Cytochrome *b* Evolution in Birds and Mammals: An Evaluation of the Avian Constraint Hypothesis

Scott E. Stanley[1] *and Richard G. Harrison*

Section of Ecology and Systematics, Cornell University

Patterns of molecular evolution in birds have long been considered anomalous. Compared with other vertebrates, birds have reduced levels of genetic divergence between groups of similar taxonomic ranks for a variety of nuclear and mitochondrial markers. This observation led to the avian constraint hypothesis, which identifies increased functional constraint on avian proteins as the cause for the reduction in genetic divergence. Subsequent investigations provided additional support for the avian constraint hypothesis when rates of molecular evolution were found to be slower in birds than in mammals in a variety of independent calibrations. It is possible to test the avian constraint hypothesis as an explanation for this avian slowdown by comparing DNA sequence data from protein-coding regions in birds and homologous regions in mammals. The increased selective constraints should lead to a reduction in the proportion of amino acid replacement substitutions. To test for such a decrease, we calculated the numbers of amino acid replacement substitutions per replacement site (d_N) and silent substitutions per silent site (d_S) for the complete mitochondrial cytochrome *b* gene using 38 avian and 43 mammalian comparisons that were phylogenetically independent. We find that d_N/d_S is significantly smaller in birds than in mammals. This difference cannot be explained by differences in codon bias affecting d_S values. We suggest that the avian slowdown can be explained, at least in part, by a decreased tolerance for amino acid substitutions in avian species relative to mammalian species.

Introduction

Over 30 years ago, Zuckerkandl and Pauling (1962) and Margoliash (1963) noted that for hemoglobin and cytochrome *c*, different mammalian lineages appeared to be evolving at approximately equal rates. This observation led to the proposition that rates of molecular evolution for each protein were the same across lineages, or that there existed a set of molecular clocks (Zuckerkandl and Pauling 1965). Perhaps no other single hypothesis has stirred such debate among evolutionary biologists in recent years, and some have argued that a molecular clock is the most important discovery in molecular evolution (Wilson, Carlson, and White 1977). Indeed, the possible utility of such a clock, should it exist, is enormous. Recent investigations, however, have demonstrated that rates of evolution do in fact differ between lineages (e.g., Britten 1986; Hasegawa and Kishino 1989; Kocher et al. 1989; Krajewski 1990; Zink and Avise 1990; Adachi, Cao, and Hasegawa 1993). Interest in molecular clocks has now turned to examining factors that might influence rates of molecular evolution within and between lineages.

Several factors have been implicated as important in determining rates of molecular evolution. A synthesis of this literature is difficult, since the work to date has relied on many different types of data from different loci and different taxa (e.g., Avise and Aquadro 1982; Kessler and Avise 1985; Kocher et al. 1989; Krajewski 1990; Adachi, Cao, and Hasegawa 1993; Martin and Palumbi 1993). Moreover, many of the data used in these analyses were collected for reasons other than assessment of rate variation between lineages and were applied to this task only secondarily. In spite of these limitations, interesting patterns of rate variation have emerged. In particular, metabolic rate has been implicated repeatedly as an important factor in determining rates of molecular evolution, and a number of mechanistic hypotheses have been proposed (see Rand 1994).

It is unlikely that rates of molecular evolution are determined by only a single factor. Nonetheless, metabolic rate has been found to correlate with rates of molecular evolution in a number of independent studies (Thomas and Beckenbach 1989; Kocher et al. 1989; Martin, Naylor, and Palumbi 1992; Adachi, Cao, and Hasegawa 1993; Nunn and Stanley 1998). Much of this work has been restricted to the mitochondrial genome due to the ease with which mitochondrial DNA sequence data can be collected. However, earlier studies of nuclear markers (e.g., allozymes and microcomplement fixation data for nuclear proteins) also pointed to a connection between metabolic rate and rates of molecular evolution (Prager et al. 1974; Avise and Aquadro 1982; Patton and Avise 1985).

The metabolic rate hypothesis (Martin and Palumbi 1993) invokes higher mutation rates as the explanation for the positive correlation between metabolic rate and rates of molecular evolution (Martin, Naylor, and Palumbi 1992; Martin and Palumbi 1993). The hypothesis is based on the observation that concentrations of mutagenic oxygen radicals and rates of DNA synthesis are positively correlated with metabolic rate (Gross, Getz, and Rabinowitz 1969; Shigenaga, Gimeno, and Ames 1989). However, despite high avian metabolic rates, rates of molecular evolution appear to be slower in birds than in mammals (Adachi, Cao, and Hasegawa 1993; Mindell et al. 1996; Nunn et al. 1996).

[1] Present address: Department of Ornithology, American Museum of Natural History, New York, New York.

Key words: cytochrome *b*, mitochondrial DNA, metabolic rate hypothesis, avian constraint hypothesis, genetic divergence, birds, mammals, evolutionary rates.

Address for correspondence and reprints: Scott E. Stanley, Department of Ornithology, American Museum of Natural History, Central Park West at 79th Street, New York, New York 10024. E-mail: scoob@amnh.org.

Mol. Biol. Evol. 16(11):1575–1585. 1999

The Avian Constraint Hypothesis

In 1974, Prager et al. (1974) noted that transferrin and albumin evolution were slower in birds than in other vertebrates as measured by microcomplement fixation, relying on fossil calibrations of immunological distances. The authors suggested that this slowdown could be due to some factor that is unique to birds, such as increased body temperature or reduced DNA content. The notion of an avian slowdown was supported by subsequent analyses of allozyme data (Avise, Patton, and Aquadro 1980*a*, 1980*b*; Avise and Aquadro 1982; Patton and Avise 1985; Barrowclough and Corbin 1987) and mtDNA restriction fragment patterns (Kessler and Avise 1985; Ovenden, Mackinlay, and Crozier 1987; Ball and Avise 1992). These studies demonstrated that for comparisons between avian taxa (i.e., species, genera, families, etc.), genetic distances are smaller on average than distances between taxonomically equivalent mammalian, amphibian, and reptilian groups. A recent review of available cytochrome *b* sequences confirmed this observation (Johns and Avise 1998).

Avise and Aquadro (1982) elaborated the avian constraint hypothesis originally proposed by Prager et al. (1974) and conducted a review of the evidence available at the time. Specifically, they proposed that avian protein evolution might be constrained by the extreme physiological environment in birds, noting that metabolic rates and body temperatures are relatively high in birds compared with other vertebrates. The logic of Prager et al. (1974) was that in the avian environment, proteins needed to be especially fine-tuned, and a greater proportion of amino acid substitutions would be maladaptive. Hochachka and Somero (1973) earlier proposed a similar effect of increased body temperature in birds, relying on known relationships between body temperature and biochemical processes (Somero 1978; Bennett and Ruben 1979).

In response to the avian constraint hypothesis, Wyles, Kunkle, and Wilson (1983) suggested that the purported slowdown was simply an artifact of errors in dating divergence events and proposed that a recent origin of the class Aves was responsible for the lack of genetic divergence between avian lineages, a sentiment echoed repeatedly by Alan Wilson and colleagues (see review by Sheldon and Bledsoe 1993). In addition, many authors have found greater levels of divergence between avian taxa in the tropics (Hackett and Rosenberg 1990; Hackett 1993), suggesting that the lack of divergence observed by the Wilson and Avise groups is an artifact of sampling in temperate regions, where avian lineages may be younger than they are elsewhere. Moreover, Shields and Wilson (1987) calibrated an mtDNA clock for species in the family Anatidae, birds which have a reasonable fossil record, and found a rate similar to the mammalian rate.

