Genetica **115:** 131–146, 2002.

Genome size and developmental complexity

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Key words: amphibians, C-value paradox, developmental rate, differentiation, fish, hierarchy, insects, metamorphosis, neoteny, nucleotype, paedomorphosis, plants

Abstract

Haploid genome size (C-value) is correlated positively with cell size, and negatively with cell division rate, in a variety of taxa. Because these associations are causative, genome size has the potential to impact (and in turn, be influenced by) organism-level characters affected by variation in either of these cell-level parameters. One such organismal feature is development. Developmental rate, in particular, has been associated with genome size in numerous plant, vertebrate, and invertebrate groups. However, rate is only one side of the developmental coin; the other important component is *complexity*. When developmental complexity is held essentially constant, as among many plants, developmental rate is the visibly relevant parameter. In this case, genome size can impose thresholds on developmental lifestyle (and vice versa), as among annual versus perennial plants. When developmental rate is constrained (as during time-limited amphibian metamorphosis), complexity becomes the notable variable. An appreciation for this rate-complexity interaction has so far been lacking, but is essential for an understanding of the relationships between genome size and development. Moreover, such an expanded view may help to explain patterns of variation in taxa as diverse as insects and fish. In each case, a hierarchical approach is necessary which recognizes the complex interaction of evolutionary processes operating at several levels of biological organization.

Introduction: The C-value enigma

The C-value enigma (less appropriately, but still more commonly, known as the 'C-value paradox') is the complex puzzle relating to the profound variation in genome size among eukaryotes. In the most general terms, the puzzle can be divided into three primary components (Gregory, 2001a):

(1) the mechanisms responsible for changes in DNA content, of which there are several,
(2) the reasons for the observed relationships between genome size and nuclear, cellular, and organismal traits, and
(3) the forces responsible for the differential maintenance and/or loss of non-coding DNA which result in a non-random taxonomic distribution of genome size variation.

The second of these topics has recently been reviewed in detail (Gregory, 2001a). Put briefly, there is a significant negative relationship between C-value (haploid genome size) and cell division rate, and a positive association with cell size. The mechanistic explanations for these relationships may be varied and complex, but the correlations themselves are best interpreted as resulting from the causal, 'nucleotypic' influence of bulk DNA on the cellular phenotype. By extension, genome size is itself potentially related to any organismal feature affected by changes in cell division rate and/or cell size. These organism-level effects, and their selective consequences, may play an important role in shaping the patterns of genome size distribution among taxa.

Development is one notable organismal feature impacted by both cell size and cell division rate. Developmental *rate*, in particular, has been investigated in numerous groups in terms of its possible contribution to the evolution of genome size (and vice versa). Plants and amphibians have long served as exemplars of the link between genome size and developmental

rate, and it is in these groups that the general notion of 'thresholds' on genome size set by developmental time constraints has been developed most thoroughly. They will also feature prominently in the present discussion.

As important as rate-related constraints may be, it should be recognized that there is a second component to development that is also relevant to the evolution of genome size. Stated directly, it is not only how quickly an organism must undergo its various developmental transformations, but also how much transforming is to be done in the amount of time available. To the extent that this transformation involves the growth, division, and differentiation of cells, genome size will be relevant not only in terms of overall rate, but also in regards to the very process of development. And in cases where developmental rate is constrained, the degree of this transformation will be the visibly relevant parameter correlated with genome size. Thus, the flip-side of *rate* on the developmental coin is *complexity*. As such, a proper understanding of the association between genome size and development requires an exploration of both elements. In this regard, the present discussion begins with a description of some commonly observed trends in plants, and the concept of rate-related thresholds they illustrate. A more inclusive view incorporating the component of developmental complexity is then developed with examples drawn from amphibians. In this case, the complexity of both the *process* and the *products* of development are considered important (though for different reasons). Finally, this broadened perspective of rate-plus-complexity is applied to some previously unexplained trends in genome size distribution among organisms such as insects and fish.

Genome size and development in plants

Plants have always been important models for theories relating to the C-value enigma. The term 'C-value' itself was coined in reference to constant *classes* of DNA content in plants (Swift, 1950), and the 'nucleotypic theory' was developed as an explanation for the relationship between genome size and plant developmental rates (Bennett, 1971, 1972). Plants continue to play a major role in the study of genome size evolution, and the relationship between DNA content and developmental traits remains a popular subject of analysis. A detailed critical review of the literature describing relationships between genome size and developmental parameters in plants would therefore be extremely useful, but lies well beyond the scope of this article (and the expertise of this author!). The following brief discussion will focus instead on a small subset of these findings, but should nevertheless provide a good general introduction to the patterns that exist in plants.

The genomes of plants have frequently been labeled as 'fluid', 'dynamic', and 'in constant flux'. The reasons for this view are varied, but the seemingly common observation of pronounced intraspecific variation in plant genome sizes has been a significant contributor. More recently, many supposed cases of intraspecific genome size variation have been attributed to simple measurement artifacts (e.g., Greilhuber, 1997, 1998). Thus, although intraspecific comparisons are perhaps the most informative from an adaptive (gradualistic) evolutionary perspective, they are also the most susceptible to experimental error.

Maize (*Zea mays*) displays legitimate intraspecific variation in genome size of up to 40% (2C·5–6 pg), caused for the most part by differences in the number of heterochromatic knobs and/or supernumerary B chromosomes (e.g., Poggio et al., 1998). Numerous ecogeographical correlates of genome size are known from this species, but most telling of all is the finding that artificial selection for earliness of flowering (i.e., rapid development) resulted in a reduction in mean genome size in an experimental population (Rayburn, Dudley & Biradar, 1994); even small changes in genome size have visible effects on development in this species. Maize is of particular interest in this regard, since its genome is believed to have doubled in size in only about 3 million years by the action of transposable elements (SanMiguel & Bennetzen, 1998; SanMiguel et al., 1998). This may have been a very fortuitous event from a human perspective, because its larger genome probably allowed maize to grow more effectively in colder conditions (Grime & Mowforth, 1982) and made it more amenable to cultivation at higher latitudes (Bennett, 1976, 1987; Laurie & Bennett, 1985; Rayburn et al., 1985) and different altitudes (Bullock & Rayburn, 1991). A similar transposable element-mediated increase in genome size has been described in populations of wild barley (*Hordeum spontaneum*), and has been interpreted in terms of positive selection for increased DNA content according to varying growing conditions (Kalendar et al., 2000). Taken together, these observations, *inter alia*, not only indicate that DNA content can exert an important influence on development in plants such as

Zea mays, but that selection at the level of the organism (either positive as in barley, or negative as with maize) may influence the evolution of elements at the genomic level and vice-versa. Such ecogeographic and developmental constraints appear applicable to a wide variety of plants, both within and among species (e.g., Bennett, 1976, 1987; Grime & Mowforth, 1982; Grime, Shacklock & Band, 1985; Mowforth & Grime, 1989).

