appear to distinguish different taxa, which may point to important evolutionary and developmental changes in the divergence of the lineage. Finally, sequence analysis may help test hypotheses on major evolutionary patterns such as the existence of developmental constraints, or conserved phylotypic stages.

Cellular, molecular and genetic heterochrony

With an increasing focus on the relative timing of developmental events, there has been an expansion in the kinds of developmental phenomena addressed by studies of heterochrony. An important recent trend has been the growing number of studies that look at heterochrony at genetic, molecular and cellular levels.

Over the past twenty years or so, it has been shown that many of the important differences among organisms are not due to the presence or absence of specific genes. For the most part, the repertoire of genes available across the Metazoa and the specific sequences of the functional region of many genes are highly conserved. Examples of initially surprising conservation include, for example, the *Hox* genes, *Pax 6,* and genes such as those found in the *distalless* complex. All of these genes are present in highly conserved form across many organisms. Instead, much of the phenotypic diversity we see is due to changes in the regulation of expression in time and space of these highly conserved genes. Changes in the timing of onset or offset or rate of expression, as well as changes in the spatial pattern of expression are critical on producing phenotypic change (e.g., Patel, 1994; Raff, 1996; Lowe and Wray, 1997; Ghazi and VijayRaghaven, 2000; Tautz, 2000; Wray and Lowe, 2000; Carroll *et al.*, 2001; Davidson, 2001; Davis and Patel, 2002; Mathis and Nicolas, 2002; Salazar-Ciudad and Jernvall, 2002 and references therein). An increasing number of papers have shown how shifts in the relative timing of onset or offset of particular genes – genetic heterochrony – may produce significant phenotypic change. This linking of molecular genetics with classical questions of how heterochrony produces phenotypic change is now providing a clear link between microevolutionary processes and macroevolutionary changes.

It is particularly interesting that seemingly subtle shifts in the timing of various processes can have many kinds of different and significant effects. For example, shifts in timing of gene expression can produce gross morphological changes such as limb elongation, differentiation of additional serial elements, or shifts in identity of elements. Shifts in timing of the appearance of a regulatory factor, or its receptor can produce switches among alternative developmental pathways and lead to dramatically different phenotypes. Timing shifts can lead to changes in patterning of specific elements, such as sensory bristles or color pattern. Finally, shifts in timing can produce accelerated maturation or delayed maturation of individual elements or of the entire organism, which may have significant functional and life history implications. Below I summarize several recent studies that demonstrate cases in which seemingly slight heterochronies in the expression of a given gene clearly correlate with important phenotypic changes.

The vertebrate limb: heterochrony and morphological change

One of the best studied model systems for evolution and development is the vertebrate limb (e.g., Shubin *et al.*, 1997; Capdevila and Belmonte, 2000; Tamura, *et al.*, 2001; Duboule, 2002; Hinchliffe, 2002; Niswander, 2002; Tickle, 2002 and references therein). Several recent studies have demonstrated ways that specific heterochronies in gene expression pattern may produce the kinds of morphological variation observed in evolution. For example, Shapiro *et al.* (2003) have studied the mechanisms producing digit loss in the Australian lizard *Hemiergis*. They show that the loss of digits is directly correlated with shifts in the timing of expression of the gene for *sonic hedgehog* (*SHH*). *SHH* is a secreted protein that is expressed in the zone of polarizing activity of the developing limb bud. It appears to be critical in normal outgrowth of the limb as well as for normal spatial patterning of the bud. In *Hemiergis* there is significant natural variation in digit number. In particular three species show consistent patterns: *H. quadrilineata* possesses two fingers and toes on each limb, *H. peronii* has individuals with either three or four digits on each limb, and *H. initialis*, exhibits the full complement of 5 digits on each. The duration of *SHH* activity varied in direct proportion to digit number. *H. initialis* exhibited the longest period of *SHH* expression, and *H. quadrilineata* the shortest. No differences in other important regulatory genes were observed, nor was there any difference in the specific location or pattern of *SHH* expression. The authors hypothesize that the truncated period of *SHH* activity correlates with a reduction in cell proliferation. They demonstrate, through BrdU staining, that there is a decrease in proliferation in the posterior parts of the limb bud in *H. quadrilineata,* which possesses only two digits. Thus, in this case a macroevolutionary event – digit loss – may be mediated by a very slight shift – heterochrony – in the length of expression of this single gene.

