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Evolutionary implications of the relationship between genome size and body size in flatworms and copepods

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Genome and body sizes were measured in 38 species of turbellarian flatworms and 16 species of copepod crustaceans. Significant positive relationships existed between genome size and body size in both groups. The slopes of these regressions indicated that increases in cell volume are reinforced by increased cell numbers, or that cell volumes show positive allometric variation with genome size. Genome sizes appear to vary in a discontinuous fashion among congeneric species in both groups, indicating that such changes have occurred rapidly, and with potentially profound effects on important morphological characters.

Keywords: allometry, C-value, cell volume, evolution, morphology.

Introduction

The evolutionary implications of the 80 000-fold variation in genome sizes ('C-values') among eukaryotes have long been debated. The fact that neither organismal complexity nor number of protein-coding genes shows any relationship with DNA content (known as the 'C-value paradox'; Thomas, 1971) has ruled out the simplest explanations for this diversity. As such, it is not surprising that competing theories have developed to account for variation in genome size. The noncoding DNA present in huge quantities in the genomes of certain species has been variably described as 'junk' which accumulates by drift (Pagel & Johnstone, 1992), as 'extinct' genes (Ohno, 1972), as 'selfish' parasitic elements which compete amongst themselves for maximum representation (Doolittle & Sapienza, 1980; Orgel & Crick, 1980; Orgel *et al*., 1980), or as a phenotypically relevant but sequence-independent property of the genome (Commoner, 1964; Bennett, 1972; Cavalier-Smith, 1978, 1982, 1985). In these cases, genome sizes are seen to be determined by natural selection operating either intragenomically or on the organismal phenotype. Thus, a primary test of the validity of these explanations for the C-value paradox is via an analysis of the linkage between changes in genome size and the phenotypes of individual organisms.

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Interspecific differences in genome size are known to be associated with variation in important cellular parameters, most notably cell volume (Mirsky & Ris, 1951). The DNA contents and volumes of cells are interrelated in all major groups including prokaryotes, protists, algae, plants, vertebrates, and invertebrates (e.g. Holm-Hansen, 1969; Pedersen, 1971; Price *et al*., 1973; Olmo & Morescalchi, 1975, 1978; Szarski, 1976; Cavalier-Smith, 1978, 1982, 1985; Shuter *et al*., 1983; Walker *et al*., 1991). Indeed, it has been asserted that 'the most reliably established fact about genome evolution is that C-values are generally positively correlated with cell and nuclear volumes' (Cavalier-Smith, 1982). Proponents of the selfish and junk DNA hypotheses have suggested that this relationship is strictly correlational, with larger cells 'tolerating' greater amounts of phenotypically neutral DNA (Orgel & Crick, 1980; Orgel *et al*., 1980; Pagel & Johnstone, 1992). Others favouring the 'nucleoskeletal hypothesis' have argued that larger cells require larger nuclei for more efficient RNA transport into the cytoplasm (Cavalier-Smith, 1978, 1982, 1985). Pagel & Johnstone (1992) argued that the nucleoskeletal hypothesis failed to gain support from their survey of genome sizes, developmental rates, and nucleocytoplasmic ratios in salamanders, and concluded that only the junk DNA hypothesis could explain the correlation between developmental rate and genome size. However, these authors ignored the possible role of DNA content in *determining* cell sizes and division rates (and hence developmental rates). Moreover, the direct

effects of ploidy shifts and B chromosomes on cell volume (Nurse, 1985) indicate that DNA content has important *direct* effects on cell size. This result, coupled with the well-established link between cell volume and key physiological characteristics such as metabolic rate (e.g. Vinogradov, 1995, 1997) and development time (e.g. Horner & Macgregor, 1983; Sessions & Larson, 1987), lends support to the selectionist hypothesis that cell volumes (and by extension, genome sizes) have substantial impacts on organismal fitness (see Gregory & Hebert, 1999). However, it is also important to know if changes in cell volume (and genome size) have larger-scale (i.e. macroevolutionary) effects which are exerted through influences on morphology as well as physiology. One such potential correlate with cell volume and C-value is body size.

