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A BIRD'S-EYE VIEW OF THE C-VALUE ENIGMA: GENOME SIZE, CELL SIZE, AND METABOLIC RATE IN THE CLASS AVES

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Abstract.—For half a century, variation in genome size (C-value) has been an unresolved puzzle in evolutionary biology. While the initial ''C-value paradox'' was solved with the discovery of noncoding DNA, a much more complex ''C-value enigma'' remains. The present study focuses on one aspect of this puzzle, namely the small genome sizes of birds. Significant negative correlations are reported between resting metabolic rate and both C-value and erythrocyte size. Cell size is positively correlated with both nucleus size and C-value in birds, as in other vertebrates. These findings shed light on the constraints acting on genome size in birds and illustrate the importance of interactions among various levels of the biological hierarchy, ranging from the subchromosomal to the ecological. Following from a discussion of the mechanistic bases of the correlations reported and the processes by which birds achieved and/or maintain small genomes, a pluralistic approach to the C-value enigma is recommended.

Key words.—Cell size, C-value paradox, DNA content, erythrocytes, flight, metabolic rate, nucleotype.

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More than 50 years ago, at a time when the chemical basis of heredity was still a subject of controversy, Boivin and the Vendrelys proposed that the apparent constancy of DNA amounts within animal species could be taken as strong support for DNA as the genetic material (Boivin et al. 1948; Vendrely and Vendrely 1949, 1950). That is, if DNA is constant in amount within every chromosome set of a species (but protein is not), then surely this implies a hereditary role for DNA. From this line of argumentation emerged the concept of the ''C-value'' (Swift 1950), a term that is now used interchangeably with ''genome size,'' at least in reference to diploid animals. (Note that C was introduced to signify the haploid, or 1C *class* of DNA. It does *not* stand for ''constant'' or ''characteristic.'')

While the DNA constancy hypothesis survived several challenges in the 1950s and 1960s, a deeper problem soon became exposed: Genome size bears no relationship to organismal complexity. If C-values are constant because DNA is the stuff of genes, then how could they be unrelated to gene number? This initially surprising puzzle became known as the ''C-value paradox'' (Thomas 1971), a term that continues to enjoy widespread use. However, the paradox dissolved with the discovery of vast quantities of noncoding DNA within eukaryotic genomes, and this terminology has therefore long been out of date. Nevertheless, several puzzling issues do remain involving the origin(s); taxonomic distribution; and cellular, organismal, and ecological correlates and associated reasons for maintenance and/or loss of this noncoding DNA. To make matters more complex, each of these questions comprises both mechanistic and evolutionary components. These various issues in combination make up the much broader ''C-value enigma'' (Gregory 2000, 2001a).

One of the most important things to be recognized regarding the C-value enigma is that although these questions are all interrelated, they are also independent. A single overarching solution to the problem of genome size evolution does not exist. It is therefore a false dichotomy to pit theories dealing with the spread of DNA (e.g. junk and selfish DNA theories) against those discussing the consequences of this spread (e.g. nucleotypic theory; for a review see Gregory 2001a). The C-value paradox may have been solved decades ago with the simple discovery of noncoding DNA, but the C-value enigma is immune to one-dimensional explanations. Instead it appears that although there are important universals, the specific treatment of the C-value enigma must vary on group-by-group basis. For example, phenomena relevant to genomic expansion or constraint in amphibians (or fish, or insects, or plants) may not be very important in homeotherms and vice versa. Of course, the same is true of any biological character.

It has long been recognized that birds possess high metabolic rates compared to other vertebrates (Welty 1955). It has also been established for some time that there is a ''comparative minuteness of the corpuscles . . . throughout the class of birds'' (Gulliver 1875, p. 475), and moreover that avian ''erythrocyte size appears to be related to the general metabolic activity of the species'' (Hartman and Lessler 1963, p. 467). For more than half a century it has been known that birds have genomes smaller than those of most other vertebrates (Vendrely and Vendrely 1949, 1950; Mandel et al. 1951; Mirsky and Ris 1951), and it was similarly shown very early that ''in the nucleated red cells of vertebrates . . . there is an approximately direct relationship between cell mass and DNA content'' (Mirsky and Ris 1951, p. 461).

Szarski (1970, p. 652) was perhaps the first to unite these independent observations by emphasizing that ''small cells and a small amount of DNA in the nucleus characterize groups with a high metabolism.'' As an extreme example, ''birds have the highest metabolism accompanied by the smallest cell size and by the smallest amount of DNA per nucleus'' (Szarski 1970, p. 652). Many general discussions of the nature and importance of the relationship between genome size and erythrocyte volume have emerged since that time (e.g., Cavalier-Smith 1978, 1985, 1991; Szarski 1970, 1976, 1983; Olmo 1983; Gregory 2001a). As related to homeotherms, Cavalier-Smith (1985, p. 128) suggests that ''strong stabilizing selection for optimal red cell volume is

a major selective force that maintains a relatively uniform cell volume in mammals and birds (and secondarily causes the uniformity in C-values).'' The special relevance of this cellular constraint in birds and bats arising from the high metabolic demands of powered flight has been discussed many times (Burton et al. 1989; Tiersch and Wachtel 1991; Baker et al. 1992; Wachtel and Tiersch 1993; Hughes and Hughes 1995; Gregory and Hebert 1999; Hughes 1999; Gregory 2000). To wit, birds and bats have smaller genomes than their relatives because they require high metabolisms, which require small cells, which require small DNA contents.