There is a helpful partitioning of developmental lifestyles in plants that also serves to emphasize the influence of genome size on distribution and development. Annuals are those plants that complete their entire life cycle within a single growing season; perennials are those that live through several such seasons. Obviously, the luxury of slow development afforded by a perennial lifestyle is not available to annuals. Therefore, the temporal-developmental constraints experienced by annuals may be expected to favor small genomes as compared to perennial relatives. Indeed, such a pattern has been reported for many genera of plants, including *Arachis*, *Brachyscome*, *Calotis*, *Crepis*, *Happlopappus*, *Papaver*, and *Vicia* (e.g., Resslar, Stucky & Miksche, 1981; Srivastava & Lavania, 1991; Singh, Raina & Singh, 1996; Naranjo et al., 1998; Watanabe et al., 1999). Annual lifestyle in most of these genera is believed to be a derived feature, and these species typically inhabit harsher, more time-limited environments. As such, genome reduction is also generally viewed as a derived feature arising adaptively to permit a more rapid mode of growth. This view has been granted substantial support by the recent phylogenetic study of Watanabe et al. (1999) which provided 'the first statistical verification of the association between a large reduction in chromosome number or genome size and the evolution of annual habit'.

That a shift from perennial to annual lifestyle is linked with an adaptive genome size reduction is of potentially great theoretical interest. Specifically, it would be useful to know whether this shift *initiates* a reduction in DNA content, by active deletion of existing non-coding elements (genome-level selection) or by favoring individuals within the species with smaller genomes (individual-level selection), or whether small genomes are 'pre-adaptations' (a term used here *faute de mieux*) for an annual lifestyle such that only species already possessing smaller genome sizes, for whatever reason, can become annuals (species-level sorting). Perhaps processes operating at all three levels are at play, in which case the importance of maintaining a hierarchical perspective in questions of genome size evolution would be clearly illustrated. Certainly, this would lend powerful support to the hierarchical views long held by many macroevolutionists (e.g., Eldredge, 1985; Lieberman & Vrba, 1995; Gould, 1998).

These associations between genome size and developmental lifestyle are not limited to comparisons of congeneric species. As a clear and general example, 'weeds' (a taxonomically diverse group of fast-growing plants defined by their nuisance to humans) are known to possess genomes much smaller than most other angiosperms (Bennett, Leitch & Hanson, 1998). More explicitly, Bennett (1976, 1987) has suggested that an ephemeral lifestyle is possible only below a threshold of 10 pg, whereas above 30 pg a perennial lifestyle is inevitable; annuals and facultative perennials fall somewhere between these two extremes. Though the exact nature of the constraints at play are not clear (e.g., to what degree genome size is shaped by, or itself determines, the evolution of a particular developmental lifestyle), it is apparent from these broad trends that 'the nucleotype profoundly affects where, when, and how plants grow' (Bennett, 1987).

The take-home message from plants is as follows: first, genome size and developmental rate are related to one another both within and among similar species. Second, there is a notion of thresholds, such that genome size is constrained by developmental lifestyle (and/or the reverse). These points are applicable in general to other groups, including the amphibians described in the following section. However, it should be recognized that in these botanical examples there has been no attempt to control for variation in developmental complexity. In fact, the thresholds based on rate alone are relevant only insofar as developmental complexity is assumed to be essentially constant among the species in question. In comparisons of conspecifics or closely allied species of plants, this assumption may be justified. But in broader comparisons of organisms with very different developmental programs, such as in the amphibians, complexity becomes an important – perhaps even dominant – parameter.

Developmental complexity in amphibians

Although genome sizes vary immensely among extant amphibia (~130-fold, from 0.95 to 120 pg), this variation is not distributed evenly among the three

living orders. In frogs (Order Anura, aka Salientia), genomes range over 20-fold in size but are invariably smaller than 20 pg, with an average C-value of less than 5 pg (Gregory, 2001b). Interestingly, this entire range can be found within a single family, the Myobatrachidae (from 0.95 pg in *Limnodynastes ornatus* to 19 pg in *Arenophryne rotunda*). All salamanders (Order Urodela, aka Caudata), on the other hand, possess genomes larger than 13 pg (Gregory, 2001b). In this group the average genome size is greater than 35 pg (range 13–120 pg), and there is only minor overlap between them and the Anura. Caecilians, legless amphibians of the Order Gymnophiona (aka Apoda), are almost completely unknown in terms of genome size (only three species, from 3.7 to 14 pg), and as such will not feature in the present discussion.

In homeotherms, there is a significant negative correlation between genome size and resting metabolic rate (Vinogradov, 1995, 1997; Gregory, 2002). This has been attributed to various causal factors, most notably the positive relationship between DNA content and erythrocyte sizes found in both classes of homeotherms (Gregory, 2000, 2001c, 2002). Genome size and cell size are also strongly positively correlated in amphibians (Olmo, 1983; Gregory, 2001a,c), and the distribution of genome size variation within the amphibian class has similarly been interpreted in terms of metabolic constraints. For example, the large genomes (and cells) of aquatic urodeles have been attributed to the low metabolic rates associated with water-breathing (e.g., Cavalier-Smith, 1991; discussed in a later section). By a similar token, the extreme genome sizes of lungfish have been explained as a necessary adaptation to aestivation during dry seasons (e.g., Cavalier-Smith, 1991; Gregory & Hebert, 1999). However, several lines of evidence suggest that metabolic constraints are, at best, of only minor significance in amphibians and lungfish.