Richardson and Oelschläger (2002) have studied hyperphalangy (the addition of extra phalangeal elements to an individual digit) in the dolphin. Hyperphalangy has repeatedly evolved in vertebrates

in which limbs are secondarily modified as flippers. These authors show that the proliferative ridge (the apical ectodermal ridge) appears to exist longer during development in the digits in which hyperphalangy is observed. They propose that this heterochrony is the means by which additional phalanges are generated. Both of these studies are cases where a heterochrony in the expression of a gene (or presumed expression in the dolphin case), have had a direct effect on the growth, and therefore form, of the final structure.

A somewhat different situation is postulated in the study by Blanco *et al*. (1998). These authors propose that the developmental basis for the elongated ankle bones in frogs is that the tarsal (ankle) region has acquired patterning characteristic of the tibia. They propose that this "tibialization" is produced by a shift in the expression of the *Hoxa-11* gene, which normally is responsible for patterning the tibia, into the region of the tarsal bones. The shift in the site of *Hoxa-11* patterning is accomplished by a shift in timing of expression – heterochrony – rather than a shift in the spatial pattern of expression. Specifically, in the hind limb as compared to the forelimb, *Hoxa-11* is expressed for a longer period, presumably through the period in which the cells destined for the ankle differentiate. Unlike the *SHH* example, where the genetic heterochrony seems to directly change the duration of cell proliferation, in this case the genetic heterochrony does not directly affect a morphological process such as growth rate or period, but instead results in a shift in imposition of regional identity.

Heterochrony and the development of alternate phenotypic pathways

Nijhout (1999) reviews the mechanisms responsible for the development of polyphenism in insects. In polyphenic organisms, identical genotypes may produce radically different phenotypes within the same species. Examples of this phenomenon include the various castes observed within social insects, or color morphs so often seen in various butterfly species. Nijhout points out that the development of any organism may be characterized as the progress through a series of stages in which specific decisions about differentiation must be made. Often critical or sensitive periods exist during which these developmental decisions are made. By slightly shifting certain events relative to sensitive periods, radically different phenotypes may be produced. In insect development, hormones generally act as switches that alter patterns of gene expression and may direct organism into alternate pathways. Nijhout summarizes a variety of ways that this switching among alternative pathways may occur, and in particular two kinds of shifts – the relative timing of secretion of the hormone, or the relative timing of a sensitive period – are means by which simple molecular heterochronies can produce alternate phenotypes. Nijhout (1999) and Moczek and Nijhout (2003) discuss ways in which such shifts produce evolutionary important changes among species.

Genetic heterochronies and phenotypic patterning

A number of authors have discussed the specific means by which shifts in the timing of gene expression my produce significant differences in phenotypic pattern. For example, Koch *et al*. (2000) study two different mutations that produce significant different morphologies in the wings of the butterflies, *Papilio glaucus* and *Bicyclus anynana*. In each case there is a naturally occurring alternative phenotype, which differs significantly from the wildtype. In *P. glaucus*, the normal yellow wing background is replaced with a black background. In *B. aynana*, wing spot patterns differ. Koch *et al*. (2000) studied wing patterning mechanisms and found that in both cases the pattern difference resulted from heterochronies in the rates of scale development. For example, in the melanic form of *P. glaucus*, the scales destined to be background scales show delayed differentiation, fail to make the yellow pigment, and melanize at the same time as normal dark spots. Thus a whole-scale color shift is produced simply by changing the rate of scale maturation. A similar mechanism appears to be working in the scales destined to contribute to the alternate wing spot pattern in *B. anynana*: those scales show a simple delay in development, and thus develop a different base color.