Thus, as subjects for the study of genome size evolution, birds present a mixed blessing. On the one hand, the physiological demands of an airborne lifestyle suggest that metabolic constraints on cell and genome sizes should be especially important, and therefore identifiable as such, in this group. On the other hand, their genome sizes cover only a very narrow (approximately two-fold) range, making direct tests of such relationships difficult within the class. Despite this rather frustrating state of affairs, several recent lines of evidence suggest that a relationship may indeed be discernable between C-value and cellular and metabolic parameters in our fine feathered friends. First, Vinogradov (1995) reported results suggestive, but not demonstrative, of a negative relationship between genome size and mass-corrected metabolic rate across numerous bird species. Such a relationship was later found within a much more restricted sample of passerines (Vinogradov 1997). Yet in mammals, among whom genome sizes are only slightly more variable (about four-fold), a clearly significant negative relationship is found between these two parameters (Vinogradov 1995). Notably, Vinogradov (1995, p. 1253) pointed out that the correlations between C-value and metabolic rate ''look similar in birds and mammals, but the ranges of both genome sizes and of metabolic-rate residuals in birds seem not broad enough to become significant.'' Second, and in keeping with these metabolic rate relationships, a strong positive correlation has recently been reported between genome size and red blood cell size in mammals, despite the narrow range in C-values and the uniquely enucleate nature of mammalian erythrocytes (Gregory 2000). There is also a strong negative correlation between erythrocyte diameter and body temperature in mammals (Salienko 1995). Third, a general inverse association between flight ability and genome size has been shown to exist among birds (Hughes 1999).

Birds have similarly been something of a tease in terms of cell size. Whereas significant positive relationships between genome size and erythrocyte size have been demonstrated for all other vertebrate classes (amphibians: Olmo and Morescalchi 1975, 1978; De Smet 1981; Horner and Macgregor 1983; reptiles: De Smet 1981; Olmo and Odierna 1982; fish: Pedersen 1971; Olmo 1983; mammals: Gregory 2000), no such relationship has been demonstrated (or even properly investigated) in birds. De Smet (1981) compared genome size and dry cell area in 15 species of birds and found a positive but nonsignificant correlation coefficient ($r = 0.34$) between the two, and although other studies have found birds to fit well along the general vertebrate regression line (Commoner 1964; Olmo 1983), the existence of a relationship between genome size and cell size within the class Aves currently remains to be shown. Given the assumptions involved in many theoretical interpretations of genome size evolution in birds, it is a substantial understatement to say that the relationship between C-value and cell size has not been examined sufficiently in this group.

To date, genome sizes have been reported for approximately 160 species of birds (Gregory 2001b), which represents less than 2% of the class. By comparison, C-values have been measured in some 300 species of reptiles (4%), 320 mammals (7%), 400 amphibians (8%), and 900 fish (3%; Gregory 2001b). Thus, in both absolute and relative terms, birds are the least-studied vertebrate class when it comes to genome size. Nevertheless, the available data are now sufficient for a proper evaluation of the relationships between genome size and cell size in this class. Such a test represents one of the primary objectives of the present study. A second goal of this study was to reexamine the relationship between C-value and metabolic parameters. The mechanistic bases for the relationships found will be discussed in some detail, as will their implications for bird evolution in particular, and for genome size evolution in general.

Materials and Methods

The present study made use of previously published data on cell and nuclear sizes, haploid genome sizes (C-values), and active and resting metabolic rates.

Cell and Nucleus Size

Cell size data were taken from three sources, including the classic survey of Gulliver (1875) and subsequent studies by Bartsch et al. (1937) and Hartman and Lessler (1963). Gulliver's (1875) contribution marked the first systematic measurements of erythrocyte sizes, and included data from all vertebrate classes (with birds being the best-represented group). Although not always properly acknowledged, these meticulous measurements of dry cell diameters—made over a period of more than 25 years—are still often used in textbooks and the primary literature. As to the accuracy of Gulliver's (1875) measurements, his values for various mammals have recently been found to differ by only 1–4% compared to those obtained using image analysis (Gregory 2000) and light and scanning microscopy (Benga et al. 2000).

In birds, unlike mammals, mature erythrocytes assume a flattened elliptical shape rather than a circular biconcave disk, such that a single mean diameter measurement is not an appropriate indicator of cell size in this class (Gregory 2000). As such, the two dry diameter measurements provided for each species were used to calculate dry elliptical areas (area $= \pi \times$ ½length $\times$ ½width) in the present study.

Gulliver's (1875) survey was by far the most comprehensive, but he provided measurements of nucleus size for only a small percentage of the species included. Bartsch et al. (1937), however, presented data on both cell and nuclear sizes for some 50 species of birds. Hartman and Lessler (1963) similarly reported both nucleus and cell sizes for their 124 species of birds, but because most of these were tropical species not commonly studied, their data were of limited use in comparisons with the available genome size and metabolic rate datasets. For the most part, comparisons between cell

size and genome size or metabolic rate involved Gulliver's (1875) data, whereas the relationship between nucleus size and cell size was based largely on the other two studies.