In their analysis of a large sample of salamander species, Licht and Lowcock (1991) found only a very weak relationship between genome size and metabolic rate, and one which appeared only under conditions of thermal stress. Although they took neither phylogeny nor body size into account, a subsequent re-analysis of these and additional data confirmed their conclusions and extended them to frogs and larval amphibians as well (T.R. Gregory, unpublished). Thus, there appears to be no obvious relationship between genome size and metabolic parameters among amphibians. Aestivation itself also seems inadequate as an explanation for the large genome sizes of some vertebrates. At first sight, the fact that the non-aestivating Australian lungfish (*Neoceratodus forsteri*, ~50 pg) has a genome less than half the size of the aestivating South American (*Lepidosiren paradoxa*, ~115 pg) and African (*Protopterus aethiopicus*, ~130 pg) lungfish species might be taken as evidence in favor of this hypothesis. On the other hand, some species of aestivating frogs such as *Scaphiopus couchii* and *Pyxicephalus adspersus* are known to reduce their metabolic rates to a low level comparable to that of the urodele *Siren intermedia* (Pinder, Storey & Ultsch, 1992). And while *Siren* has a large genome (~55 pg), *Scaphiopus* and *Pyxicephalus* have very small genomes, even for anurans (both ~1.5 pg) (Gregory, 2001b). Not only this, but these desert-dwelling frogs may remain in aestivation for 7–10 months per year (Pinder, Storey & Ultsch, 1992)! Clearly, it is possible to maintain an aestivating lifestyle without the burden of huge quantities of non-coding DNA; that selection would favor a 75-fold increase in genome size to achieve this end seems highly doubtful to say the least. It also bears mentioning that *Scaphiopus* emerges from aestivation to breed during brief seasonal wet periods, and that its larvae are forced to develop very quickly in ephemeral pools (Pinder, Storey & Ultsch, 1992). In this species, at least, developmental (but not metabolic) constraints on genome size are probably quite important. Considerable evidence has accumulated to suggest that this is true of amphibians in general.

Developmental rate and constant complexity

Table 1 provides a summary of the numerous surveys that have revealed correlations between interspecific differences in developmental parameters and variation in genome size among amphibians. While some of the earlier studies did not adequately control for important variables such as temperature, more recent ones have not only confirmed the results of previous work, but have also provided corrections for the non-independence of interspecific data. The common observation reported in each of these studies is that genome size is negatively correlated with bulk developmental rate in a variety of amphibian taxa. In some cases, a relationship is detectable among congeneric species, as with members of the genus *Rana* (Oeldorf, Nishioka & Bachmann, 1978). This latter observation agrees well with Vinogradov's (1999) recent report of a very tight linkage between genome size and temperature-controlled cell cycle duration in four species of *Rana*.

Table 1. Summary of previous studies reporting a significant positive relationship between haploid genome size (C-value) and developmental time (DT) in amphibians

Study	Taxonomic sample	Developmental parameters	C-value range (pg)	DT range (days)	P?
Goin, Goin and Bachmann, 1968	21 species of frogs (5 families)	Minimum duration of larval phase, temperatures not specified	1.4–7.4	14–365	No
Bachmann, 1972	7 species of frogs (3 families)	Average embryonic development, 13.6–31.7°C (corrected to 20°C)	1.4–6.7	1.1–2.8	No
Oeldorf, Nishioka and Bachmann, 1978	25 species of frogs (6 families)	Embryonic (two cell stage to gill circulation stage), corrected to 20°C	1.5–9.0	2.1–9.0	No
Horner and Macgregor, 1983	5 species of frogs, 4 species of salamanders (various families)	Average embryonic development, 18–23°C	1.4–83.0	2–50	No
Pagel and Johnstone, 1992	24 species of salamanders (7 families)	Average embryonic development, temperatures not specified	15.1–82.6	20–250	Yes
Camper et al., 1993	20 species of bufonoid frogs (5 families)	Average overall development, temperatures not specified	2.5–9.0	21–85	No
Jockusch, 1997	15 species of salamanders (family Plethodontidae)	Average embryonic development, 13°C	14.1–68.9	39–234	Yes
Chipman et al., 2001	7 species of frogs (4 families)	Embryonic (32-cell stage to closure of neural tube), various temperatures and corrected to 23°C	2.95–9.0	0.6–1.1	No

Genome size data have been updated according to Gregory (2001b). The types and numbers of species used, and the developmental conditions and parameters measured are given, as are the ranges in C-value and DT. 'Embryonic development' refers to the time from oviposition to hatching, 'larval phase' indicates the time from hatching to the juvenile stage, and 'overall development' includes both embryonic and larval development. Whether or not phylogenetic information (P) was incorporated into the study is also indicated.

Based on these observations, it is perhaps tempting to ascribe much of the variation in amphibian genome size to constraints derived from this simple relationship with developmental rate. Goin, Goin and Bachmann (1968), for example, drew particular attention to the fact that frogs inhabiting ephemeral pools have smaller genomes than those found in more long-lasting aquatic environments. Yet, Horner and Macgregor (1983) point out several examples in which organisms possessing vastly different genome sizes (and developmental rates) successfully develop, survive, and reproduce in the very same ponds. Thus, they conclude that 'ecological arguments as a means of rationalizing differences in genome size and corresponding differences in developmental rate are, except in the extreme limits of the ranges, rather unconvincing' (Horner & Macgregor, 1983). On the other hand, this cohabitation may in fact be facilitated, at least among anurans, by the partitioning of niches according to different rates of larval maturation (e.g.,

Oeldorf, Nishioka & Bachmann, 1978; Camper et al., 1993) or other differences in reproductive strategy (e.g., Morescalchi, 1990). It is also interesting that frog species that breed in temporary bodies of water, such as *Scaphiopus couchii* (1 pg), *Spea (Scaphiopus) hammondii* (1 pg), *Bufo calamita* (4 pg), and *Rana temporaria* (4 pg), are able to accelerate the onset of metamorphosis in response to the threat of drying. By contrast, *Rana esculenta* (7 pg) does not breed in temporary ponds, and is not capable of such adaptive developmental rate modulation (Loman, 1999). Physiological and developmental adaptations to life in an ephemeral pool are, therefore, not unknown in anurans, and the possibility of a related nucleotypic adaptation should not be dismissed.

The appropriate interpretation of these relationships is, as with plants, one of thresholds. Genome size is clearly not the only factor that contributes to variation in developmental rate, but the important point is that 'development can be slowed down independently of genome size but genome size sets a limit to the genetical acceleration of development' (Bachmann, Chambers & Price, 1985). Put most directly, 'above 10 pg of DNA per nucleus fast development is not found' (Oeldorf, Nishioka & Bachmann, 1978). Clearly, this is reminiscent of the relationship between DNA content and development in plants.