Three factors determine the role of genome size in explaining body size variation among a group of allied taxa. The first factor is simply the extent of genome size variation among these taxa; homeothermic vertebrates show little variation, whereas members of some invertebrate classes show several hundred-fold variation in genome size (Li, 1997). As a result, genome size-related cell volume shifts are of little import in explaining body size diversity in the former lineages, but may be important in the latter. A second factor which influences the extent of this effect is the scaling of the relationship between genome size and cell volume. There is variability — even among closely allied lineages — in the nature of the response. Whereas some groups show a negative allometric relationship so that large changes in genome size produce only slight changes in cell size, others show positive allometry so that dramatic changes in cell size accompany modest genome size shifts (Cavalier-Smith, 1982). The third factor which determines the organismal impact of genome size diversity is the regulation of mitotic divisions. Some invertebrate taxa show cell number determinism, so shifts in cell volume necessarily have an impact on body size. By contrast, some vertebrate lineages, such as amphibians, show a compensatory reduction in the number of cell divisions in response to increased cell size so that body size remains static (Fankhauser, 1955). Although not yet demonstrated, it is possible that other lineages show a positive correlation between the number of mitotic cycles during development and cell size, leading to a reinforcement of body size shifts induced by cell size changes. Because of the complexity of factors which determine body size, efforts to ascertain the role of genome size logically commence with an examination of both the extent of genome size diversity among taxa and the strength of the relationship between this diversity and body size itself. In those cases where genome sizes and body sizes are positively correlated, it can subsequently be determined if this arises solely from effects on cell size, or from joint shifts in cell size and cell number.

The present study aims to extend understanding of the relationship between variation in body and genome sizes by examining two taxonomically and structurally divergent groups of invertebrates which have previously been the subject of only limited work. The turbellarian flatworms are a speciose lineage whose members show sufficiently marked variation in size to justify their partition into micro- and macroturbellarians. Lacking an external skeleton, turbellarians show body shapes which vary from tubular to planar sheets. Past work on genome size diversity in turbellarians has been restricted to a few large-bodied species which show exceptionally large genomes for invertebrates (Hebert & Beaton, 1990). The copepod crustaceans are also taxonomically diverse, but show much more limited variation in body structure and size. Intensive studies have been carried out on genome size variation in species belonging to two closely allied genera of copepods, *Calanus* and *Pseudocalanus* (Robins & McLaren, 1982; McLaren *et al*., 1988, 1989). This work has established that body size variation among species in these genera is tightly correlated with differences in genome size, but no effort has yet been made to place these results in a broader taxonomic context. Thus, the primary goal of the present study was to elucidate the nature of the relationship between body size and DNA content across a wide taxonomic sample within these two groups of invertebrates.

Materials and methods

Collection, measurement and preparation

Individuals from 38 species of turbellarian flatworms (Phylum Platyhelminthes, Class Turbellaria, various Orders) and 16 species of copepods (Phylum Arthropoda, Class Crustacea, Order Calanoida) were collected from natural populations and identified according to Kolasa (1991) and Wilson (1959), respectively (Tables 1 and 2). The body sizes of flatworms were calculated as cylindrical volumes (in mm^3) using measurements of body length and width in mature individuals (body volume = $[\pi \cdot (\frac{1}{2}\ \text{width})^2 \cdot \text{body length}]$). This cylindrical volume was halved to account for the flatness of some species which did not possess an approximately circular cross-section (see Table 1). Adult copepod body sizes were measured simply as body length (in mm) from head to tail.