However, the calibration of Shields and Wilson (1987) is based on a single calibration point for the *Anser* and *Branta* divergence, making it impossible to assess the variance associated with the rate they calibrated. The timing of that divergence was also questionable (Shields and Wilson 1987). In contrast, earlier work by Patton and Avise (1985) on birds in the same family (Anatidae) showed low levels of divergence within this avian family compared with other nonavian vertebrate taxa which they believed to be of approximately the same age. More recently, Mindell et al. (1996) approached this question using a relative-rates approach. They showed that when compared with mammals, birds do appear to evolve more slowly for 13 mitochondrial genes (with *Gallus gallus* representing birds) and 6 nuclear genes (with *Gallus gallus* and *Anas platyrhynchos* representing birds), and they interpreted their results as support for the constraint hypothesis, referred to by Mindell et al. (1996) as the body temperature hypothesis. (While Mindell et al. [1996] referred to the hypothesized explanation for the avian slowdown as the ''body temperature hypothesis,'' we use the more general term ''avian constraint hypothesis,'' since in its original form [Prager et al. 1974; Avise and Aquadro 1982] this hypothesis suggested that several factors in addition to body temperature, including DNA content, blood pH, and blood P_{CO_2}, might conspire to influence rates of molecular evolution in birds.) Additionally, Adachi, Cao, and Hasegawa (1993) showed that rates of mtDNA evolution in vertebrates could be ranked from slow to fast in the order of fishes, amphibians, birds, and mammals. Furthermore, Nunn et al. (1996) calibrated rates of evolution for cytochrome *b* in the Albatross family (Diomedeidae: Procellariiformes) and found slower rates in this avian family than in mammals. These results, combined with the earlier work of the Wilson and Avise groups demonstrating reduced levels of divergence between avian taxa, lend convincing support for an avian slowdown.

The avian constraint hypothesis, therefore, appears to have two distinct sources of evidence that have contributed to its development. One sort of evidence derives from comparisons of genetic distances between avian taxa and taxa from other vertebrate classes. These comparisons suggested that divergences were consistently lower between avian taxa than between taxa of the same rank in other vertebrates classes. However, comparisons based on taxonomic rank are less than ideal due to differences in the conventions used by avian and mammalian taxonomists. Evidence derived from direct comparisons of rates of molecular evolution for avian and nonavian groups has emerged more recently. These results suggest that rates of evolution are lower in birds than in mammals, but higher in birds than in other vertebrate groups. Thus, the avian slowdown appears to hold only relative to mammals. The avian constraint hypothesis must now be cast as a bird-versus-mammal hypothesis, and takes on new meaning in the face of evidence that rates of molecular evolution and metabolic rates are positively correlated, because birds tend to have higher metabolic rates than do similar-sized mammals.

While rates of molecular evolution do appear to be slower in birds than in mammals, the specific predictions of the constraint hypothesis have not been critically evaluated. Here, we use cytochrome *b* (cyt-*b*) se-

quences from birds and mammals to test predictions that follow from the avian constraint hypothesis. We use multiple phylogenetically independent comparisons of cyt-*b* sequences from birds and mammals to determine if increased selective constraint is a viable explanation for the avian slowdown.

Materials and Methods

In order to test for increased selective constraints in birds relative to mammals for the mitochondrial protein cyt-*b*, we used phylogenetically independent comparisons of complete cyt-*b* genes for both published and unpublished data sets. For the published sequences, we attempted to examine all cyt-*b* data sets that included complete gene sequences from multiple bird and mammal taxa. We restricted our analyses to data sets that included sequences that were sufficiently divergent to provide enough variation for meaningful comparisons, but not so much variation that relevant information about the sequences was lost or obscured by multiple mutation events (see below). In addition, we avoided including multiple data sets for a given taxon within birds or mammals once that taxon was already represented in our analysis (i.e., we did not consider additional rodent data sets once two large rodent cyt-*b* data sets were in our analysis). Otherwise, every effort was made to include all data sets that met our criteria.

For each comparison, we calculated maximum-likelihood estimates for the numbers of silent, or synonymous, substitutions per synonymous site (d_S) and replacement, or nonsynonymous, substitutions per nonsynonymous site (d_N) between two sequences using the computer program codeml in the PAML package (Yang 1998). The likelihood algorithm was the F3 $\times$ 4 maximum-likelihood method described by Goldman and Yang (1994). This method accounts for the nature of the genetic code, transition/transversion bias, and different base frequencies at codon positions. It uses multiple iterations of the likelihood calculation to arrive at the final d_N and d_S estimates (Yang 1998). We then calculated the ratio of nonsynonymous to synonymous substitutions (d_N/d_S). It follows from the avian constraint hypothesis that increased selective constraints operating on birds would lead to a reduction in the d_N/d_S ratio, since fewer replacement substitutions would be tolerated (i.e., d_N would be reduced).

Phylogenetically independent pairs of avian and mammalian taxa were chosen based on the results of phylogenetic analyses of complete cyt-*b* sequences. In most cases, we relied on the published phylogenies that accompanied the sequence data for identifying phylogenetically independent comparisons. Exceptions include ground squirrels (species in the genera *Cynomys* and *Spermophilus*), tropicbirds (*Phaethon rubricauda* and *Phaethon lepturus*), and loons (*Gavia immer* and *Gavia stellata*), from which cyt-*b* sequences are first presented in this study. For the ground squirrels, a phylogeny was generated using the sequences presented here, together with other ground squirrel taxa, using a parsimony analysis with equal weights given to all character state transformations(results not shown). Phylogenetically independent pairs were then chosen based on this tree. It was not necessary to construct phylogenetic hypotheses for the tropicbirds and loons, since only two taxa from the same genus were sequenced for these orders, and they are clearly sister taxa relative to the other avian taxa in our analysis. For published phylogenies, if multiple phylogenetic methods were used in the original analyses, we relied on the results of parsimony analyses over those of other methods. If sequences from other genes were also used in the phylogenetic analyses of the original published studies, we relied on the results of the combined analyses. In one case (for the bovid sequences), the original study did not include a parsimony analysis and relied solely on distance-based methods for phylogeny reconstruction. To be consistent, we carried out an unweighted parsimony analysis on the bovid sequences and used the results of that analysis to select phylogenetically independent pairs of bovid taxa for comparison.

In addition to independence, we applied two other criteria in deciding which sequences would be compared. For two sequences to be included in a pairwise comparison, there had to be at least one nonsynonymous substitution between them. We also restricted our analysis to comparisons between taxa for which the estimates of d_S were <1. While this is a somewhat arbitrary cutoff, it was necessary to have some threshold value to avoid pairs of sequences for which the number of synonymous substitutions per site was high enough to make estimates of d_S suspect. If two sequences have accumulated multiple substitutions per site, d_N/d_S ratios between pairs of taxa may be misleading, despite attempts to correct for multiple substitutions. This effect is also manifest in the form of increased variance for d_S estimates as d_S gets large (Tanaka and Nei 1989).