Overlap between the three studies was minimal, but it was nevertheless apparent that variation did exist from one study to another. However, it was not possible to determine a reliable correction factor based on this limited overlap (no more than five species for any two studies). As such, data from each study were analyzed separately wherever sample sizes permitted. This provided internal consistency to the datasets and allowed for an independent assessment of the relationships based on three distinct taxonomic samples.

Metabolic Rate

Data on resting and active metabolic rates were taken from the compilation of Bennett and Harvey (1987). As in Vinogradov (1995, 1997), these were converted from kcal/day to ml O_2 h^{-1} under the assumption that 1 L O_2 = 4.8 kcal, and then computed as mass-specific rates (ml O_2 $h^{-1}g^{-1}$) using the associated body size data given in Bennett and Harvey (1987). Wherever multiple values were available for a single species, only the lowest mass-specific value was used (as in Bennett and Harvey 1987). Active metabolic rate represents oxygen consumption during activity as measured or calculated by any of several techniques (see Bennett and Harvey 1987).

Genome Size

The present analysis made use of genome size data from 18 different studies standardized using a value of 1.25 pg for chicken and/or 3.50 pg for human (for original sources, see Gregory 2001b). It is important to note that these data have been measured in several different ways (flow cytometry, Feulgen densitometry, reassociation kinetics, biochemical analysis), with varying degrees of reliability. Nevertheless, multiple reports for a single species tend to differ only slightly among studies once standardized (Tiersch and Wachtel 1991), such that they could simply be averaged to give a single value. In one notable exception, values for the 18 species measured by De Smet (1981) were not included because they were generally inconsistent with those of other authors and because the two internal standards used (chicken and human) gave discordant results. Any residual error in the genome size data resulting from methodological differences among studies will be random with respect to the other parameters with which they are being compared and is therefore not considered statistically problematic.

Statistical Analyses

Relationships among C-values, nucleus and cell sizes, and resting and active metabolic rates were assessed using least-squares regression and Pearson correlation analysis of log-transformed data. Regressions involving metabolic rate data were corrected for body mass using the regression residuals of all parameters against body mass. Cell size, and to a lesser extent C-value, tended to correlate positively with body mass (a fact first noted in Gulliver 1846), so these parameters were also mass corrected when used in comparisons with metabolic rate.

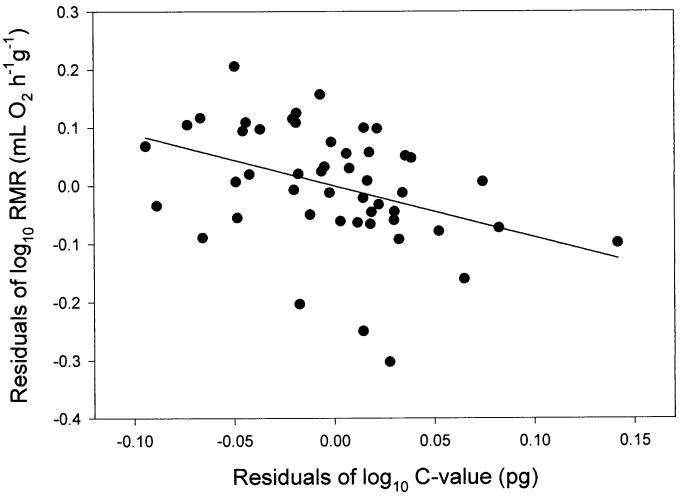

FIG. 1. Relationship between regression residuals of C-value versus body mass and resting metabolic rate (RMR) versus body mass in 50 species of birds ($r = -0.39$, $P < 0.005$). Genome size data from Gregory (2001b); metabolic rate data from Bennett and Harvey (1987).

To date, a phylogeny including all the bird species used in the present analysis is unavailable. As such, the most desirable methods of correcting for phylogenetic nonindependence of data (namely analyses of phylogenetically independent contrasts) were not possible. As an alternative, mean group data were evaluated at each of the specific, generic, familial, and ordinal levels (Vinogradov 1997; Gregory 2000) using the taxonomy of Howard and Moore (1994) and with species names updated from older references according to Peters and successors (1931–1987). Family means were based on generic means, such that each genus contributed only once to the family mean. Similarly, ordinal means were based on familial means. Regression residuals used in metabolic rate correlations were not averaged for higher-level analyses, but were computed anew for each level (Vinogradov 1997). Calculations and statistical analyses were carried out using Statistix version 1.0 (Analytical Software, Chicago, IL), SigmaPlot version 4.0 (SPSS, Inc., Chicago, IL), and Quattro Pro version 9.0 (Corel Corp. Ltd., Ottawa, Canada).

RESULTS

C-Value and Metabolic Rate

A significant negative relationship exists between C-value and mass-specific metabolic rate at the species level ($r = -0.39$, $P < 0.005$; Fig 1). This relationship is even stronger at the ordinal level ($r = -0.52$, $P < 0.05$), but appears marginal at the generic and familial levels (all $P \leq 0.06$). In contrast, C-value was not significantly correlated with active metabolic rate at any taxonomic level (all $P > 0.18$).