In preparation for Feulgen staining, one or more individuals of each species of flatworm were placed onto a microscope slide in a few drops of water, and squashed using a cover slip. A second cover slip was used to smear the cells across the slide, and a few additional drops of water were added as necessary to disperse the cells and

Table 1 Diploid (2C) genome sizes, body sizes, and source locations for 38 species of turbellarian flatworms. Body volume measurements which were corrected for flatness are indicated by an asterisk (*)

Order and species	Genome size (pg)	Body size (mm^3)	Source
Order Alloeocoela			
Bothrioplana semperi	9.40	0.86*	Ausable River, Ontario
Hydrolimax grisea	5.10	77.72	Detroit River, Ontario
Otomesostoma auditivum	41.04	2.68	Churchill, Manitoba
Prorhynchus stagnalis	8.56	0.408*	Gull Lake, Michigan
Order Catenulida			
Myostenostomum tauricum	0.38	0.003	Detroit River, Ontario
Rhynchoscolex simplex	3.36	0.62	Detroit River, Ontario
Stenostomum arevaloi	0.62	0.19	Detroit River, Ontario
Stenostomum beauchampi	0.46	0.004	Ojibway Park, Ontario
Stenostomum brevipharyngium	0.12	0.001	Detroit River, Ontario
Stenostomum grande	1.50	0.17	Point Pelee, Ontario
Stenostomum unicolor	0.40	—	Detroit River, Ontario
Order Kalypthorhynchia			
Gyratrix hermaphroditus	5.48	0.106	Point Pelee, Ontario
Order Macrostomida			
Macrostomum gilberti	1.16	0.002*	Point Pelee, Ontario
Macrostomum sensitivum	0.34	0.007*	Ausable River, Ontario
Microstomum lineare	0.96	0.926	Ausable River, Ontario
Order Tricladida			
Dugesia polychroa	2.64	43.57*	Lake St. Clair, Ontario
Dugesia tigrina	3.76	12.56*	Windsor, Ontario
Foviella affinis	3.60	—	New Brunswick
Hymanella retenuova	8.92	9.92*	Ojibway Park, Ontario
Phagocata woodworthi	7.52	64.12*	Michigan
Polycelis nigra	4.12	8.41*	England
Procerodes litoralis	2.12	2.65*	New Brunswick
Procotyla fluviatilis	5.24	51.29*	New Brunswick
Uteriporus vulgaris	2.28	—	New Brunswick
Order Typhloplanida			
Bothromesostoma sp.	7.54	2.01	Churchill, Manitoba
Castrella pinguis	2.68	0.29	Detroit River, Ontario
Dalyellia viridis	2.06	5.08	Rondeau Park
Gieysztoria sp.	1.26	0.23	Point Pelee, Ontario
Krumbachia hiemalis	3.34	1.54	Ausable River, Ontario
Mesostoma arctica	10.20	2.26	Churchill, Manitoba
Mesostoma ehrenbergii	32.70	33.66*	Rondeau Park, Ontario
Olisthanella truncula	0.24	0.06	Point Pelee, Ontario
Phaenocora sp. 1	1.44	0.70*	Churchill, Manitoba
Phaenocora sp. 2	3.22	—	Rondeau Park, Ontario
Rhyncomesostoma sp.	4.32	—	Ojibway Park, Ontario
Strongylostoma elongatum	1.66	—	Stony Point, Ontario
Strongylostoma radiatum	1.30	—	Point Pelee, Ontario
Typhloplana viridata	1.96	0.014	Windsor, Ontario

avoid clumping. The squashes were air-dried and stored in the dark until staining. Copepods were fixed at the time of collection in a 3:1 (v:v) solution of methanol and glacial acetic acid and stored in 70% ethanol at 4°C until staining.

Feulgen staining

Flatworm squashes and unfixed whole-body copepods were fixed for 60 min in a solution of 3:1 (v:v) methanol and glacial acetic acid followed by a 30-min wash in

Table 2 Diploid (2C) genome sizes, body sizes, and source locations for 16 species of copepods