After applying these criteria, not all cyt-*b* sequences used in the original studies were included in our final analysis, since we used only sequences from the phylogenetically independent pairs with appropriate sequence divergence between them. In some cases, this greatly reduced the number of sequences that were included, particularly when many of the taxa in a given data set tended to be either extremely closely or extremely distantly related, or when the structure of the tree did not provide many pairs of taxa in the form of sister lineages (i.e., when a tree was largely pectinate).

To determine if d_N/d_S ratios for birds and mammals were significantly different, we compared the ratios for all bird pairs to the ratios for all mammal pairs using the nonparametric Mann-Whitney *U*-test. A nonparametric test was used because the data were severely heteroscedastic and could not be transformed to meet the assumptions of normality and equal variances.

Results and Discussion

After applying our selection criteria, we were able to include 37 bird and 43 mammal comparisons (i.e., 74 and 86 cyt-*b* sequences, respectively) in our analysis. The avian and mammalian taxa included in the d_N/d_S

Table 1
d_S, d_N, and d_N/d_S Ratios for 37 Pairs of Avian Taxa

Species in Pair (with GenBank accession numbers)	d_S	d_N	d_N/d_S Ratio
Galliformes			
Alectoris graeca (Z48772)[a] vs. *Alectoris rufa* (Z48775)[a]	0.2918	0.0080	0.028
Alectoris magna (Z48776)[a] vs. *Alectoris philbyi* (Z48774)[a]	0.2094	0.0056	0.027
Procellariiformes			
Thalassarche bulleri (U48945)[b] vs. *Thalassarche cauta* (U48953)[b]	0.0648	0.0024	0.035
Thalassarche chrysostoma (U48954)[c] vs. *Thalassarche impavida* (AF076093)[c]	0.0906	0.0012	0.013
Phoebetria palypebrata (U48943)[b] vs. *Phoebetria fusca* (U48942)[b]	0.0855	0.0024	0.028
Diomedea amsterdamensis (U48948)[b] vs. *Diomedea sanfordi* (U48946)[b]	0.1653	0.0059	0.035
Phoebastria immutabilis (U48949)[b] vs. *Phoebastria albatrus* (U48952)[b]	0.1665	0.0048	0.029
Fulmar glacialis (U74348)[c] vs. *Fulmar glacialoides* (AF076055)[b]	0.1786	0.0024	0.013
Halobaena caerulea (AF076057)[c] vs. *Pachyptila desolata* (AF076068)[c]	0.6437	0.0135	0.021
Procellaria aequinoctialis (U74350)[c] vs. *Procellaria westlandica* (AF076078)[c]	0.2028	0.0024	0.012
Puffinus huttoni (AF076084)[c] vs. *Puffinus puffinus* (U74355)[c]	0.2229	0.0037	0.016
Calonectris diomedea (U74356)[c] vs. *Calonectris leucomelas* (AF076045)[c]	0.1609	0.0012	0.008
Puffinus bulleri (AF076081)[c] vs. *Puffinus pacificus* (AF076088)[c]	0.1563	0.0012	0.008
Puffinus griseus (U74353)[c] vs. *Puffinus creatopus* (AF076083)[c]	0.1614	0.0062	0.038
Pterodroma hasitata (U74332)[c] vs. *Pterodroma cahow* (U74331)[c]	0.1386	0.0024	0.017
Pterodroma incerta (U74335)[c] vs. *Pterodroma magentae* (U74338)[c]	0.0850	0.0012	0.014
Pterodroma externa (U74339)[c] vs. *Pterodroma inexpectata* (U74346)[c]	0.2289	0.0012	0.005
Pterodroma cookii (U74345)[c] vs. *Pterodroma hypolueca* (AF076079)[c]	0.4136	0.0085	0.021
Pelecanoides urinatrix (AF076076)[c] vs. *Pelecanoides magellanicus* (AF076075)[c]	0.6017	0.0109	0.018
Oceanodroma leucorhoa (AF076064)[c] vs. *Oceanodroma furcata* (AF076063)[c]	0.8151	0.0131	0.016
Halocyptena microsoma (AF076058)[c] vs. *Oceanodroma tethys* (AF076066)[c]	0.4505	0.0047	0.010
Sphenisciformes			
Eudyptes chrysocome (AF076051)[c] vs. *Eudyptes chrysolophus* (AF076052)[c]	0.3820	0.0019	0.005
Pygoscelis papua (AF076090)[c] vs. *Pygoscelis antarctica* (AF076089)[c]	0.3020	0.0183	0.061
Gaviiformes			
Gavia immer (AF158249)[d] vs. *Gavia stellata* (AF158250)[d]	0.6842	0.0151	0.022
Pelecaniformes			
Phaethon rubricauda (AF158251)[d] vs. *Phaethon lepturus* (AF158252)[d]	0.4473	0.0090	0.020
Passeriformes			
Diphyllodes resublica (U15200)[e] vs. *Diphyllodes magnificus* (X74255)[e]	0.3314	0.0048	0.014
Epimachus meyeri (U15206)[e] vs. *Epimachus fastuosus* (X74253)[e]	0.3397	0.0060	0.018
Lophorina superba 005 (U25733)[e] vs. *L. superba* 004 (U25732)[e]	0.0434	0.0012	0.028
Manducodia comrii (U15207)[e] vs. *Phonygammus keraudrenii* (X74252)[e]	0.8778	0.0185	0.021
Paradisaea rubra (U25736)[e] vs. *Paradisaea* (*raggiana*) *augustaevictoriae* (U25738)[e]	0.2069	0.0024	0.012
Paro lawesi 106 (U25734)[e] vs. *P. lawesi* 103 (U25735)[e]	0.0241	0.0012	0.048
Trogoniformes			
Apaloderma narina (U94798)[f] vs. *Apaloderma vittatum* (U89200)[f]	0.9425	0.0085	0.009
Trogon collaris (U94808)[f] vs. *Trogon personatus* (U89201)[f]	0.7083	0.0198	0.028
Trogon comptus (U94804)[f] vs. *Trogon melanurus* (U94805)[f]	0.3505	0.0149	0.043
Trogon curucui (U94801)[f] vs. *Trogon violaceous* (U94802)[f]	0.2186	0.0114	0.052
Pharomachrus antisianuc (U89204)[f] vs. *P. pavoninus* (U94800)[f]	0.2867	0.0083	0.029
Harpactes ardens (U94796)[f] vs. *Harpactes diardii* (U94797)[f]	0.7019	0.0086	0.012

NOTE.–Values of d_N, d_S, and d_N/d_S ratios are from outputs generated using PAML (Yang 1998).
[a] Randi (1996).
[b] Nunn et al. (1996)
[c] Nunn and Stanley (1998).
[d] This study.
[e] Nunn and Cracraft (1996).
[f] Espinosa de los Monteros (1998).

comparisons are presented in tables 1 and 2, respectively, along with the GenBank accession numbers for their cyt-*b* sequences and the references for the studies in which they were originally published.