Also as with plants, thresholds such as these apply to comparisons of developmentally similar species. It seems unlikely, for example, that they could explain the nearly non-overlapping genome sizes of frogs and salamanders. In fact, such correlations between genome size and developmental rate may only appear when other factors – namely, the number of 'steps' to be taken during development – are held essentially constant. To take the opposite perspective, if developmental *time* is held constant (a constraint that could occur for any number of environmental reasons), then a frog might be expected to have a smaller genome than a salamander if it had to proceed through more developmental steps in the same time period, provided that these 'steps' involve cellular processes influenced by genome size (like division, growth, and differentiation). It is relevant in this regard that 'salamanders seem to have the simplest patterns of development and the fewest modifications of the generalized amphibian development' (Duellman & Trueb, 1994). Of course, this is a rather simplistic comparison. The developmental programs of both frogs and salamanders are quite varied, and a more detailed examination of their associations with C-value is needed before anything conclusive can be said in this context about amphibian genome size evolution. In this case, the relevant comparison is between genome size and the complexity of the developmental process itself, especially with regards to metamorphosis. Relationships between C-value and morphological complexity (i.e., of the products of development) will be considered in a later section.

Developmental process: direct development

The experience of metamorphosis may be foreign to humans, but the phenomenon is not; even most school children are familiar with the basic observation of a tadpole transforming into a frog, or a caterpillar becoming a butterfly. Metamorphosis is easily recognized, even at this simplest level, as a period of profound morphological change. Particularly relevant to genome size evolution is the fact that this process generally occurs rapidly with respect to overall lifespan, and therefore involves intensive differentiation in a short period of time. Given that animals in the midst of metamorphosis may be especially vulnerable to predation or other stresses, this can be a highly time-limited process, and therefore one requiring rapid cell division and differentiation. Clearly, such a constraint would have direct implications for the C-value that such an animal could maintain. That is, organisms with such complex developmental programs experience strongly rate-limited periods of differentiation (both sides of the coin, as it were), and would be expected to possess small genomes. This notion is eminently testable in amphibians, given that metamorphosis is not a ubiquitous feature among either frogs or salamanders. Indeed, certain taxa in both groups have evolved 'direct development', involving the loss of an independent larval stage. This represents an important change in the complexity of the developmental program, and is therefore of relevance to the present thesis.

In the study by Camper et al. (1993), direct developers (with larval development confined to within the egg, and ending in the hatching of a froglet) were found to possess significantly smaller genomes than so-called 'intermediate' and 'aquatic' developers, both of which have a distinct larval stage. 'Aquatic' developers (in which eggs are layed directly into the water, or into a foam nest above the water, with free-swimming larvae unattended by adults) had genomes that spanned almost the entire range observed. Notably, it is within this latter type of frog that most correlations between genome size and developmental

rate have been examined. In other words, developmental complexity is held roughly constant in this group (i.e., all have larvae and must metamorphose), and it is not surprising that they show a wide range in genome size, which in turn is associated with bulk developmental rate. The comparison of frogs with *different* developmental programs is very informative in this context, since it shows the confinement of complex development to within the egg – where development *time* is held largely constant because of limited egg resources (even though direct-developers typically have larger eggs) – to be associated with a smaller genome size relative to 'intermediate' and 'aquatic' developers with a distinct larval stage.

In salamanders of the family Plethodontidae, the situation may take the opposite form. According to Jockusch (1997), the evolution of direct development among some terrestrial salamanders does not affect the overall relationship between genome size and embryonic development time. However, if all that is measured is time spent in the egg, it may be of little relevance whether a larval or juvenile salamander hatches out, since these are not greatly different from one another (the same is clearly not true of larval vs. juvenile frogs, so the effects of direct development on genome size may be different in the two groups). What *does* seem relevant in this case is the loss of metamorphosis from larva to adult later in the developmental program which, as mentioned previously, may be time-limited because of vulnerability to predation and other external factors.

The lungless salamander family Plethodontidae is divided into two subfamilies, Desmognathinae and Plethodontinae, the latter of which consists of the tribes Hemidactyliini, Bolitoglossini, and Plethodontini. Most members of the subfamily Desmognathinae possess distinct aquatic larval stages with complete metamorphosis, and their genome sizes are all at the bottom of the urodele range (14–17 pg). One notable exception to this is *Desmognathus aeneus*, which is now believed to be a direct developer (Marks & Collazo, 1998). Unfortunately, the genome size of *D. aeneus* is not yet known; it would be very interesting if it turned out to deviate significantly from the mean for the tribe (although there may still be time-related developmental constraints based on its subsistence on yolk reserves early in life).

Most species in the tribe Hemidactyliini also typically have a biphasic lifestyle, but in several cases, most notably among troglobitic salamanders, metamorphosis has been lost such that larval characters are retained into adulthood. In this group, genome sizes range from 21 to 32 pg. Species in the tribes Bolitoglossini and Plethodontini, by contrast, all display direct development with eggs hatching into miniature adults. The genome sizes of plethodontines range from 18 to 69 pg and those of bolitoglossines vary from 21 to 76 pg (Gregory, 2001b). The bolitoglossines will be discussed in more detail in a later section, but it is clear from even this cursory review that the evolution of direct development has been associated with considerable expansion of genome size in plethodontid salamanders. Again, this is the opposite pattern to that apparent in direct developing frogs, but it nevertheless indicates that change in the complexity of the developmental program (i.e., loss of metamorphosis) is linked to variation in genome size.

Developmental process: neoteny

In a survey of 27 species of plethodontids, ranging in C-value from approximately 14 to 76 pg, Sessions and Larson (1987) examined the relationships between genome size and limb regeneration rate. Two distinct parameters were measured in this study:

(1) *growth rate*, measured as area of re-growth per unit time, and which 'addresses the quantity of regenerated tissue', and
(2) *differentiation rate*, quantified according to a defined classification of regenerative stages, and which 'addresses the changing morphology and appearance of mature tissue types in the regenerate' (Sessions & Larson, 1987).