Species	Genome size (pg)	Body size (mm)	Source
Order Calanoida			
Diaptomus forbesii	7.62	1.54	Saskatoon, Saskatchewan
Diaptomus insularis	3.82	0.90	Saskatoon, Saskatchewan
Diaptomus leptopus	5.54	2.08	Saskatoon, Saskatchewan
Diaptomus nudus	6.66	1.60	Saskatoon, Saskatchewan
Diaptomus sicilis	3.54	1.20	Alberta
Eurytemora composita	1.58	1.16	Churchill, Manitoba
Hesperodiaptomus n. sp.	11.08	3.23	Churchill, Manitoba
			Cow Creek, Oregon
			Thunder Basin, Wyoming
Hesperodiaptomus arcticus	9.34	3.23	Churchill, Manitoba
			Cow Creek, Oregon
Hesperodiaptomus nevadensis	11.42	3.15	Saskatoon, Saskatchewan
Hesperodiaptomus shoshone	6.22	—	Rexburg, Idaho
Hesperodiaptomus victoriaensis	8.74	2.70	Churchill, Manitoba
Heterocope septentrionalis	10.90	3.35	Churchill, Manitoba
Leptodiaptomus tyrrelli	2.66	1.60	Churchill, Manitoba
Leptodiaptomus wilsonae	6.50	1.26	Churchill, Manitoba
Limnocalanus macrurus	3.26	2.00	Detroit River, Ontario
Osphranticum labronectum	4.90	1.25	Houston, Texas

distilled water. Copepods stored in 70% ethanol were passed through a graded ethanol dilution series (up to distilled water). After washing in distilled water, all samples were hydrolysed at room temperature for 30 min in 5N HCl. The samples were then rinsed for 1 min in 0.1N HCl in order to prevent the carry-over of strong acid, and were stained in freshly prepared Schiff leucofuchsin sulphurous acid reagent for 100 min. Staining was followed by three 5-min rinses in fresh bisulphite solution, a 10-min wash in gently running tapwater, and three 2-min rinses in distilled water. Flatworm slides were allowed to air-dry and were then stored in the dark until DNA quantification. Whole-body copepods were stored in 20% ethanol at 4°C until dissection, at which time the epidermal layers (which are known to consist of diploid cells; McLaren *et al*., 1989) were removed, placed on standard microscope slides, and allowed to air-dry.

Eight internal standards of known diploid genome size were included in each staining run, and consisted of laboratory cultures of one cladoceran species (*Daphnia pulex*, 0.74 pg) and two ostracods (*Cypridopsis vidua*, 1.4 pg and *Cyprinotus incongruens*, 1.8 pg), erythrocytes from two fish species (*Poecilia sphenops*, 1.8 pg and *Oncorhynchus mykiss*, 5.2 pg) and one amphibian species (*Xenopus laevis*, 6.3 pg), and two turbellarian species (*Mesostoma arctica*, 10.2 pg and *M. ehrenbergii*, 32.7 pg) (Fig. 1).

DNA quantification

The absorbencies (optical densities, OD) of Feulgen-stained nuclei were determined using a Wild-Leitz DADS scanning microspectrophotometer equipped with an argon lamp, a 546-nm (20 nm bandwidth) interference filter, a 0.1 μm stage-stepping interval, and a 0.4 μm scanning aperture. Nuclei were measured at 100× objective magnification (immersion oil, $n_D = 1.518$). As pilot studies indicated that DNA content variation among nuclei in a single individual was small, genome size estimates were derived from the analysis of 10 nuclei per individual. To ensure that variation amongst individuals was considered, nuclei from a minimum of three individuals were examined from each species. Damaged or overlapping nuclei were not analysed. The areas of individual nuclei were measured simultaneously with OD, and multiplied by background-corrected optical densities to determine an integrated optical density (IOD) for each nucleus. The regression of the relationship between mean nuclear IOD and DNA content determined from the known standards (all $r^2 \geq 0.95$) was used to calculate an absolute diploid genome size (in pg) for each specimen of unknown C-value.