Values for d_N, d_S, and the resulting d_N/d_S ratios for each pairwise comparison between birds and mammals appear in tables 1 and 2, respectively. Histograms for d_N and d_S values for birds and mammals show that d_N is reduced in birds, while d_S appears to be similar in both groups (fig. 1), leading to a reduction in d_N/d_S ratios in birds (fig. 2). The mean d_N/d_S ratios were 0.023 (SE = 0.0022) for birds and 0.056 (SE = 0.0069) for mammals, with the d_N/d_S values arcsine transformed, as is appropriate when dealing with ratios (Sokal and Rohlf 1981, pp. 427–428). The results of the Mann-Whitney *U*-test are significant, with birds having significantly smaller d_N/d_Sratios relative to mammals ($z = -5.274$; $P < 0.0001$). These results are consistent with the avian

Table 2
d_S, d_N, and d_N/d_S Ratios for 43 Pairs of Mammalian Taxa

Species in Pair (with GenBank accession numbers)	d_S	d_N	d_N/d_S Ratio
Cetacea			
Stenella longirostris (X92524)[a] vs. *Tursiops truncatus* (X92526)[a]	0.2571	0.0062	0.024
Delphinapterus leucas (X92531)[a] vs. *Monodon moncero* (X92532)[a]	0.3369	0.0209	0.062
Balaenoptera borealis (X75582)[a] vs. *Balaenoptera edeni* (X75583)[a]	0.1447	0.0132	0.091
Balaenoptera acutorostrata (X75753)[a] vs. *Balaenoptera bonaerensis* (X75581)[a]	0.2337	0.0094	0.040
Balaena glacialis (X75587)[a] vs. *Balaena mysticetus* (X75588)[a]	0.2418	0.0097	0.040
Artiodactyla			
Camelus dromedarius (U06426)[b] vs. *Camelus bactrianus* (U06427)[b]	0.4724	0.0134	0.028
Lama glama (U06429)[b] vs. *Lama guanicoe* (U06428)[b]	0.0526	0.0026	0.049
Lama pacos (U06425)[b] vs. *Vicugna vicugna* (U06430)[b]	0.0680	0.0025	0.037
Dasyuromorphia			
Dasyurus hallucatus (M99460)[c] vs. *Sarcophilus harrisii* (M99465)[c]	0.7307	0.0202	0.028
Phascolosorex dorsalis (M99462)[c] vs. *Neophascogale lorentzii* (U07585)[d]	0.6567	0.0209	0.032
Myoictic melas (U07584)[d] vs. *Myoictic wallace* (AF010265)[e]	0.5135	0.0179	0.035
Dasykaluta rosamondae (M99456)[e] vs. *Paranthechinus apicalis* (M99457)[e]	0.9859	0.0333	0.034
Ningaui ridei (U07586)[e] vs. *Ningaui yvonneae* (U07587)[e]	0.8765	0.0169	0.019
Antechinus stuartii (M99454)[f] vs. *A swainsonii* (M99453)[f]	0.4625	0.0273	0.059
Planigale maculata maculata (U07590)[e] vs. *Planigale maculata sinualis* (U10318)[e]	0.4107	0.0131	0.032
Carnivora			
Eumetopias jubatus (X82311)[g] vs. *Zalophus californicus* (X82310)[g]	0.2039	0.0148	0.073
Arctocephalus gazella (X82292)[g] vs. *Arctocephalus forsteri* (X82293)[g]	0.2671	0.0150	0.056
Ursus arctos (X82308)[g] vs. *Thalarctos maritimus* (X82309)[g]	0.0559	0.0119	0.212
Panthera leo (X82300)[g] vs. *Panthera tigris* (X82301)[g]	0.5727	0.0195	0.034
Mephitis mephitis (X94927)[h] vs. *Spilogale putorius* (X94928)[g]	0.9751	0.0252	0.026
Rodentia			
Cynomys leucurus (AF157838)[i] vs. *Cynomys mexicanus* (AF157841)[i]	0.7346	0.0212	0.029
Spermophilus perotensis (AF157840)[i] vs. *Spermophilus spilosoma* (AF157846)[i]	0.1752	0.0052	0.030
Spermophilus mohavensis (AF157925)[i] vs. *Spermophilus tereticaudus* (AF157941)[i]	0.1244	0.0120	0.097
Spermophilus mexicanus mexicanus (AF157852)[i] vs. *Spermophilus mexicanus parvidens* (AF157870)[i]	0.1570	0.0027	0.017
Spermophilus elegans (AF157839)[i] vs. *Spermophilus undulatus* (AF157912)[i]	0.3746	0.0130	0.035
Spermophilus columbianus columbianus (AF157882)[i] vs. *Spermophilus columbianus ruficaudus* (AF157942)[i]	0.0879	0.0026	0.030
Spermophilus armatus (AF157850)[i] vs. *Spermophilus beldingi* (AF157881)[i]	0.2873	0.0079	0.028
Spermophilus townsendi townsendi (AF157935)[i] vs. *Spermophilus townsendi nancyi* (AF157932)[i]	0.0140	0.0027	0.192
Spermophilus townsendi idahoensis (AF157880)[i] vs. *Spermophilus townsendi mollis* (AF157938)[i]	0.1499	0.0039	0.026
Spermophilus townsendi vigilis (AF157888)[i] vs. *Spermophilus brunneus brunneus* (AF157883)[i]	0.2550	0.0080	0.031
Spermophilus erythrogenys (AF157855)[i] vs. *Spermophilus fulvus* (AF157908)[i]	0.1266	0.0038	0.030
Spermophilus relictus 1 (AF157867)[i] vs. *Spermophilus relictus* 2 (AF157876)[i]	0.0385	0.0036	0.095
Spermophilus xanthoprymnus (AF157902)[i] vs. *Spermophilus dauricus* (AF157899)[i]	0.7501	0.0239	0.032
Spermophilus musicus (AF157900)[i] vs. *Spermophilus pygmaeus* (AF157910)[i]	0.1883	0.0117	0.062
Spermophilus variegatus (AF157854)[i] vs. *Spermophilus atricapillus* (AF157944)[i]	0.2895	0.0197	0.068
Spermophilus lateralis (AF157887)[i] vs. *Spermophilus saturatus* (AF157916)[i]	0.2803	0.0130	0.046
Spermophilus adocetus (AF157843)[i] vs. *Spermophilus annulatus* (AF157851)[i]	0.7703	0.0212	0.028
Arvicanthus abyssinicus 1 (AF004566)[j] vs. *Arvicanthus abyssinicus* 2 (AF004567)[j]	0.0207	0.0039	0.189
Arvicanthus dembeensis (AF004568)[j] vs. *Arvicanthus niloticus* 1 (AF004569)[j]	0.1037	0.0102	0.098
Arvicanthus niloticus 2 (AF004570)[j] vs. *Arvicanthus niloticus* 4 (AF004572)[j]	0.1931	0.0074	0.038
Arvicanthus simalicus 1 (AF004573)[j] vs. *Arvicanthus simalicus* 2 (AF004574)[j]	0.0385	0.0036	0.095
Arvicanthus sp. 3 (AF004577)[j] vs. *Arvicanthus* sp. 11 (AF004585)[j]	0.4830	0.0127	0.026
Arvicanthus sp. 9 (AF004583)[j] vs. *Arvicanthus* sp. 10 (AF004584)[j]	0.2219	0.0124	0.056

NOTE.—Values of d_N, d_S, and d_N/d_S ratios are from outputs generated using PAML (Yang 1998).