An apparent negative relationship between C-value and limb growth rate disappeared when corrected for phylogenetic associations, but a significant negative correlation between genome size and differentiation rate remained throughout (see also Harvey & Pagel, 1991). As an interesting corollary, Jockusch (1997) found that while limb growth rate was not correlated with embryonic development time, limb differentiation rate was (as Sessions & Larson (1987) had suggested it might be). Moreover, this relationship has survived both increases and decreases in genome size within the family (Sessions & Larson, 1987; Jockusch, 1997), thereby arguing strongly in favor of a causative interpretation of the relationship (vs. a 'coincidental' or 'coevolutionary' one; see Gregory, 2001a). In this case, it is not the bulk growth of tissue, but rather the complexity of tissue differentiation, that is associated with genome size. According to Sessions and

Larson (1987), "evolutionary changes in genome size may be an important correlate of concerted developmental perturbations that underlie morphological repatterning in urodeles". Based on these findings, Vignali and Nardi (1996) have suggested that rather than causally determining overall growth rate, changes in genome size may act to decouple the processes of growth and differentiation. When growth and differentiation proceed at substantially different rates, a likely evolutionary outcome is heterochrony.

Heterochronic changes resulting in sexually mature organisms displaying formerly juvenile characteristics are known collectively as *paedomorphosis*. This can occur by two primary routes:

(1) *progenesis* – the precocious development of adult sexual organs while still in the larval stage, or
(2) *neoteny* – the retardation of somatic development relative to sexual maturation and/or the truncation of the developmental program, as by the loss of metamorphosis and retention of larval morphology (Gould, 1977).

Obviously, changes in genome size are expected to have little relevance to the former of these processes. The latter process, which is by far the more common among amphibians, is potentially related in an intimate way to variation in genome size.

A general relationship between neoteny and large genome size was pointed out early on by Commoner (1964), who noted that among amphibians 'relatively low DNA values ... occur only in the genera in which metamorphosis is obligate. Genera in which neoteny is possible or obligate exhibit extraordinary DNA values'. Recall also that most frogs undergo much more intensive metamorphosis than salamanders, and that their haploid genomes cover the substantially narrower range of about 1–19 pg (vs. 13–120 pg in salamanders). Notably, the association between obligate neoteny and large genome size appears to have evolved independently three times among salamanders (in the Sirenidae, Amphiumidae, and Proteidae). Neoteny is a derived feature in each of these groups and so too are their large genome sizes, as apparent from fossil osteocyte volume data (Thompson & Muraszko, 1978). However, the reasons for this association remain somewhat arcane.

Of the obligately neotenic salamanders, Cavalier-Smith (1991) says: "As in lungfish, their exceptionally large genomes are purely the result of selection for exceptionally large cells'. As mentioned previously, he argues that this arises of necessity when a switch is made to less efficient water-breathing among non-metamorphosing aquatic salamanders. This can be dispelled by the simple observation that terrestrial salamanders (at least, those with direct development) also have very large genomes, and non-metamorphosing amphiumids – which also possess enormous C-values – though aquatic, have lungs and breathe air.

Wake and Marks (1993) explain the association between neoteny and large genome size in more direct, but nonetheless metabolic, terms: "Large genomes slow down biological processes, and it may be impossible for large-genomed species to have a 'normal' salamander life history, with an aquatic larval period and a terrestrial adult, because they may not be able to accomplish metamorphosis, which involves a speeding-up of metabolic processes." However, it has already been noted that the relationship between genome size and metabolic rate in salamanders is weak at best. Under extreme physiological conditions (such as at high temperatures), such metabolic concerns may come into play (Shahbasov & Ganchenko, 1990; Licht & Lowcock, 1991), but they are not generally applicable to normal metabolic processes. That metamorphosis requires an acceleration of metabolism comparable to that induced by severe environmental stress seems rather unlikely. Indeed, the relationship between genome size and neoteny in salamanders is likely to defy any such one-dimensional explanations. Instead, it may be necessary to adopt a more hierarchical view of the co-evolution of the organism and the genome.

Metamorphosis in amphibians is regulated proximately by hormones released from the thyroid gland, and in general the initial loss of metamorphosis in salamanders can be attributed to a failure in the hypothalamo-pituitary-thyroid axis (Duellman & Trueb, 1994). This, it seems, can result from mutations in a very small number of genes (e.g., Shaffer & Voss, 1996). In his 1951 poem *The Axolotl and the Ammocoete*, Garstang described facultative neoteny as follows:

They change upon compulsion,
if the water grows too foul,
For then they have to use their lungs,
and go ashore to prowl:
But when a lake's attractive,
nicely aired, and full of food,
They cling to youth perpetual,
and rear a tadpole brood.

In more technical terms, metamorphosis is favored under conditions of r-selection (low food avail-

ability, high predation and/or competition, faster development), while the maintenance of an aquatic (larval) lifestyle is beneficial under K-selection (lots of food, few predators, low competition, slow development) (Gould, 1977; Duellman & Trueb, 1994; Martin & Gordon, 1995; see Whiteman, 1994 for a detailed review of this and other mechanisms). The longer conditions remain favorable and metamorphosis is eschewed, the more likely it becomes that a gene of relevance to a key metamorphogenic pathway will mutate without immediate selective consequences (Gould, 1977; Martin & Gordon, 1995). At some point, the accumulation of such mutations (e.g., those affecting both thyroid hormone production and sensitivity) will render metamorphosis impossible, and an obligate neotene will result.

It is notable that obligate neotenes have larger genome sizes than facultative neotenes, which in turn have higher C-values than normally metamorphosing salamanders (and again, frogs, with their intensive metamorphosis, have the smallest genomes of all). Martin and Gordon (1995) suggest that as salamanders pass through evolutionary stages of facultative, inducibly obligate, and finally uninducibly obligate neoteny, their genomes expand in size. In this regard, they suggest that there is a positive linear relationship between the phylogenetic age of a neotenic lineage and its typical genome size, such that DNA content appears to increase at an approximately constant rate following the onset of neoteny (they even propose a 'junk DNA molecular clock' based on this steady accumulation). This genomic expansion, they argue, is primarily a product of the repeated duplication and extinction of 'adult' genes no longer relevant in the permanently larval animal.