Some cyclopoid copepods exhibit chromatin diminution, whereby large amounts of DNA present in the early zygote are deleted from the somatic cell line (Dorward & Wyngaard, 1997). In these cases, basal

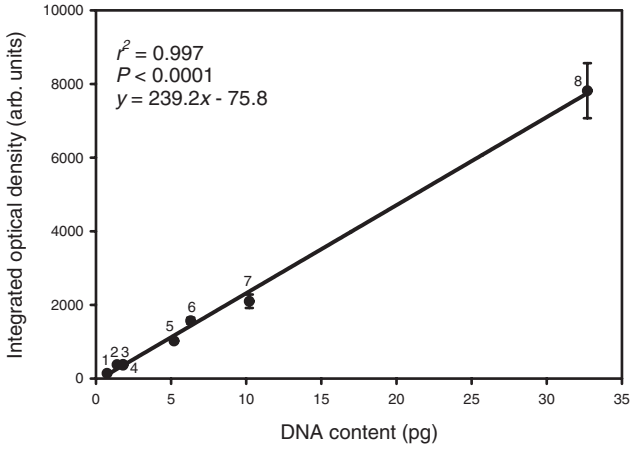

Fig. 1 Standard curve of the type used to calculate genome sizes of the flatworms and copepods in the present study. The standards of known diploid (2C) DNA content were as follows: 1, *Daphnia pulex* (0.74 pg); 2, *Cypridopsis vidua* (1.4 pg); 3, *Cyprinotus incongruens* (1.8 pg); 4, *Poecilia sphenops* (1.8 pg); 5, *Oncorhynchus mykiss* (5.2 pg); 6, *Xenopus laevis* (6.3 pg); 7, *Mesostoma arctica* (10.2 pg); 8, *Mesostoma ehrenbergii* (32.7 pg). Unknown genome sizes were calculated from the regression line of the relationship between DNA content and integrated optical density (IOD).

genome sizes estimated from the survey of gametes differ from those obtained using diploid somatic cells. However, this phenomenon has not been found in any calanoid copepod studied to date. Moreover, in cases where differences in DNA content exist between somatic and gametic tissues, it is desirable to use somatic cell DNA contents rather than sperm DNA contents in comparisons which seek to examine the relationship between genome size and body size.

Results and discussion

Genome size, cell volume and body size

The diploid (2C) genome sizes of the flatworm species examined in the present study varied more than 300-fold, from 0.12 pg in *Stenostomum brevipharyngium* to 41.04 pg in *Otomesostoma auditivum*. Although variation was apparent between allied species, some orders contained mostly large-genome species, whereas others included only species with small genomes (see Table 1; Fig. 2a). Genome sizes among copepods were much less diverse, showing only 7-fold variation, and ranging from 1.58 pg in *Eurytemora composita* to 11.42 pg in *Hesperodiaptomus nevadensis* (see Table 2).

Body size was positively correlated with genome size in both flatworms ($r^2 = 0.50$, $P < 0.0001$) and copepods ($r^2 = 0.53$, $P < 0.003$) (Fig. 2a,b). Similar relationships have been described for other invertebrate groups