[a] Arnason and Gullberg (1996).

[b] Stanley, Kadwell, and Wheeler (1994).

[c] Krajewski et al. (1997*a*).

[d] Krajewski et al. (1993).

[e] Krajewski et al. (1997*b*).

[f] Krajewski et al. (1992).

[g] Arnason et al. (1995).

[h] Ledje and Arnason (1996).

[i] This study.

[j] Ducroz, Volobouev, and Granjon (1998).

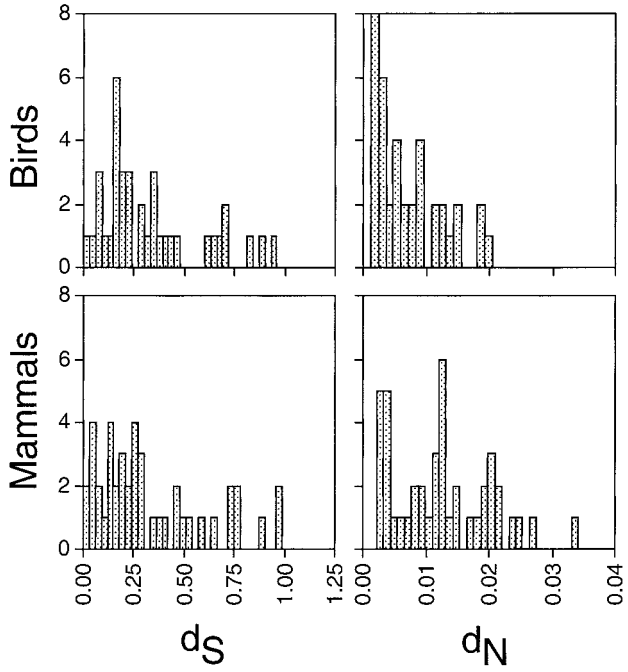

FIG. 1.—Histograms of d_S and d_N for both birds and mammals used in this study. The values for d_S and d_N for birds and mammals are from tables 1 and 2. Note that the distributions of d_S is similar for both birds and mammals, but d_N values for birds appear to be reduced relative to those for mammals.

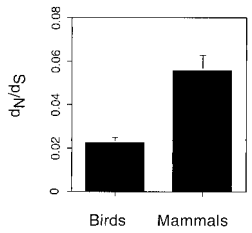

FIG. 2.—Mean values for d_N/d_S ratios for 37 birds and 43 mammal pairwise comparisons, with error bars representing standard errors. Values are taken from tables 1 and 2.

constraint hypothesis, providing the first direct evidence for increased selective constraint operating on avian proteins relative to orthologous proteins in mammals. While d_N/d_S ratios do vary within birds and mammals, avian values are consistently lower. However, evidence for increased functional constraint in birds does not by itself demonstrate that higher body temperature or any physiological trait in birds is the source of this additional constraint. Clearly, it would be interesting to know if differences in body temperatures, which range from 31°C to 40°C in mammals (Altman and Dittmer 1974) and from approximately 38°C to 43°C in birds (King and Farner 1961), could account for this increased constraint. Hochachka and Somero (1973) suggested that higher temperatures could increase the demands on the ordering of enzyme-substrate systems and that enzymes operating at high temperatures may require more rigid higher-order structures. This would imply that avian physiology may impose stricter requirements on proteins, which could lead to reduced tolerance for amino acid replacements in avian proteins and produce the pattern observed here.

Plots of d_N Versus d_S

That birds and mammals have different d_N/d_S ratios demonstrates that the two groups are accumulating synonymous and nonsynonymous substitutions at different rates. It does not, however, demonstrate the nature of those differences, since some information is lost in the averaging process. In order to investigate differences in the rates of synonymous and nonsynonymous substitutions in birds and mammals in more detail, we plotted d_N versus d_S for all bird and mammal comparisons (fig. 3). If synonymous and nonsynonymous substitutions identified in pairwise comparisons were effectively neutral, then they would accumulate linearly with time and would do so at a rate proportional to the synonymous and nonsynonymous mutation rates. Thus, plots of d_N against d_S would be linear (provided the estimates of d_N and d_S were accurate) and would go through the origin, because any two sequences would have d_N and d_S values of 0 when they first diverged. Differences in functional constraint would simply affect the slope of a regression line for these plots.

When we plot d_N versus d_S for the taxa considered here, we find that the slope of the regression line for birds is significantly lower than the slope for mammals (fig. 3; $P = 0.031$), consistent with the difference in ratios. The difference in d_N/d_S ratios that we observe also appears to be related to a lower y-intercept for birds. The y-intercept for mammals is significantly greater than the y-intercept for birds ($P = 0.043$). In other words, the reduced d_N/d_S ratios in birds can be explained by both a reduction in the rate of accumulation of nonsynonymous substitutions (leading to a shallower slope) and an apparent delay in the accumulation of nonsynonymous substitutions until more synonymous substitutions have occurred (leading to a lower y-intercept).

It is possible that part of this pattern is due to the effects of polymorphisms for cyt-*b*, which are still sorting in some of the species of mammals that we included in our analysis. When two closely related haplotypes are compared, a significant portion of the history since their divergence covers a period when polymorphisms are still segregating in both populations. This proportion is larger for more closely related haplotypes than for more distantly related ones (fig. 4). Recently, it was shown that d_N/d_S ratios are higher within species than between species for some mammals (Nachman et al. 1996; Rand and Kann 1996; Kennedy and Nachman 1998). This is to be expected if some nonsynonymous substitutions are deleterious. As such, these substitutions would be present as polymorphisms but would never go to fixation and never add to divergence. If more of the mammalian comparisons than the bird comparisons used in this analysis involved closely related haplotypes, then d_N/d_S ratios might be higher in mammals for this reason alone, not because of differences in levels of constraint.

Without knowing something about levels of polymorphism in the species considered here, we cannot di-

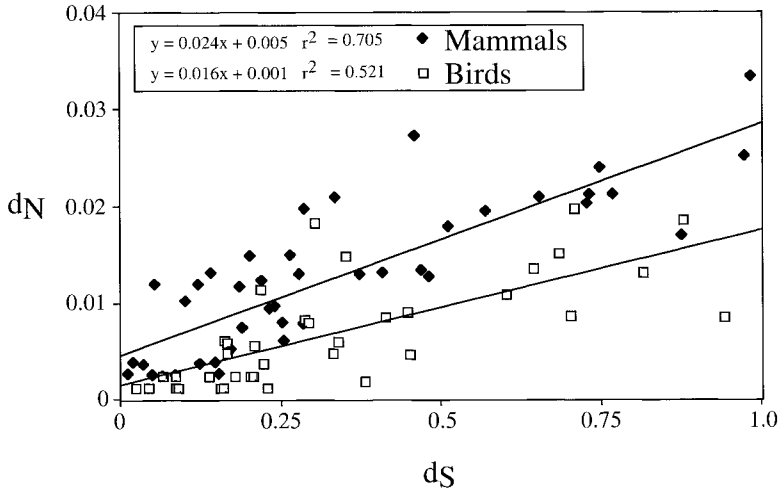

FIG. 3.—Plots ofd_N versus d_S for bird and mammal cyt-*b* sequences. The values for d_S and d_N for birds and mammals are from tables 1 and 2. The graph has linear regression lines for both birds and mammals. The equations for the regression lines are in the upper left-hand corner of the graph.