Cell Size and Metabolic Rate

Based on the cell size data of Gulliver (1875), there is a highly significant negative relationship between cell size and

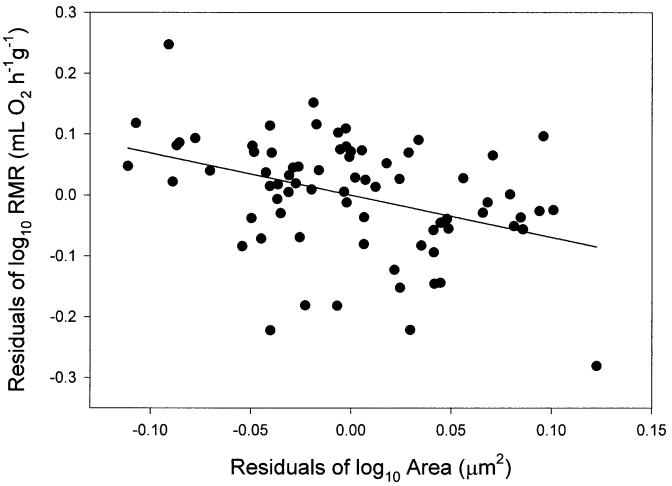

FIG. 2. Relationship between regression residuals of cell size versus body mass and resting metabolic rate (RMR) versus body mass in 74 species of birds ($r = -0.45$, $P = 0.0001$). Cell size data from Gulliver (1875); metabolic rate data from Bennett and Harvey (1987).

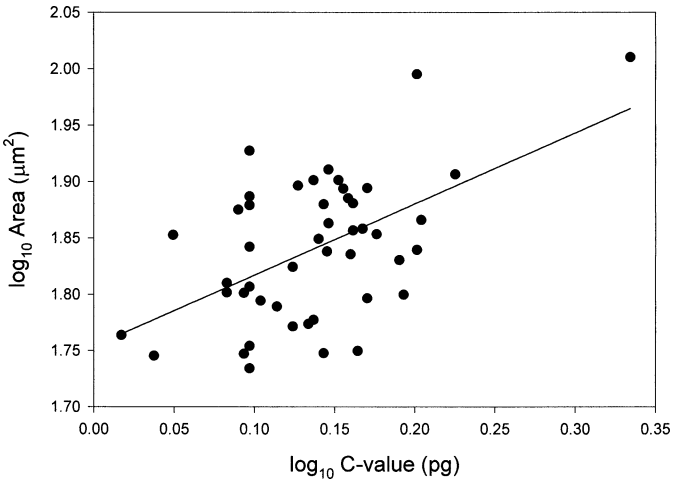

FIG. 3. Relationship between C-value and erythrocyte size in 47 species of birds ($r = 0.53$, $P < 0.0001$). Genome size data from Gregory (2001b); cell size data from Gulliver (1875).

mass-specific resting metabolic rate (Fig. 2). This becomes even stronger as taxonomic level increases (species: $r = -0.39$, $P = 0.0006$; genera: $r = -0.44$, $P = 0.0001$; families; $r = -0.53$, $P = 0.0002$; orders: $r = -0.75$, $P = 0.0003$). For the smaller datasets of Bartsch et al. (1937) and Hartman and Lessler (1963), correlations were not significant at the species level (all $P > 0.15$), but achieved significance at each of the generic, familial, and ordinal levels in both cases (all $P < 0.04$).

A significant negative correlation was found between cell size and active metabolic rate at the species level using data from Gulliver (1875; $r = -0.54$, $P < 0.03$, $n = 17$), and at the species and generic levels using data from Bartsch et al. (1937; all $r > -0.70$ to -0.85, all $P < 0.03$). However, the significance disappeared at higher taxonomic levels in both cases. Sufficient data were not available for this analysis using the data of Hartman and Lessler (1963).

C-Value and Cell Size

Within the taxonomically diverse survey of 47 bird species available based on the data of Gulliver (1875), a highly significant positive correlation was identified between C-value and erythrocyte size (Fig. 3). This correlation remained significant at the generic and familial levels (all $r > 0.46$, all $P < 0.005$). The correlation coefficient remained the same at the ordinal level ($r > 0.46$), but the relationship became marginal due to the small sample size ($n = 16$, $P < 0.07$). A similar relationship was found using data from Bartsch et al. (1937) but the very narrow range in genome sizes in this small sample (1.33–1.63 pg, $n = 11$) was such that significance was contingent on the inclusion of the single high value available (*Tyto alba;* without this value the overall range was less than 12%). The data of Hartman and Lessler (1963) were not useful in this analysis, because genome sizes were available for only five of the species measured in that study.

Nucleus Size and Cell Size

Strong positive correlations were observed between nucleus size and cell size in all three datasets (Fig. 4) and at all taxonomic levels (all $r > 0.50$, all $P < 0.05$). The single exception occurred at the ordinal level using the data of Hartman and Lessler (1963; $r = 0.40$, $P = 0.10$), although this appeared to be based on the values from the sole representative of the order Tinamiformes, *Crypturellus soui*. The removal of this one species from the dataset restored the significance of this higher-level correlation ($r = 0.55$, $P < 0.03$).