Many other examples of genetic heterochrony producing important phenotypic change exist. Kim *et al*. (2000) study patterns of expression of the *hairy* gene, one of the pair-rule genes important in setting up initial segmentation pattern, in three different species of *Drosophila*. They find quantitative differences in the rate of gene expression and show that *hairy* expression varies with regard to absolute time in the three species, and also relative to cell cycle-dependent morphological differentiation. This result demonstrates not only shifts in developmental rate, but also dissociation of *hairy* expression and other events. The genetic heterochronies may correlate with changes in number or patterning of segments. Skaer *et al*. (2002) found that differences in bristle pattern in two closely related species of blowfly appeared to be due to changes in the timing of expression of important genes, rather than the spatial pattern of expression. Villani and Demason (1999, 2000) and Wiltshire *et al*. (1994) provide examples of cases in which leaf morphological variation in *Pisum sativum* is generated largely through changes in the timing of expression of several genes, rather than shifts in the genes expressed or the spatial pattern of gene expression.

Evolution of major morphological changes

Kozmik *et al*. (2001) study the evolution of expression of the homeobox gene *Vent*, an evolutionarily conserved marker for ventral (= lateral plate) mesoderm in chordates. They compare expression of this gene in *Amphioxus* (*AmphiVent*) and vertebrates such as *Xenopus* and teleosts. These authors show that *AmphiVent* and the vertebrate *Vent* genes are evolutionarily conserved in their spatial expression. In vertebrates, however, the ventral mesoderm differentiates much earlier than in *Amphioxus* and likewise expression of *Vent* is accelerated in vertebrates relative to *Amphioxus*. Kozmik *et al*. (2001) hypothesize that the precocial appearance of mesoderm is an important evolutionary innovation of the vertebrates. Vertebrate embryos, unlike *Amphioxus*, are relatively large and therefore must differentiate an efficient embryonic circulatory system, the major fate of the ventral mesoderm, quite early in their development. Thus, they propose that this genetic heterochrony is associated with major functional innovations in evolution.

In a similar manner, Hinman *et al*. (2000) hypothesize that the evolution of the biphasic body plan in ascidians was accompanied by a temporal shift in the otherwise conserved expression of the *cdx* gene. The primitive condition in ascidians was a free swimming larvae that possessed both a feeding and a locomotor apparatus. In the common ascidian condition the mobile larvae is non-feeding, while in the adult the feeding apparatus is present but the locomotor axial structures degenerate. Hinman *et al*., (2000) show that in the ascidians *Hec-cdx* is expressed biomodally with the expression in the hind gut separated from expression in the

posterior central nervous system. In other animals these expression domains overlap temporally. They argue these results (as well as results from expression of other genes) suggest that "the generation of the novel ascidian biphasic body plan was not accompanied by a deployment of these genes into novel pathways but a heterochronic shift in tissue-specific expression" (p. 215).

Integrated studies

In a series of studies Smith and colleagues have examined heterochronies in craniofacial development in marsupial and placental mammals at multiple levels, combining many of the approaches discussed above (Clark and Smith 1993; Smith 1994, 1996, 1997, 2001b&c, 2002; Nunn and Smith, 1998; Vaglia and Smith 2003). Marsupials are born at a highly embryonic state and complete most development while attached to the teat, nursing. The morphological configuration of the neonate is highly distinctive (Fig. 1 A,B). Smith has investigated some of the specific heterochronies that have produced this characteristic morphology and allow independent function of the neonate at an embryonic state. These studies include phylogenetic studies in which the sequence of organogenesis of cranial bones, muscles, and features of the sensory and central nervous systems was examined in a wide range of marsupial and placental mammals. This work demonstrated that in marsupials not only are the muscle and bones of the oral and facial apparatus accelerated in development but also that there is a significant delay in the differentiation of central nervous system tissues, in particular in the region of the forebrain. The origin of these differences in development was studied in later papers and it was shown that the differentiation of neural crest from the neural plate was accelerated relative to the timing of appearance in other vertebrates (Fig. 1 C,D). The shifts in the timing origin of neural crest demonstrates that even at the earliest stages of cranial development, significant heterochronies distinguished marsupials from other vertebrates. Current work is extending this investigation of heterochrony to an examination of the expression pattern of major genes important in patterning the craniofacial region (Fig. 1 E,F).