rectly test for this effect. We can, however, determine qualitatively if the comparisons at lower levels of sequence divergence (which presumably involve closely related species) tend to be elevated for mammals. To this end, we constructed LOWESS scatterplots (Cleveland 1981) for the d_N-versus-d_S plots using the SYSTAT (1992) computer program. (The LOWESS smoothing method differs from standard regression methods in that it does not assume a model.) We then compared the LOWESS plots with the regression lines to see where the values in the plots deviated from linearity (i.e., where the LOWESS plot deviated from the regression line; fig. 5). If differences between birds and mammals for the relative amounts of polymorphism to divergence

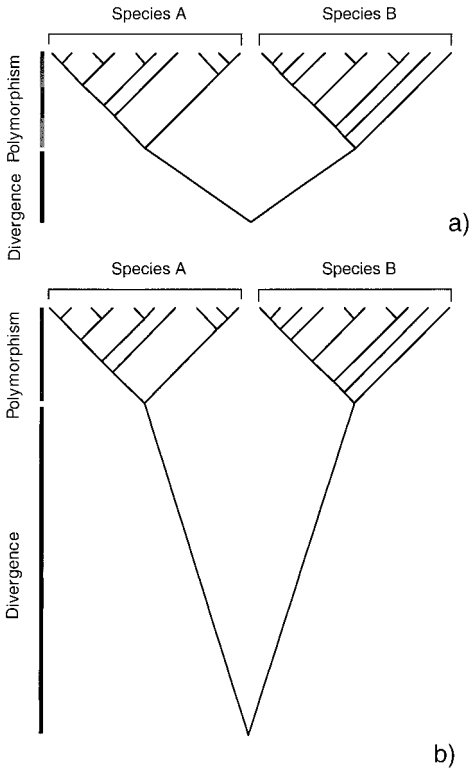

FIG. 4.—Two hypothetical gene genealogies for two species. *a*, The two species are close relatives, with proportionally more of the history separating them represented by polymorphisms. *b*, The two species are more distantly related, and divergence represents a larger proportion of their separate histories.

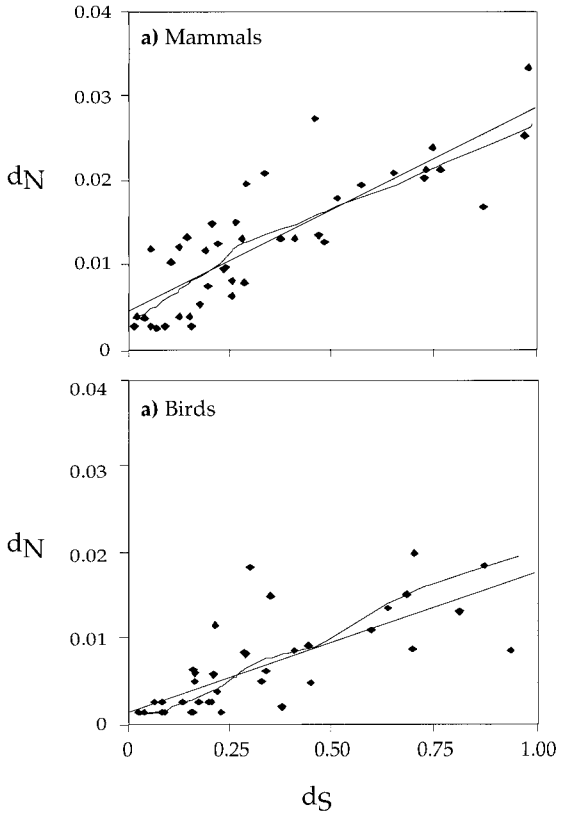

FIG. 5.—Plots of d_N versus d_S for bird and mammal cyt-*b* sequences. The values for d_S and d_N for birds and mammals are from tables 1 and 2. Both graphs include regression lines (from fig. 3) and LOWESS plots. The sliding window for the LOWESS plots was set to include 50% of the data points (i.e, the tension value was 0.5).

were affecting our comparisons of d_N/d_S ratios in the manner described above, then we would expect the LOWESS plot for mammals to be above the regression line in the lower left region of the plot (i.e., where levels of divergence are low), since the localized clustering of points above the regression line would bring the LOWESS plot above the regression line in this region of the graph. This does not appear to be the case for the data considered here. For both birds and mammals, the LOWESS plots are below the regression line where divergence is low.

This exercise demonstrates the utility of comparing d_N/d_S ratios, as well as plots of d_N versus d_S. Comparing ratios will be informative whether or not the data are linear. Thus, ratios will be informative under a broader array of conditions. However, plots of d_N against d_S provide information that will not emerge from comparing the distributions of d_N/d_S ratios for different groups.

Alternative Explanations for Reduced d_N/d_S Ratios in Birds

While our results are consistent with increased functional constraint reducing d_N values in birds, it is also useful to consider other possible causes for the reduction in avian d_N/d_S ratios. One concern is that for both the bird and the mammal data, d_N/d_S ratios are dominated by the single avian and mammalian data sets that make up the majority of comparisons (the tube-nosed seabird sequences for birds and the ground squirrel sequences for mammals). It would not be necessary to propose an avian-versus- mammalian explanation for this pattern if it were, in fact, simply a tube-nosed-seabird-versus-ground-squirrel phenomenon. To determine if our results were robust to the removal of these clades, we repeated our analysis on a reduced avian and mammalian data set, leaving out tube-nosed seabirds and ground squirrels. Again, avian d_N/d_S ratios (mean ± SE: 0.027 ± 0.0037) are lower than mammalian d_N/d_S ratios (0.058 ± 0.0093) in a Mann-Whitney *U*-test ($z = -3.366$; $P = 0.0008$). This suggests that the reduced d_N/d_S ratios that we observe in birds are a general avian phenomenon, since several distantly related groups of birds and mammals are represented in our analysis.

It could also be argued that d_S is affected by selection in different ways in birds and mammals. Evidence that selection might be operating on synonymous substitutions has continued to accumulate (Ikemura 1981; Grosjean and Freirs 1982; Shields et al. 1988; Akashi 1995), suggesting that in some cases synonymous substitutions are not neutral. Here, we interpret differences in d_N/d_S ratios as evidence for selection operating on replacement substitutions. If, in fact, d_S is under different forms of selection in birds and mammals, it is possible that the difference we see in these ratios is not due to selection acting to reduce d_N in birds, but to some force reducing d_S in mammals. An obvious candidate for this effect is codon bias, which could reduce d_S in mammals if it were more severe in mammals than in birds.