In addition to gene duplication, genomes may increase in size by the action of transposable elements. In the case of some plants (e.g., maize and barley), this can occur rapidly, and possibly in response to changes in developmental parameters at the organism level. In salamanders, a shift to neoteny may relax constraints on the expansion of self-propagating genomic elements by eliminating the profound and strongly time-limited differentiation of tissues that metamorphosis entails. In this simplest case, large genome sizes would be a secondary byproduct of the interaction of the organismal phenotype with evolutionary processes operating within the genome. And while even this one-way interaction requires a hierarchical interpretation, it may be more reasonable to suggest a complex evolutionary feedback between the various genome- and organism-level evolutionary processes at play. Specifically, increases in genome size may be favored in response to selection (perhaps for reasons of metabolic efficiency, although see above), which would promote the expansion of non-coding regions and secondarily affect the development of the organism. More reasonably, the same prolonged absence of metamorphosis that allows regulatory genes to mutate could also permit the expansion of the genome, since metamorphosis is likely an important factor in limiting genome size growth in amphibians. At some stage, genome size may become too large to permit the restructuring of the soma in a short period of time, thereby acting in concert with mutations in coding genes to eliminate the process of metamorphosis entirely. Moreover, because transposable elements insert preferentially into non-genic heterochromatic regions (Dimitri & Junakovic, 1999), there would be yet another feedback loop whereby an initial increase in non-coding DNA content could promote the further expansion of non-coding elements (e.g., Nardi et al., 1999). Clearly, the interaction of the genomic and organismal phenotypes is a complex one involving cause and consequence at both of these levels in the biological hierarchy. Additionally, and independently of the complexity of the developmental process itself, the impacts of C-value on cell size and differentiation rate can apply directly to the *products* of development. Perhaps nowhere is this more apparent than in the evolution of amphibian brain morphology.

Developmental products: big genomes and simple brains

It has already been pointed out many times that erythrocyte size is positively correlated with genome size in each of the vertebrate classes, and that the sizes of these cells are influenced causally by bulk DNA content (see Gregory, 2001a,c for reviews). In both frogs and salamanders (at least), there is also a strong positive relationship between C-value and the sizes of neurons (e.g., Roth, Blanke & Wake, 1994). Coupled with the negative relationship between genome size and cell division rate (Gregory, 2001a) and the constraints on skull anatomy among amphibians, this means that:

> Animals with large genomes and large cells have fewer cells, including nerve cells, owing to reduced cell-proliferation rates. Brains of relatively large-celled amphibian taxa develop slowly and exhibit retarded differentiation of neuronal tissue,

> including degree of cell migration and formation of anatomically distinct nuclei and layers. As a consequence, brains of animals with large genomes have an 'immature' or pedomorphic appearance when compared with related taxa having smaller genomes and cells (Roth, Blanke & Wake, 1994).

In short, large genomes mean simple brains in both frogs and salamanders. However, this simplification is not merely the product of a small skull containing a limited number of large cells; it is a symptom of an 'organism-wide syndrome' of paedomorphosis caused by a truncation of development related to increased genome size (Roth, Nishikawa & Wake, 1997).

What is particularly interesting in this context is the fact that these simplifications of the brain are derived features of both frogs and salamanders. That is, brains in these lineages are believed to have been more complex in the past, and to have become simplified as a consequence of genome size increases in both groups (Roth & Schmidt, 1993; Roth et al., 1993). Obviously, this simplification of the brain runs counter to the progressive, anthropocentric view of evolution still held by many people. It also poses a significant challenge to those who continue to assert that noncoding DNA is without influence on the organismal phenotype. This latter point is especially important when one considers the large genome sizes of many species of miniaturized salamanders of the plethodontine tribe Bolitoglossini. In these animals, which have very small brain volumes, the increase in genome size has had a profound effect on organismal fitness. In fact, large genomes have forced bolitoglossine salamanders to abandon an active predation strategy in favor of an ambush strategy and to evolve associated features like a fast projectile tongue (Roth & Schmidt, 1993). In this case, evolution at the level of the genome has influenced the organism at the level of ecology.

It is something of a puzzle why this expansion of the genome, with its substantial (and, at least initially, adverse) impacts on the organismal phenotype, was not effectively countered by natural selection. For the most part, the spread of selfish DNA is implicated as a primary cause (Roth & Schmidt, 1993; Roth, Blanke & Wake, 1994; Roth, Nishikawa & Wake, 1997). However, it is questionable whether transposable element activity itself could produce an upward mutation pressure sufficiently strong to overcome the inevitable countervailing forces of organism-level selection. Positive selection for increased genome size based on a shift to a metabolically 'frugal' strategy has also been invoked (Szarski, 1983; Roth et al., 1990), although this hypothesis encounters the problems described above regarding genome size and metabolic rate in amphibians. Once again, the proper explanation may involve a complex evolutionary feedback between changes in genome size and the rates, processes, and products of development.

Amphibians: summary

According to Roth et al. (1993), "it is widely believed that living amphibians (Lissamphibia) are paedomorphic compared with their hypothesized sister taxon, the Palaeozoic temnospondyls". Furthermore, 'small genome size is plesiomorphic in amphibians, and large genomes have evolved independently at least twice within frogs and salamanders' (Roth, Blanke & Wake, 1994). Within frogs, small genomes are characteristic of direct developers which undergo a complex but time-limited development within the egg. In salamanders, on the other hand, both direct development and neoteny are associated with larger genomes. The combination of more intensive metamorphosis in frogs and the different implications of direct development may go a long way towards explaining the nearly nonoverlapping genome size ranges between anurans and urodeles.

A third general increase in genome size is found in the lungfish, and each of these is clearly a derived feature, as illustrated by fossil cell size data (Thomson, 1972; Thomson & Muraszko, 1978). The enormous genomes of aquatic urodeles and lungfish have typically been interpreted in metabolic terms, but in neither case does this seem justified. Instead, both are probably related to a relaxation of constraints on genome size based on a simplification of the developmental program. In neotenic urodeles, the loss of metamorphosis and its time-limited tissue differentiation is an obvious factor. In lungfish, the role of simplified development has been more difficult to establish, but there is now evidence that lungfish may in fact be neotenic (Joss, 1998). That these two major evolutionary trends – paedomorphosis and genome size increase – occurred together should, by now, come as little surprise. Indeed, it has been asserted that 'large genome size, with concomitant slow cell cycles and large cell sizes, has been an important proximal factor in the heterochronic evolution in salamanders' (Wake & Roth, 1989). The same is true of lungfish (Bemis, 1984).

The complexity of the developmental products can also be influenced by these effects on cell size and division rate. Amphibian brain complexity, in particular, is directly associated with these parameters, and therefore indirectly (but still causally) with genome size. Thus, developmental complexity in the amphibians takes two different meanings – one relating to the process of development, the other to the products – both of which are linked to variation in genome size. These two types of complexity are not necessarily related to one another (i.e., a more complex amphibian brain does not imply a fundamentally more complex developmental process), but both are affected by the same cell size/division rate influences of bulk DNA content. Of course, such influences are not limited to amphibians.