An attempt was also made to evaluate the relationship between C-value and nuclear area. However, the very limited range in the present data (2.0-fold in genome size and 1.7-fold in nuclear area), combined with small sample sizes (all $n \leq 13$), provided little resolving power. As such, no sig-

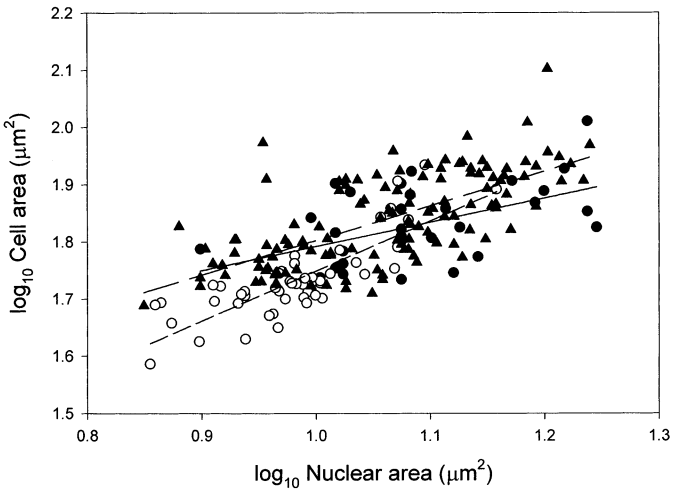

FIG. 4. Relationship between nucleus size and cell size using data from three independent studies. Closed circle: data from Gulliver (1875; solid line: $r = 0.51$, $P < 0.003$, $n = 32$); open circle: data from Bartsch et al. (1937; short-dashed line: $r = 0.87$, $P < 0.0001$, $n = 50$); triangle: data from Hartman and Lessler (1963; long-dashed line: $r = 0.68$, $P < 0.0001$, $n = 124$).

nificant relationship could be identified here (all $P > 0.15$), but see below.

Discussion

Genomes, Cells, and Metabolic Rates

The strongest physical correlate of metabolic rate in birds, as in mammals, is body size. The precise causal nature of this relationship has remained elusive, but in birds it apparently involves surface area–related differences in heat loss (Bennett and Harvey 1987). In both mammals and birds, a significant negative correlation has been identified between nuclear DNA content and body mass–corrected resting (or basal) metabolic rates (Vinogradov 1995, 1997; present study). In mammals, as much as 20% of the total variance in metabolic rate can be attributed to differences in genome size, at least as analyzed at higher taxonomic levels (Vinogradov 1995). Indeed, the relationship is strong enough to suggest that mammals could possibly employ changes in genome size to fine tune metabolic rates over and above the effects of body size (Vinogradov 1995). Genome size–metabolic rate relationships can even be identified within the rodent order (Vinogradov 1995), but had previously only been found within the passerine order in birds (Vinogradov 1997). Positive correlations between cell size and C-value are also easily recognized within the mammalian class (Gregory 2000). In birds, relationships of this sort have been considerably more difficult to establish, given the meager twofold range in genome sizes within the entire class. Of course, this limited range in C-values has itself long been viewed as adaptive. Indeed, the point is often made that the range is small and the values all so low because all birds tend to have high metabolic rates and therefore to be constrained to small cells, and thus small genomes. The statistically significant positive relationship between genome size and erythrocyte size, along with the negative relationships between metabolic rate and both cell size and genome size, found in the present study provide a new and powerful empirical basis for this interpretation (Figs. 1–3).

Based on the results of his original analysis, Vinogradov (1995, p. 1257) suggested that ''more accurate future measurements (or more vast datasets) will allow demonstration of the nucleotypic effect in birds.'' This prediction has been confirmed with the larger and more variable dataset of the present study. Particularly relevant was the inclusion of some larger genome (and cell) sizes that lend greater breadth to the data analyzed (e.g., ostrich, which has both the largest genome and the largest cells so far reported for birds, but upon which the significance of the relationships reported was not dependent). Higher-level analyses were also more diverse in the present study as compared with Vinogradov (1995), with 49 (vs. 16) families, and 16 (vs. 10) orders represented. Despite these differences in sample sizes and taxonomic ranges, the results of the two studies were very similar, with both showing the same patterns of correlational strength at the various taxonomic levels (i.e., weakest at the family level, strongest at the order level). However, only with the present sample sizes did the relationships achieve statistical significance.

The positive relationship between C-value and nucleus size has been well established over a wide range of vertebrate taxa. In combined analyses of vertebrates at large, birds fall along the same general line as members of other groups (e.g., Vialli 1957; Manfredi Romanini 1973; De Smet 1981; Olmo 1983). This situation mirrors that described previously for genome size versus cell size relationships, but in contrast with cell size, such a correlation with nucleus size within birds could not be demonstrated in the present study. It may also be relevant that whereas living erythrocytes in birds are quite flattened (such that a measure of two-dimensional area provides a good estimate of overall size), nuclei are cylindrical in vivo. The measure of dry nuclear area used in the present analysis may therefore be less than ideal for a comparison involving so little variance as to make it reliant on the accuracy of each datum. This is unfortunate, because most theories relating genome size to cell size do so via the intermediate of nucleus size, in the context of either coevolution (Cavalier-Smith 1978, 1985, 1991) or, more commonly, causation (for a review and critical discussion of the competing theories, see Gregory 2001a). In any case, it is worth noting that positive genome size-nucleus size correlations can be identified within the slightly less narrow ranges of these parameters in both reptiles (erythrocytes: De Smet 1981; Olmo and Odierna 1982) and mammals (leukocytes: Manfredi Romanini 1985).

A significant relationship between either genome size or cell size and active metabolic rate also failed to materialize in the present analysis. In this case, insufficient sample sizes are unlikely to provide a satisfactory explanation. However, it is not clear whether the lack of a demonstrable correlation reflects the various difficulties of measurement (Bennett and Harvey 1987), or whether active metabolic rate is legitimately unrelated to cell (and genome) size. The data of the present study are far too limited to justify speculation on this topic, and in any case, the findings concerning resting metabolic rate are sufficient to permit a discussion of avian genome size evolution in the context of flight-related constraints.