In order to test for such a difference in codon bias, we calculated the effective number of codons (N_c) according to the method described by Wright (1990), modified for the vertebrate mitochondrial genetic code. For mitochondrial genes, this index of codon bias ranges from 60, when all codons (excluding stop codons) are used in equal frequency, to 20, when only a single codon is used for each amino acid. Thus, greater bias is indicated by a lower effective number of codons. We used the average frequency of each codon for all sequences in the bird and mammal data sets (figs. 6 and 7) as a measure of codon usage for each group. Based on this analysis, we found that birds have a lower N_c, and thus a greater codon bias. For birds, N_c was 41.4, whereas for mammals, it was 43.4. It is not easy to establish how significant these differences are, but simulations conducted by Wright (1990) show that given a fixed codon usage pattern, it is possible to arrive at slightly different values of N_c for proteins approximately the same length as cyt-*b* (380 codons in birds and 379 codons in mammals). When the true N_c value was set at 41 for proteins that were 400 codons in length, Wright (1990) found that the standard error for N_c estimates based on 100 simulations using this method was approximately ±0.25. Thus, a difference of 2.0 does appear to represent a significant difference in the amounts of codon bias in birds and mammals for cyt-*b*.

UUU (F)	6.3					UGU (C)	1.1
UUC (F)	22.8					UGC (C)	2.6
Total	29.1	UCU (S)	2.7			total	3.7
		UCC (S)	8.2				
UUA (L)	6.5	UCA (S)	10.2	UAU (Y)	3.1	UGA (W)	10.4
UUG (L)	0.4	UCG (S)	0.5	UAC (Y)	10.5	UGG (W)	0.6
Total	6.9	Total	21.6	Total	13.6	total	11.0
				CAU (H)	3.6		
				CAC (H)	8.7		
CUU (L)	5.9	CCU (P)	3.6	Total	12.3	CGU (R)	0.8
CUC (L)	16.3	CCC (P)	9.7			CGC (R)	1.9
CUA (L)	33.5	CCA (P)	10.9	CAA (Q)	7.0	CGA (R)	5.1
CUG (L)	3.1	CCG (P)	0.5	CAG (Q)	1.0	CGG (R)	0.2
Total	58.8	Total	24.7	Total	8.0	Total	8.0
AUU (I)	8.9			AAU (N)	3.3		
AUC (I)	22.4			AAC (N)	17.7		
Total	31.3	ACU (T)	4.3	Total	21.0		
		ACC (T)	12.3				
AUA (M)	7.8	ACA (T)	10.8	AAA (K)	8.9	AGU (S)	0.3
AUG (M)	1.5	ACG (T)	0.3	AAG (K)	1.0	AGC (S)	1.6
Total	9.3	Total	27.7	Total	9.9	Total	1.9
				GAU (D)	1.7		
				GAC (D)	4.7		
GUU (V)	1.7	GCU (A)	5.3	Total	6.4	GGU (G)	2.8
GUC (V)	6.0	GCC (A)	14.1			GGC (G)	8.8
GUA (V)	8.1	GCA (A)	7.6	GAA (E)	6.0	GGA (G)	10.6
GUG (V)	0.7	GCG (A)	0.1	GAG (E)	1.0	GGG (G)	2.2
Total	16.5	Total	27.1	Total	7.0	Total	24.4

FIG. 6.—Codon usage chart for all avian cytochrome *b* sequences used in this study, excluding stop codons. Numbers represent average codon usage for all 74 sequences.

This difference in codon usage indices is not surprising. As figures 6 and 7 reveal, there are differences between the bird and mammal cyt-*b* sequences in nucleotide composition at third codon positions. For example, the C-to-U usage at third positions is markedly different for 11 of the amino acids (F, S, Y, L, P, I, T, N, V, A, and D), with more C's at third positions in birds. While it is difficult to quantify this effect beyond

UUU (F)	11.7					UGU (C)	0.6
UUC (F)	15.5					UGC (C)	2.9
Total	27.2	UCU (S)	4.8			total	3.5
		UCC (S)	5.1				
UUA (L)	10.5	UCA (S)	10.3	UAU (Y)	6.6	UGA (W)	11.2
UUG (L)	0.5	UCG (S)	0.5	UAC (Y)	8.5	UGG (W)	0.7
Total	11.0	Total	20.7	Total	15.1	total	11.9
				CAU (H)	4.3		
				CAC (H)	8.0		
CUU (L)	9.5	CCU (P)	4.7	Total	12.3	CGU (R)	0.8
CUC (L)	9.7	CCC (P)	6.4			CGC (R)	1.5
CUA (L)	26.1	CCA (P)	11.0	CAA (Q)	5.8	CGA (R)	5.4
CUG (L)	2.2	CCG (P)	0.6	CAG (Q)	0.5	CGG (R)	0.1
Total	47.5	Total	22.7	Total	6.3	Total	7.8
AUU (I)	18.7			AAU (N)	4.9		
AUC (I)	20.5			AAC (N)	10.8		
Total	39.2	ACU (T)	5.6	Total	15.7		
		ACC (T)	7.5				
AUA (M)	12.4	ACA (T)	12.4	AAA (K)	8.9	AGU (S)	0.9
AUG (M)	2.5	ACG (T)	0.4	AAG (K)	0.6	AGC (S)	2.1
Total	14.9	Total	25.9	Total	9.5	Total	3.0
				GAU (D)	3.9		
				GAC (D)	6.9		
GUU (V)	3.8	GCU (A)	5.1	Total	10.8	GGU (G)	3.4
GUC (V)	5.0	GCC (A)	7.4			GGC (G)	8.3
GUA (V)	8.5	GCA (A)	10.7	GAA (E)	5.4	GGA (G)	11.4
GUG (V)	0.7	GCG (A)	0.3	GAG (E)	0.6	GGG (G)	1.5
Total	18.0	Total	23.5	Total	6.0	Total	24.6

FIG. 7.—Codon usage chart for all mammalian cytochrome *b* sequences used in this study, excluding stop codons. Numbers represent average codon usage for all 86 sequences.

the calculations presented above, we feel that it only serves to support our interpretation of increased constraint in birds. The concern would be whether a difference in codon usage could by itself explain the decreased ratio of replacement to silent substitutions that we observe in birds. However, increased selective constraint on silent sites in the form of increased codon bias in birds would presumably reduce the number of silent substitutions (d_S in our case) and thus increase our estimates of d_N/d_S ratios. Since we find that d_N/d_S ratios are, in fact, lower for birds, our interpretation of increased constraint is simply more conservative in the face of increased codon bias.

It is also possible that synonymous mutations are mildly deleterious in both birds and mammals for some reason other than codon bias. If this is the case, then differences in population size between birds and mammals could also affect d_S values differentially in the two vertebrate classes. This would be a concern here if, in general, population sizes were smaller in birds than in mammals. This follows from the nearly-neutral theory (Ohta 1972, 1992), which predicts that in populations with small effective sizes, substitution rates for mildly deleterious mutations will be faster. This could lead to an increase in d_S in birds relative to mammals if synonymous substitutions were nearly neutral in both. We are not able to test this possibility directly, since accurate estimates of N_e are generally not available for the taxa in our analysis. However, there is no obvious reason to suspect that the population sizes for the avian taxa we consider here are consistently smaller than those for the mammalian taxa. In fact, many of the mammals in our analysis are likely to have small population sizes (e.g., whales and large carnivores).