Insects: more on metamorphosis

Arthropods represent, by far, the dominant forms of multicellular life on the planet, yet the genome sizes of several groups (e.g., arachnids, myriapods) remain entirely unknown. And while crustaceans and insects are the best-studied groups among invertebrates, together they currently total only about 500 measured species (Gregory, 2001b). It is therefore no understatement to say that very little is known about genome size evolution in animals at large. Nevertheless, studies of genome size variation and its phenotypic impacts are not entirely lacking for invertebrates. In copepod crustaceans, for example, significant negative relationships have been reported between genome size and developmental rate (McLaren, Sévigny & Corkett, 1988; White & McLaren, 2000; G.A. Wyngaard, manuscript in preparation). Positive associations between genome size and body size have been reported in this and other invertebrate groups as well (see Gregory, Hebert & Kolasa, 2000). In short, relationships between genome size and both the rates and products of development are known to exist in invertebrates just as they do among plants and amphibians.

The current lack of insect genome size data is the most surprising, given the extreme diversity of this group. Of the mere 300 or so insect C-values measured to date, most belong to the orders containing flies (Diptera), beetles (Coleoptera), grasshoppers (Orthoptera), and aphids (Hemiptera); in most cases, only a few families are included. Several entire insect orders are represented by fewer than *five* measured species, and many orders can currently claim no representation whatsoever. As such, discussions of genome size evolution in insects are probably somewhat premature. Regardless, hints of some interesting patterns are already visible. If these prove reliable, then the current emphasis on constraints derived from developmental rate/complexity will be granted substantial support.

The 32 insect orders are divided into three broad categories according to the nature of their developmental programs (particularly with reference to wing development). The Apterygota display *ametabolous* development, such that they emerge as miniature adults and undergo no metamorphosis. The Exopterygota possess a *hemimetabolous* developmental program, in which they grow via a series of nymphal moults and display only partial metamorphosis. Finally, the Endopterygota, which display *holometabolous* development, have distinct larval stages and proceed through a complete metamorphosis. In this regard, the insects are similar to the amphibians, although insect metamorphosis, when it occurs, is considerably more intensive than even that of frogs.

Most of the insect genome sizes measured to date fall within the range of 0.2–1.5 pg. Notable exceptions include crickets and grasshoppers (Orthoptera, 1.5–16 pg), dragonflies (Odonata, 1.5–2 pg), true bugs (Hemiptera, 0.2–6.2 pg), stick insects and leaf insects (Phasmida, 2–8 pg), and cockroaches (2–3 pg). The common unifying feature of each of these orders is that they display hemimetabolous development lacking complete metamorphosis. None of the holometabolous orders so far analyzed (Diptera, Coleoptera, Hymenoptera, Lepidoptera) appears to possess members with genome sizes greater than 2 pg. In keeping with this, the firebrat *Thermobia domestica* (Thysanura), the only ametabolous insect measured to date, has an estimated genome size of 5.6 pg (Gregory, 2001b). It is also informative in this regard that while aphids (Hemiptera, suborder Homoptera) tend to have small genomes (0.2–0.7 pg), this probably results from additional constraints imposed by body size (Finston, Hebert & Foottit, 1995) (or indeed, rapid overall development). Cicadas – homopterans not so constrained – do not share the small genomes of aphids (∼4.5 pg; J.S. Johnston, unpublished). Members of the suborder Heteroptera also appear to have larger genomes (1.2–6.2 pg). In summary, and as a preliminary estimate only, it would appear that a genome size of about 2 pg represents the threshold for metamorphosis in insects. Again, the existence of such a threshold parallels the situations described previously for both plants and amphibians.

This view clearly makes some easily testable predictions (and at the very least, may stimulate the acquisition of more insect genome size data!). Specifically, as-yet-unstudied hemimetabolous exopterygote orders would be expected to contain at least some species whose genome sizes exceed the 2 pg threshold. Holometabolous endopterygote orders, by contrast, might be expected to consist entirely of species with relatively small genomes. Members of the apterygote orders, because of their ametabolous development, would be expected to display relatively high maximum genome sizes. These latter groups are of additional interest because they are considered primitive in many respects and may shed light on the historical patterns of genome size evolution in the Hexapoda. Based on the genome size of the firebrat, it is not immediately obvious that ancestral insects possessed small genomes. It is entirely possible that the genomes of holometabolous insects have been secondarily reduced as an adaptation for metamorphosis (similar to the genome reduction in a shift to an annual lifestyle among plants). Alternatively, small genomes may be the ancestral condition, with all lineages except those with complete metamorphosis experiencing an increase (similar to meatmorphosing v.s. neotenic amphibians). Or, as some recent molecular evidence seems to suggest, genome sizes in insects could, in part, be determined by rates of DNA loss, such that small genome sizes are set essentially at random, and then serve as 'pre-adaptations' for the evolution of complex development in some lineages (e.g., Petrov et al., 2000). Any of these possibilities would be very interesting if borne out by further observation.

Finally, it should be emphasized that metamorphosis is not the only relevant parameter in discussions of insect genome size evolution. Aphids, as mentioned above, have incomplete metamorphosis but tiny genomes. In these and other insects, additional selective pressures such as that for small body size and/or rapid overall life cycle may contribute significantly to the resulting C-value. Yet again, the notion here is one of thresholds beyond which metamorphosis would be impeded, but below (or in the absence of) which genome size may be quite malleable in response to various evolutionary forces.

'Hinegardner's rule': process and products in fish

It has been argued by some authors that red blood cell size is directly related to swimming performance in fish (e.g., Lay & Baldwin, 1999). If this were true, then physiological (especially metabolic) constraints might be expected to play a prominent role in influencing fish genome sizes. However, it is obvious that this is not the case, given that many excellent swimmers (e.g., salmonids, tuna, etc.) have genomes larger than sedentary or slow-moving forms like pufferfish, sea horses, and flounders (Gregory, 2001b,c).

It remains perhaps a little known fact that many groups of fish emerge as distinct larvae and undergo a process of metamorphosis during their development. Thus, a comparison of direct development versus metamorphosis is possible, as with the amphibians, in the context of fish genome sizes (but would be a paper unto itself). As a preliminary example, one may compare metamorphosing lampreys (1.3–2.1 pg) with direct-developing hagfish (2.3–4.6 pg). And, of course, there is the notion mentioned above that lungfish – whose genomes are nothing less than obese – may share many developmental properties with neotenic urodeles (Joss, 1998). The genome sizes of the extraordinarily diverse teleosts also have historically been viewed in the light of 'complexity', although generally in only vague terms.