Genome Size and Flight: A Causal Connection?

Several lines of evidence of varying degrees of credibility indicate that the association between small genome size and flight is more than coincidental. Least conclusive but most obvious is the fact that volant vertebrates have small genomes. Birds tend to fly, and they also possess the smallest C-values among the terrestrial vertebrate classes. So too with bats, which have long been recognized as possessing C-values substantially lower than the mammalian mean (e.g., Manfredi Romanini et al. 1975; Burton et al. 1989). Although all but one of the bat species thus far analyzed are members of the Microchiroptera, the flying fox (*Pteropus giganteus*) displays an equally small genome (Manfredi Romanini et al. 1975). Thus, flight and small genomes may have evolved together on at least three independent occasions among vertebrates. More persuasive is the finding that within both mammals and birds there is a significant inverse association between genome size and resting metabolic rate, commensurate with the high metabolic requirements of flight (Vinogradov 1995, 1997; present study). Nevertheless, broad comparative and

correlational analyses can provide only circumstantial support for a proposed causal link between these two features.

To test this association more directly, Hughes (1999) classified some 39 families of birds for which genome sizes were available according to whether they were strong fliers (n = 17), moderate fliers (n = 15), weak fliers (n = 5), or flightless (n = 2). In this case, mean genomes sizes were shown to correspond very well to these categories, with increasing flight ability consistently associated with smaller genomes. These results were strongly supported by a more detailed phylogenetic analysis which showed that when flightless taxa or taxa with reduced flying ability were compared to sister taxa, the former in each case (n = 6) had larger mean genome sizes (Hughes 1999). According to Hughes (1999, p. 202), ''these results suggest that after an initial genome size reduction in the ancestors of modern birds, genome sizes have increased in taxa lacking strong flying ability. Such an increase is possibly due to a relaxation of selective pressures arising from the metabolic demands of active flight.'' Thus, the results of the present study and those of Vinogradov (1995, 1997), combined with the phylogenetic arguments of Hughes (1999), indicate quite strongly that a flighted lifestyle is associated causally with small genome size, as has generally been assumed. However, the mechanistic and evolutionary explanations for this relationship may prove far more complex (and theoretically informative) than previously appreciated.

C-Value Constraints in Birds

In general, correlations between genome size and metabolic properties at the whole organism level are assumed to be mediated via effects on cell size. In terms of metabolic rate, erythrocyte size—or more specifically, surface area:volume (SA:V) ratio—is believed to exert the predominant influence (e.g., Szarski 1970, 1983). Thus, Szarski (1983) envisioned a continuum of wasteful and frugal evolutionary strategies along which cell and genome sizes are reduced or expanded according to each organism's physiological requirements. It is clear that birds represent the extreme in cellular efficiency: ''Since the erythrocyte is the most important carrier of oxygen and CO_2, its surface area to size ratio is a determining factor in the exchange of these gases with the tissues. Thus, a small corpuscle offers the possibility of a greater rate of exchange than a larger one. Likewise an elliptical body is more efficient than a spherical one of the same volume. Avian erythrocytes are efficient in both of these respects'' (Hartman and Lessler 1963, p. 471).

Based on this hypothesis, a comparison between birds and mammals reveals two potentially puzzling observations. First, birds do not have substantially higher metabolic rates than mammals of the same size, and second, bird erythrocytes are actually *larger* than those of most mammals, yet mammalian genomes are larger than those of birds. As Cavalier-Smith (1978, p. 261) suggested, ''mammals can tolerate an average C-value double that of reptiles and birds only because they alone are able to eliminate their erythrocyte nuclei to compensate for their increased volume''. Yet, although enucleation allows small cells to coexist with large genomes, it does not prevent the nucleotype from exerting its influence at some stage of cell differentiation (Gregory 2000).

The fact that birds maintain higher metabolic rates than most mammals primarily as a result of their smaller body size (and not because of smaller erythrocytes) emphasizes the importance of miniaturization in avian evolution. Indeed, one of the most distinguishing modifications to occur in the evolution of birds from theropod dinosaurs was a pronounced reduction in body size (Sereno 1999). Clearly DNA itself contributes very little to body mass, but because the mass of every cell would be impacted by changes in DNA content, a reduction in genome size related to selection for small body size may be expected in birds. It is relevant in this context that erythrocyte size correlates positively with body mass among birds (Gulliver 1846, 1875; Hartman and Lessler 1963; present study). However, this effect would not be restricted to this one cell type. Most simply, larger erythrocytes would mean larger blood vessels and other associated tissues. More importantly, the sizes of cell types other than erythrocytes have also been found to correlate positively with genome size, such as neurons in amphibians (Roth et al. 1994), epithelial cells in mammals (r = 0.60, P = 0.01, n = 17; data from Olmo 1983; Gregory 2001b), and various other nonanimal cells (for a review, see Gregory 2001a). In birds, the sizes of cells as diverse as hepatocytes, kidney tubule cells, thyroid cells, and epithelial cells appear to correlate strongly with erythrocyte size across species (all $r >$ 0.99, all $P < 0.0001$, n = 6; data from Nitecki 1972), which indicates that a mechanism that changes the size of erythrocytes would be likely to exert similar effects throughout the body. It may therefore be a mistake to base all interpretations of cell and genome size reduction in birds on a simple SA:V effect in erythrocytes. A body mass effect would be consistent with the similar relative metabolic rates among birds and mammals, despite the fact that enucleate mammalian erythrocytes are smaller than those of birds (whereas the sizes of other somatic cells in mammals would correspond to their larger genome sizes).