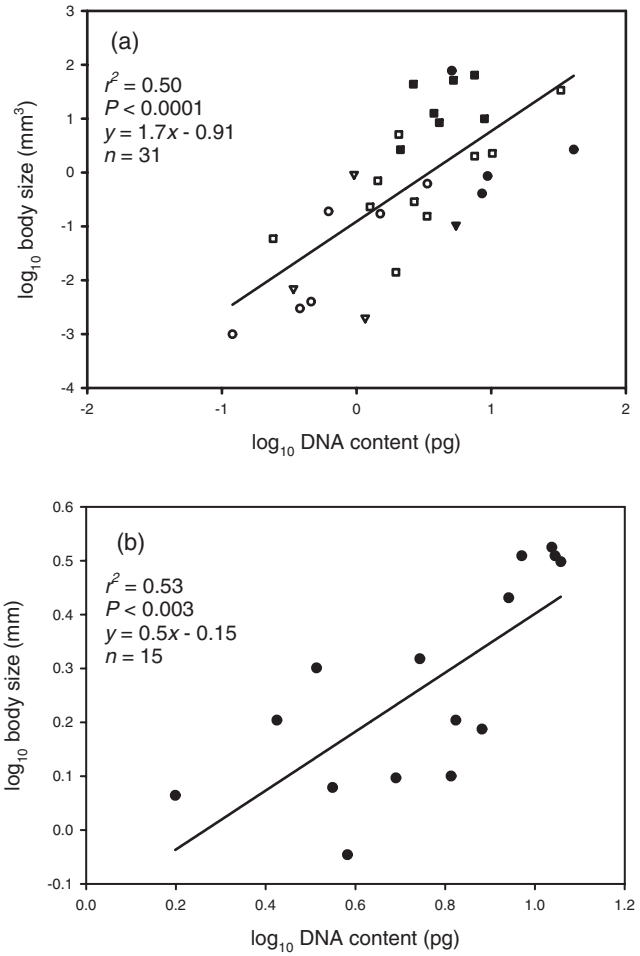

Fig. 2 Relationships between diploid genome size and body size in (a) turbellarian flatworms (body size expressed as volume in mm^3), and (b) copepods (body size expressed as length in mm). Body size measurements were not available for all species. Different symbols represent different taxonomic groups of (a) turbellarians (● = Order Alloeocoela, ○ = Order Catenulida, ▼ = Order Kalypthorhynchia, ▽ = Order Macrostomida, ■ = Order Tricladida, □ = Order Typhloplanida), and (b) copepods (Order Calanoida).

including aphids (Finston *et al*., 1995), flies (Ferrari & Rai, 1989) and molluscs (Hinegardner, 1974), indicating that the relationship persists among both hard- and soft-bodied organisms. Most notably, a positive association between body size and genome size has been reported for members of the copepod genera *Calanus* (McLaren *et al*., 1988) and *Pseudocalanus* (Robins & McLaren 1982). These latter groups provide particularly valuable information because they exhibit determinate cell numbers (McLaren & Marcogliese, 1983). Thus, changes in body size in these genera are necessarily the result of alterations in cell volume, rather than cell number.

Because length was measured in the copepods studied, ''body size'' simply represents the sum of the

diameters of adjacent cells aligned in the direction of measurement. Because cell volume varies with the cube of diameter, and because cell volume and genome size are approximately linearly related (although the relationship is believed to be somewhat weaker than unity — see below), body length would be expected to vary with the cube-root of genome size (i.e. slope of log–log relationship equal to ~1/3) in cases where cell volume was the only parameter responsible for its variation. By contrast, the copepods analysed in this study showed a slope of 0.5 (Fig. 2b). Among species of both *Pseudocalanus* and *Calanus*, the slope was equal to ~0.9 (McLaren *et al*., 1988, 1989). Hence, both studies suggest a higher than expected slope of the relationship between genome size and body size, and in the latter case, because of cell determinism, this must reflect positive allometries between genome size and cell size.

Because volumes were measured for flatworms, body size would be expected to vary directly with genome size (i.e. slope of log–log relationship equal to ~1.0) if cell volume shifts alone were responsible for variation in body size and showed a linear relationship with genome size. In the present study, the relationship between body size and genome size among turbellarian flatworms showed a slope of 1.7 (Fig. 2a). Thus, in both groups studied here, body size scaled with genome size more strongly than predicted on the basis of a linear relationship between cell volume change and genome size. This is true even within single genera of flatworms (e.g. *Stenostomum*, slope = 2.20) and copepods (e.g. *Diaptomus*, slope = 0.64; *Hesperodiaptomus*, slope = 0.44). It is therefore clear that in these groups either cell numbers shift in concert with changes in cell volume or cell volumes shift in a positively allometric fashion with changes in genome size.