The nature of developmental time

Most work on heterochrony has been divorced from an explicit discussion of the ways that embryos actually assess time. There are probably several reasons for this. First, it is clear that there is no single mechanism for time assessment (e.g. Satoh, 1982; Ambros & Horvitz, 1984, 1987; McClung, Fox & Dunlop, 1989; Reiss, 1989; Cooke & Smith, 1990; Gorodilov, 1992; Yasuda & Schubiger, 1992;

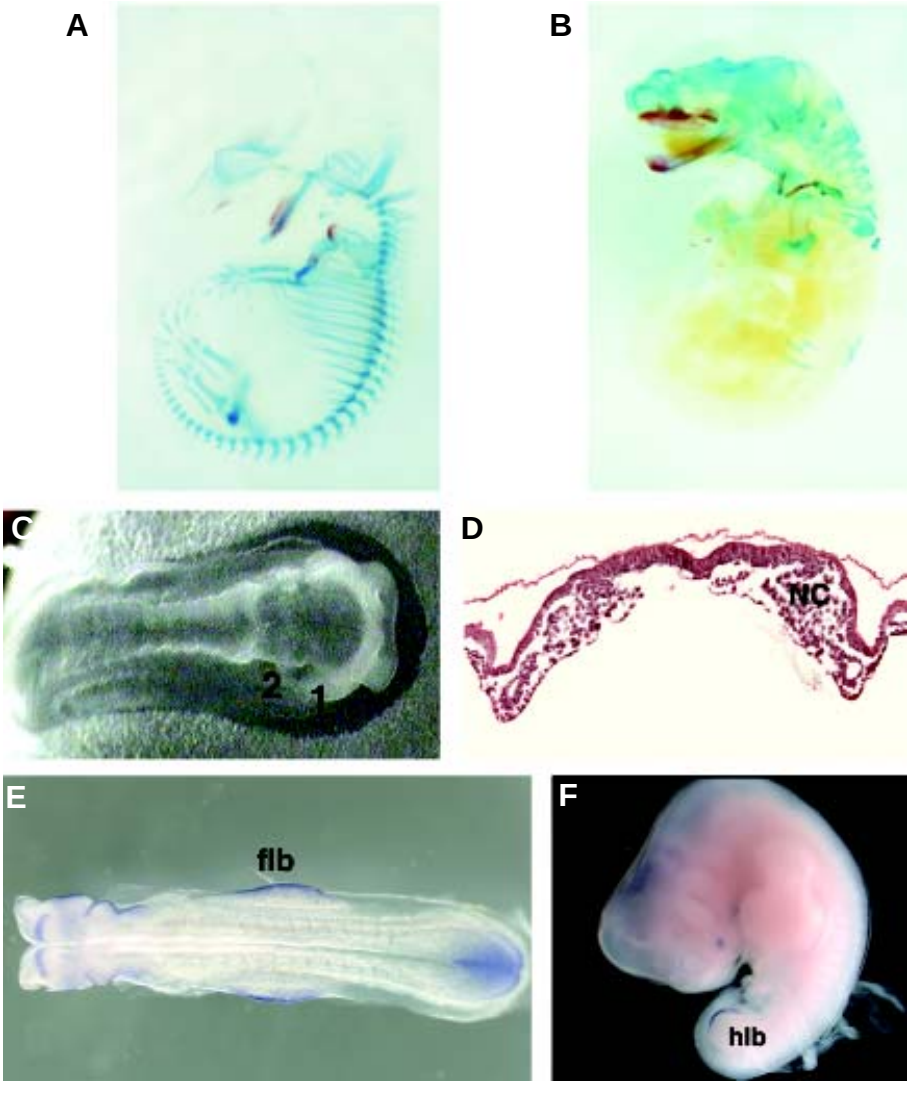

Fig. 1. Illustrations of heterochrony in marsupial mammals. (A) *A day fourteen embryonic mouse. Blue represents cartilage and red bone. Note the development of vertebrae along the entire length of the body axis, and a similar state of development in forelimb and hind limb. Note also that bone is only beginning to ossify in the facial region.* **(B)** *A two-day postnatal* Monodelphis domestica *(marsupial) embryo. Note extreme gradient in vertebral development from the anterior to posterior part of the body, and the disproportionately large forelimb. Cartilage around the nasal capsule, bones around the oral cavity, and tongue muscles are exceedingly well developed.* **(C)** *A stage 24 (about 10 days embryonic)* Monodelphis *embryo. Note the flat neural plate. At this stage the brain is undifferentiated, yet the neural crest has begun migration into the first and second arches (1 & 2).* **(D)** *A section through the first arch region of a stage 23* Monodelphis *embryo. Neural crest cell migration is underway (NC), yet the neural tube has not yet begun to close and there is little differentiation of mesoderm.* **(E)** Fgf8 *staining of a stage 25* Monodelphis *embryo. Note staining at the midbrain/hindbrain boundary and the precocial staining along ridge of body destined to form forelimb bud (flb).* **(F)** Fgf8 *staining in a stage 31* Monodelphis *embryo. Although the forelimb bud is well differentiated, the hind limb bud is just beginning to show* Fgf8 *staining (hlb), characteristic of the apical ectodermal ridge. In most other amniotes, forelimb and hind limb buds exhibit* Fgf8 *staining at nearly the same time.*

Power & Tam, 1993; Ffrench-Constant, 1994; Hall & Miyake, 1995; Howe *et al.*, 1995; Kai *et al.*, 1995; Palmeirim *et al.*, 1997; Pourquié, 1998; Stern & Vasiliauskas, 1998; Dale & Pourquié, 2000; Jiang e tal., 2000; Johnson & Day, 2000; Day *et al.*, 2001; Vasiliauskas and Stern, 2001; Crawford, 2003). Different organisms at different stages in their life history track developmental time using many different types of measures.