In the first large-scale survey of animal genome sizes ever conducted, Mirsky and Ris (1951) commented that 'higher' animals tended to possess lower DNA contents than 'primitive' ones (their now-outdated anthropocentric terminology notwithstanding). Nearly two decades later, Hinegardner (1968) stated much more explicitly that "the more advanced species of fishes have less DNA than the primitive ones", and thus that "evolution and specialization in the teleosts have been accompanied by loss of DNA". However, as many authors have pointed out, 'specialization' is a decidedly nebulous term which can mean any manner of things, and would therefore be impossible to equate with a change in genome size in either direction (e.g., Cavalier-Smith, 1985; John & Miklos, 1988). In specialized parasites, for example, developmental rate may constrain genomes to small size. In homeotherms, high metabolic rate may have a similar effect. In other cases, *increases* in genome size may be favored (as related to body size, more efficient metabolism, or some other such correlated trait). Thus, in this most general sense, the existence of a relationship between genome size and 'specialization' is dependent entirely on definition. However, in regards to the original subjects of Hinegardner's (1968, 1976) discussion, the teleost fish, there seems to be a legitimate pattern worthy of exploration.

On 'fishiness'

The absolute range in genome sizes among fish (0.35–130 pg, from pufferfish to lungfish) is deceiving, given that only the dipnoans (50–130 pg) and chondrichthyes (2.5–16 pg) have very large genomes, whereas the average value for teleosts is a mere 1.3 pg (range 0.35–7 pg, with the largest values found primarily among ancient polyploids). Nevertheless, 'Hinegardner's rule' appears to apply to teleosts, with families of generalized ('fishy') fish (e.g., salmonids) possessing larger genomes on average than those with more unique and specialized morphologies (e.g., sea horses) (see Hinegardner, 1968, 1976 for details and illustrations).

This notion has generally been met with a mixture of confusion and criticism. Ohno (1974), for example, suggested that "one can not even make an intelligent conjecture as to the underlying reason for this apparent connection between nonfunctional DNA and generalized body form (in fish)". On a more critical note, Cavalier-Smith (1985) has argued that Hinegardner's (1976) description of specialization among teleosts in terms of 'fishiness' is far too subjective to be useful. Indeed, he goes so far as to state that "unless a specific measure of specialization applicable to all eukaryotes can be suggested, the suggestion that genome size is inversely related to specialization will remain in the realm of metaphysics rather than science". While it is clearly not true that a single parameter must apply to *all eukaryotes* to be considered a relevant correlate of genome size, the criticism of a vague concept of 'specialization' is well taken. More importantly, Cavalier-Smith (1985) points out several cases in which 'specialized' fish species (e.g., lungfish) have very large genomes. However, if one defines specialization in terms of developmental complexity, rather than 'fishiness', then this discussion can be reclaimed in the name of science.

As an explanation for the trend of smaller genomes of less 'fishy' fish, Hinegardner (1976) surmised that as an organism becomes specialized in one set of traits, it ceases to make use of other traits which, along with the genes coding for them, are eventually lost. This shedding of unused genes, he suggests, would eventually result in a significant reduction in genome size. This obviously cannot be the case, for two main reasons. First, the loss of newly irrelevant morphological traits is seldom carried out via the loss of the actual genes coding for their production, and instead involves gene silencing or some other regulatory effect. Not only this, but the production of novel specialized characters might, at some stage, actually involve the *addition* of new genes (e.g., by duplication and divergence of function). Second, and much more importantly, genes make up only a small fraction of most eukaryotic genomes, so the gain and loss of genes is only mildly relevant to overall genome size change.

To reiterate a point made earlier, in organisms with similar levels of developmental complexity, developmental *rate* becomes the evident constraint on genome size (as within groups of frogs, salamanders, or plants). But when time is limited (i.e., rate is held roughly constant), the important variable is developmental *complexity*, such that more complex development involving more individual 'steps' requires smaller genome size (as with frogs vs. salamanders, or in biphasic vs. neotenic salamanders). These are simply flip-sides of the same coin. It has already been shown that in cases where 'specialization' means an overall reduction in developmental complexity, larger genome sizes are the result (as among lungfish and salamanders). If, however, 'specialization' involves a time-limited increase in developmental complexity, then *reduced* genome sizes would be expected. That is, if Hinegardner's 'non-fishy' fish are so because their developmental programs include additional steps not found in generalized species, but developmental time is limited, then this could explain why some 'specialized' fish have smaller genomes. Thus, in amphibians and lungfish genomes have become large in association with *paedomorphosis*, the maintenance of juvenile characters in the adult by the deletion of developmental steps, while in fish the proposed mechanism is exactly the reverse – that the small genomes of specialized fish are associated with the *terminal addition* of more developmental steps. Although obviously in need of further testing, this (admittedly speculative) idea based on developmental complexity may help to explain some heretofore puzzling patterns in genome size variation among teleost fishes.

Concluding remarks

The concept of developmental complexity as related to genome size, though applicable to a variety of interesting questions, does not provide a solution to the C-value enigma. Of course, neither will any other single idea or observation. The C-value enigma, as stated at the very beginning of this discussion, is a complex

and multifaceted puzzle immune to one-dimensional explanations. If the notion of developmental complexity is to shed light on the issue beyond its already fairly broad applicability, it will do so by illustrating the importance of a hierarchical approach to genome size evolution. The genome itself must be recognized as a legitimate level of biological organization with its own phenotype forged by a complex evolutionary history. More importantly, there is an intimate relationship – with causation proceeding in both directions – between the phenotype of the genome and the phenotype of the organism. Variation in genome size can constrain, and simultaneously be constrained by, the evolution of the organism in which it finds itself. Developmental complexity is but one venue in which this fascinating interaction takes place.

Acknowledgements

Supported by Natural Sciences and Engineering Research Council of Canada (NSERC) post-graduate and University of Guelph Alumni Doctoral scholarships to T.R. Gregory, and by an NSERC research grant to Paul Hebert. Thanks to Dmitri Petrov for the invitation to contribute to this issue and for constructive criticism, to Spencer Johnston and Grace Wyngaard for stimulating discussions and access to unpublished data, to Sarah Adamowicz for help with the painful process of abridging a previously much more detailed manuscript, and to the two anonymous reviewers who provided helpful comments, questions, and criticisms.

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