The relationship between cell volume and genome size can be represented by the equation $V = kC^{\alpha}$ where V = volume, C = C-value, k is a constant and α is the slope of the log–log relationship. In the absence of shifts in cell number, the scaling between genome size and cell size is critical to the determination of effects on body size. Negative allometric relationships ($\alpha = 0.83$) have been reported in angiosperms, unicellular algae, and anuran amphibians (Holm-Hansen, 1969; Price *et al*., 1973; Olmo & Morescalchi, 1978; Cavalier-Smith, 1982). However, in other groups such as the urodele amphibians, there is a strongly positive allometry ($\alpha = 1.85$) (Olmo & Morescalchi, 1975; Cavalier-Smith, 1982). The difference in scaling between anurans and urodeles is so profound that a fivefold shift in genome size is accompanied by more than an order of magnitude increase in cell volumes in urodeles, but only a twofold increase in cell volume in anurans. The strong relationships between genome size and body size detected in this study demand either a strongly positive allometric relationship between C-value and cell volume or alternatively the reinforcement of cell size shifts by additional mitotic cycles. Given the evidence for a positive allometric relationship in the copepod genera which have been studied intensively, it is parsimonious to assume that much of the body size variation detected in the present survey of these organisms has a similar derivation. However, verification of this possibility requires a determination of the extent of variation in cell numbers among taxa. Similar surveys of cell number and size are required for the Turbellaria.

Patterns of genome size variation

The DNA contents of closely related species of invertebrates often vary in a 'quantum' fashion with genome sizes varying as multiples of some basal C-value. Such quantum variation has been demonstrated in species of *Calanus* and *Pseudocalanus* (McLaren *et al*., 1989), and for other invertebrate groups such as polychaetes (Gambi *et al*., 1997), tardigrades (Garagna *et al*., 1996), anemones (Rothfels *et al*., 1966), aphids (Finston *et al*., 1995) and other insects (Hughes-Schrader & Schrader, 1956). The same pattern of discontinuous variation occurs among prokaryotes (Wallace & Morowitz, 1973; Li, 1997) and some plants (Rees, 1972; Narayan, 1982, 1983). These discontinuous jumps in genome size are clearly not the result of polyploidy, because chromosome numbers in many of these groups are static.

The taxonomic focus of the present study was not sufficiently narrow to permit a detailed investigation of the prevalence of quantum shifts, but the large differences in genome size among some allied species suggest that this phenomenon occurs in both groups. For example, genome sizes among members of the genus *Stenostomum* closely approximated a quantum series with steps of 0.12 pg. Similarly, members of the genus *Macrostomum* appear to follow a series with a basal unit of 0.3 pg, and among the two members of *Dugesia* examined, there is the suggestion of a series with steps of 1.3 pg. Other genera, including *Phaenocora* and *Mesostoma* suggest doubling and tripling series, respectively (see Table 1). A similar situation existed among the copepods, with genome sizes among members of the genus *Hesperodiaptomus* showing an approximation to a quantum series with a basal unit of ~3 pg (see Table 2). This latter value is consistent with the patterns described for other copepod genera (*Calanus* and *Pseudocalanus*), whose genome sizes vary in a discontinuous series with a basal value of ~4 pg (McLaren *et al*., 1989). A more intensive sampling of species within the genera studied here will be required before

the presence of quantum shifts can be established conclusively, however.

Assuming determinate cell numbers and the lowest α values reported (0.83), a doubling in genome size would lead to a 20% increase in body length (or a 75% increase in body volume). Although changes in body size of this magnitude are substantial, the actual effect of quantum shifts in genome size will be much more profound in groups where there is a positively allometric scaling of cell volume with genome size. The basal genome sizes of the species in question are also relevant, as the doubling of a larger genome will have a greater effect on *absolute* body size than would the doubling of a smaller genome.

It is clear that changes in genome size among congeneric species, particularly in cases where shifts in genome size occur in a discontinuous series, can affect important morphological parameters. The fact that quantum shifts in genome size are common within many groups, and that such shifts can have profound effects on body size via effects on cell volume, suggests that changes in genome size play an important role in explaining the enormous morphological diversity of invertebrates. It is also clear that the evolutionary role of genome size variation is complex and is unlikely to be understood without a large-scale investigation into its occurrence in a variety of organisms.

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