Second, while some processes may be strictly "time based", it is also likely that many events in development are simply dependent on the occurrence of prior events. For an embryo, scheduling may be a matter of sequence relative to other events rather than clock time. Order is imposed by the integration of processes into specific sequences. The fact that many events depend on induction, or cell or tissue interactions, or the complex interactions within various gene cascades means that there is a fundamental directionality to development. However, these kinds of control processes are better defined as scheduling rather than timing mechanisms. The fact that control may be sequence based rather than time based (in a strict sense) may provide further justification for the use of sequence approaches to heterochrony.

However, in the future it is likely that studies of heterochrony will explicitly address the modification of timing mechanisms. For example work on *C. elegans* (Ambros and Horvitz, 1984, 1987; Slack and Ruvkun, 1997) suggest that "heterochronic genes" exist, which may have sweeping phenotypic effects. Explicit study of heterochronies of known time keeping mechanisms are also likely. One means of scheduling has been referred to as an "hour glass" mechanism (e.g. Cooke and Smith 1990, Ffrench-Constant 1994; Pourquie, 1998, Day *et al.*, 2001) in which the decay or accumulation of a product to a threshold level provides a measure of time elapsed. These kinds of mechanisms have been proposed for the control of very early processes (e.g., gastrulation), which may be signaled by the dilution of certain cytoplasmic factors as a result of cell division. Heterochronies in such hour glass mechanism can be easily accomplished by shifts in the threshold or the initial levels of critical substances. A second time keeping mechanism relies on oscillatory feedback mechanisms, perhaps deriving from cell cycles. The best known of these is the "somite clock" (e.g., Pourquie, 1998; Dale and Pourquie, 2000 and refs. above), but others such as oscillations of K+ channels in mice (Day *et al.,* 2001) have also been proposed. As yet potential heterochronies in such mechanisms have not yet been explored. One potentially productive line of investigation would be the role of heterochronies in the somite clock in vertebrates with widely varying somite numbers (e.g., Richardson, *et al.,* 1998).

Summary

Heterochrony is but one way that development may be modified in order to produce evolutionary changes, but it is an exceedingly important one. The concept of heterochrony has accompanied the general field of evolutionary and development since the late nineteenth century. In this review I have attempted to present some of the most recent advances in our view of heterochrony. In the past decade the concept has been revitalized, and studies of heterochrony in explicit phylogenetic contexts as well as at the level of the whole organism, organ and organ system, cell, molecule and gene are allowing new linkage between classic questions of mechanisms of evolutionary change, with the most current advances in developmental biology.

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