Finally, it is useful to consider a potential constraint on bird genome sizes that is (mostly) unrelated to metabolism. In both frogs and salamanders, there is a significant positive correlation between genome size and the size of neurons (Roth et al. 1994). Variation in brain size is limited, however, such that the number of neurons that can be contained within a given brain is dictated largely by their individual size. Thus, genome size is inversely correlated with brain complexity in amphibians (Roth et al. 1994). Concomitant with their reduction in overall body size, bird evolution involved a substantial increase in relative brain size (Sereno 1999). Besides the fact that brain tissue is metabolically very costly, this means that birds have come to have small heads filled with complex brains—something that would not be possible when neurons (and genomes) are large. (Indeed, were it not for their small genomes, the term ''bird brain'' may have come to carry even more derogatory weight.)

It therefore seems likely that many different but mutually compatible selective pressures have operated to produce the small genomes characteristic of birds. It is also of interest that genome size is unrelated to chromosome number in both birds (e.g., Bachmann et al. 1972; Venturini et al. 1986) and

bats (Burton et al. 1989), indicating that these flight-related nucleotypic constraints operate independently of changes in the karyotype.

Genomic Baggage: Lost or Never Loaded?

According to Sereno (1999, p. 2143), ''cooption of structures originally evolved for another purpose has played a larger role than was previously thought in early avian evolution''. Anatomical features formerly ascribed only to birds—such as feathers, modifications of the sternum and ribs, and enlargement of the forebrain—have all been found in related nonflying taxa (Padian 1998; Sereno 1999). In other words, these features evolved prior to, and only later became directly implicated in the evolution of, flight. If the still-controversial proposition that ornithischian dinosaurs had high metabolic rates proves true (e.g., Fisher et al. 2000), then this process of cooption would likely have extended to physiological, cellular, and genomic features as well. For the reasons discussed above, it is apparent that the evolution of flight has been associated with the evolution of small genome size. What is not clear, however, is whether birds (and bats) evolved smaller genomes from initially larger ancestral C-values or whether small ancestral genomes represented a preadaptation for flight, with genomic expansion simply halted early in these lineages (Tiersch and Wachtel 1991; Wachtel and Tiersch 1993). Following Welty's (1955) analogy of birds as flying machines, it is unclear whether they have jettisoned their genomic baggage or whether it was never loaded in the first place. The mechanism(s) by which bird and bat genomes have become (or remained) small is also unclear. In short, *why* birds have small genomes is understood, but the questions of *how* and *when* this was achieved are still subjects of debate.

Until only a few years ago, the avian family tree was believed to have suffered a pronounced pruning of diversity during the Cretaceous-Tertiary (K-T) mass extinction, followed by a period of rapid ramification (e.g., Feduccia 1995). More recently, both fossil and molecular evidence have shown that major bird diversification occurred long before the meteor impact at the end of the Cretaceous, and that many ($>$20) avian lineages survived the K-T extinction event (e.g., Hedges et al. 1996; Cooper and Penny 1997; Sereno 1999; van Tuinen and Hedges 2001). Thus, hypotheses regarding the origin of low DNA amounts in birds based on severe population bottlenecks, such as that of Ota and Nei (1995) dealing specifically with a loss of immunoglobulin pseudogenes, can no longer be considered tenable.

It has been pointed out many times that introns (but not exons) are significantly smaller in chickens than in humans (Duret et al. 1995; Hughes and Hughes 1995; Oliver and Marín 1996; Deutsch and Long 1999; Hughes 1999; E. Waltari and S.V. Edwards, unpubl. ms.). In a direct intron-by-intron comparison of the two species, Hughes and Hughes (1995) found a negatively allometric correlation between intron lengths in chickens versus humans. That is, short introns were so in both species, but long introns were smaller in chickens than in humans, an observation that has been interpreted as evidence for a gradualistic postflight reduction in genome size among birds (Hughes and Hughes 1995; Hughes 1999).

In contrast to this hypothesis, Vinogradov (1999) suggested that rodents have even smaller introns on average than chicken and thus humans are not representative of mammals in general. However, although rodents do possess smaller introns than humans, it appears that only GC-rich chicken introns are longer than those of rodents (and humans), and in all cases these are the shortest category of introns to begin with (Oliver and Marín 1996). These observations may indeed suggest that bird (i.e., chicken) introns do not differ significantly in size from most mammalian introns (Vinogradov 1999), or may simply indicate that the streamlining of bird genomes has not been indiscriminate, and has instead focussed primarily on a shortening of the longest introns (Hughes and Hughes 1995; Hughes 1999).

It is important to recognize that this comparison between chickens and humans (or rats) is not particularly relevant to the question of small genomes in birds. The dubious reliability of a two-species comparison notwithstanding, the much more important analysis would not involve mammals at all, but reptiles (Vinogradov 1999). To date, a large-scale comparison of bird and reptile intron sizes has not been conducted. However, the fact that within reptiles the amount of single-copy DNA, as well as highly repetitive and middle repetitive segments, increases proportionately with genome size (Olmo et al. 1981) may be significant in this respect. In a direct comparison between chicken and alligator intron sizes conducted recently, evidence was found that suggests a reduction in intron sizes predating the evolution of birds (E. Waltari and S.V. Edwards, unpubl. ms). It bears mentioning in this regard that reptilian genomes are generally only slightly larger than (and overlap with) those of birds (Gregory 2001b), which may also imply the evolution of small genome size early in the reptilian lineage.

More generally, intron size and genome size are positively correlated between species of *Drosophila* (Moriyama et al. 1998), within the class of mammals (Ogata et al. 1996), and across eukaryotes in general (Vinogradov 1999). Of course, introns themselves are not at issue with regard to the metabolic demands of flight; differences in intron lengths can at best be interpreted only as symptoms of a larger genomic syndrome (Gregory and Hebert 1999). The tight constraints placed on genome size in birds and bats make them particularly useful as subjects in which to examine this notion of globally active genomic mechanisms.

In their study of bat genomes, Baker et al. (1992) found that although mechanisms that tend to increase genome size operate in all mammalian genomes, bats appear to possess more efficient means by which to contain this expansion. Moreover, this pattern applies to each of repetitive rDNA genes, C-band heterochromatin, and microsatellites (Baker et al. 1992; Van Den Bussche et al. 1995). Thus, genome size in bats is controlled by broad-based regulatory forces that maintain low copy numbers of repetitive DNA families (Van Den Bussche et al. 1995). Microsatellites are even rarer in avian genomes (Primmer et al. 1997), and repetitive DNA in general appears scarce in birds (Eden et al. 1978; Epplen et al. 1978; Wagenmann et al. 1981; Venturini et al. 1987; Bloom et al. 1993). This general fact about bird genomes is

even displayed in miniature within the major histocompatibility complex of chicken (Parham 1999). But again, whether a broader taxonomic sampling among birds will support these generalizations remains to be seen (Edwards et al. 1999).

Only one family of repetitive DNA appears to provide a direct clue to the evolution of small bird genomes. As Holmquist (1989, p. 477) argued, the ''almost total lack of SINEs [short interspersed nuclear elements] in the avian genome but not in other reptiles or vertebrates indicates that mass excision of SINEs occurred,'' which ''is considered to be a relatively recent event restricted to this one Sauropsidian lineage.'' Regardless, it is apparent that a constraining mechanism(s) similar to (but even stronger than) that in bats operates on repetitive DNA in birds. Again, from an evolutionary point of view it not possible to state conclusively whether these mechanisms act to retain a newly shrunken genome size or whether they have simply preserved a smaller ancestral C-value. Perhaps the most likely scenario is one of feedback between the various relevant physiological and anatomical features, and between these features and cell and genome size. Short of estimating genome sizes of other flighted vertebrates (e.g., pterosaurs) and primitive birds (especially *Archeopteryx*) from measurements of fossil cell sizes—as has been done for lungfish, amphibians, and plants (Thomson 1972; Thomson and Muraszko 1978; Masterson 1994)—it may be impossible to settle this issue (Tiersch and Wachtel 1991).

Conclusions

The genome size constraints in birds discussed in the present study can serve to illustrate the necessity of pluralistic approaches to complex evolutionary problems. In this case, relevant aspects are to be found at each level in the biological hierarchy, from the subchromosomal mechanisms of DNA content change to their impacts at the cellular and organismal levels to constraints derived from ecology. Thus, ''a hierarchical perspective is required that recognizes genome size increase [and decrease] as an independent event that has wide implications'' (Roth et al. 1994, p. 4800).

Influences in the reverse direction, of organismal traits on avian genome structure, have also been proposed before. Examples include the suggestion that flight should favor a high AT bias (Pettigrew 1994) or that a high metabolism such as that found in birds may increase the rate of oxidative mutagenesis, thereby confounding phylogenetic analyses (Martin and Palumbi 1993). However, neither of these propositions appears to have survived direct scrutiny (Mooers and Harvey 1994; Van Den Bussche et al. 1998). In contrast, the partitioning of homeotherm genomes into GC-rich isochores has long been accepted as a consequence of such a multilevel interaction (Bernardi 2000). Based on the results of the present study, it is apparent that overall size can be added to the list of genomic features influenced by higher levels of biological organization in birds.

It is important to recognize that the type of organism-level constraints showcased in the present study need not be ubiquitous to be important, and that these may vary among groups according to developmental, physiological, and ecological lifestyle. For example, whereas developmental constraints appear to feature prominently in the evolution of amphibian genome sizes (e.g., Sessions and Larson 1987; Jockusch 1997), this is all but irrelevant in mammals and birds (e.g., John and Miklos 1988; Hughes 1999). However, metabolic rate constraints are probably quite strong in homeotherms, especially among those capable of powered flight (Vinogradov 1995, 1997; Hughes 1999; Gregory 2000; present study), but are at best only very weak among members of the anamniote classes (Licht and Lowcock 1991).

The C-value enigma remains an interesting and challenging puzzle at the crossroads of molecular biology, cytogenetics, cell biology, anatomy, physiology, and ecology. As such, genome size evolution can serve as a valuable molecular microcosm of the evolutionary process at large. However, until the importance of each of these fields to the issue of genome size evolution is properly appreciated, this puzzle will continue to go unsolved and its insights unrecognized.

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