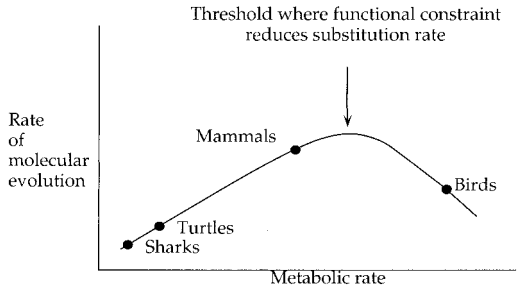

FIG. 8.—Diagram of how increases in metabolic rate can lead to higher rates of molecular evolution until some point at which increases in functional constraint act to reduce the substitution rate, lowering the overall rate of molecular evolution in birds to below that in mammals. Axes are not drawn to any particular scale, and the points on the graph are intended to represent hypothetical values.

The Metabolic Rate Hypothesis

Previous studies have found a positive correlation between metabolic rates and rates of molecular evolution in mitochondrial DNA. That rates are slower in birds than in mammals seems to contradict those results. It might appear that the avian constraint hypothesis (Prager et al. 1974; Avise and Aquadro 1982) and the metabolic rate hypothesis (Martin and Palumbi 1993) are mutually exclusive, since both make different predictions with respect to rates of molecular evolution in birds. It is important to keep in mind, however, that rates of molecular evolution are determined by both mutation rates and substitution rates (i.e., the fixation rates of mutations once they have occurred). Therefore, it is possible that mutation rates in birds are, in fact, higher on average than are those in mammals, but that increased functional constraint in birds acts to reduce substitution rates and that this reduces overall rates of molecular evolution. This scenario, depicted in figure 8, makes it possible for both the metabolic rate hypothesis and the avian constraint hypothesis to be compatible explanations for the patterns observed to date.

Acknowledgments

S.E.S. was supported by a National Science Foundation Predoctoral Fellowship. This research was supported by a National Science Foundation Doctoral Dissertation Improvement Grant and a Frank M. Chapman Memorial Fund Research Grant to S.E.S., a National Science Foundation Grant (DEB-9221504) to R.G.H., and a contribution from the Lewis B. and Dorothy Cullman Program for Molecular Systematics, a joint initiative of the New York Botanical Garden and the American Museum of Natural History. For comments on the manuscript, we thank C. Aquadro, G. Barrowclough, N. Lovejoy, C. Willett, D. Winkler, and two anonymous reviewers. For helpful advice on methods of analysis, we thank R. DeSalle, K. Kim, A. Kondrashov, S. Li, S. Remold, C. Ross, and Z. Yang.

LITERATURE CITED

ADACHI, J., Y. CAO, and M. HASEGAWA. 1993. Tempo and mode of mitochondrial DNA evolution in vertebrates at the amino acid sequence level. Rapid evolution in warm blooded vertebrates. J. Mol. Evol. **36**:270–281.

AKASHI, H. 1995. Inferring weak selection from patterns of polymorphism and divergence at ''silent'' sites in Drosophila DNA. Genetics **139**:1067–1076.

ALTMAN, P. L., and D. S. DITTMER, eds. 1974. Biology data book. Federation of American Societies for Experimental Biology, Bethesda, Md.

ARNASON, U., K. BODIN, A. GULLBERG, C. LEDJE, and S. MOUCHATY. 1995. A molecular view of pinniped relationships with particular emphasis on the true seals. J. Mol. Evol. **40**: 78–85.

ARNASON, U., and A. GULLBERG. 1996. Cytochrome *b* nucleotide sequences and the identification of five primary lineages of extant cetaceans. Mol. Biol. Evol. **13**:407–417.

AVISE, J. C., and C. F. AQUADRO. 1982. A comparative summary of the genetic distances in the vertebrates. Evol. Biol. **15**:151–185.

AVISE, J. C., J. C. PATTON, and C. F. AQUADRO. 1980*a*. Evolutionary genetics of birds II. Conservative protein evolution in North American sparrows and relatives. Syst. Zool. **29**:323–334.

———. 1980*b*. Evolutionary genetics of birds III. Comparative molecular evolution in New World warblers and rodents. J. Hered. **71**:303–310.

BALL, M. R., and J. C. AVISE. 1992. Mitochondrial DNA phylogeographic differentiation among avian populations and the evolutionary significance of subspecies. Auk **109**:626–636.

BARROWCLOUGH, G. F., and K. W. CORBIN. 1987. Genetic variation and genetic differentiation in the Parulidae. Auk **95**: 691–702.

BENNET, A. F., and J. A. RUBEN. 1979. Endothermy and activity in vertebrates. Science **206**:649–654.

BRITTEN, R. J. 1986. Rates of DNA sequence evolution differ between taxonomic groups. Science **231**:1393–1398.

CLEVELAND, W. S. 1981. LOWESS: a program for smoothing scatterplots by robust locally weighted regression. The American Statistician **35**:54.

DUCROZ, J. F., V. VOLOBOUEV, and L. GRANJON. 1998. A molecular perspective on the systematics and evolution of the genus Arvicanthis (Rodentia, Muridae): inferences from complete cytochrome b gene sequences. Mol. Phylogenet. Evol. **10**:104–117.

ESPINOSA DE LOS MONTEROS, A. 1998. Phylogenetic relationships among the trogons. Auk **115**:937–954.

GOLDMAN, N., and Z. YANG. 1994. A codon-based model of nucleotide substitution for protein-coding DNA sequences. Mol. Biol. Evol. **11**:725–736.

GROSJEAN, H., and W. FREIRS. 1982. Preferential codon usage in prokaryotic genes: the optimal codon-anti-codon interaction energy and the selective codon usage in efficiently expressed genes. Gene **18**:199–209.

GROSS, N. J., G. S. GETZ, and M. RABINOWITZ. 1969. Apparent turnover of mitochondrial deoxyribonucleic acid and mitochondrial phospholipids in the tissues of the rat. J. Biol. Chem. **244**:1552–1562.

HACKETT, S. 1993. Phylogenetic and biogeographic relationships in the neotropical genus Gymnopithys: Formicariidae. Wilson Bull. **105**:301–315.

HACKETT, S. J., and K. V. ROSENBERG. 1990. Comparison of phenotypic and genetic differentiation in South American antwrens: Formicariidae. Auk **107**:473–489.

HASEGAWA, M., and H. KISHINO. 1989. Heterogeneity in tempo and mode of mitochondrial DNA evolution among mammalian orders. Jpn. J. Genet. **64**:243–258.

HOCHACHKA, P. W., and G. N. SOMERO. 1973. Strategies of biochemical adaptation. Saunders, Philadelphia.

IKEMURA, T. 1981. Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes: a proposal for a synonymous codon choice that is optimal for the *E. coli* translation system. J. Mol. Biol. **151**:389–409.

JOHNS, G. C., and J. C. AVISE. 1998. A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome *b* gene. Mol. Biol. Evol. **15**:1481–1490.

KENNEDY, P., and M. W. NACHMAN. 1998. Deleterious mutations at the mitochondrial ND3 gene in South American marsh rats (Holochilus). Genetics **150**:359–368.

KESSLER, L. G., and J. C. AVISE. 1985. A comparative description of mitochondrial DNA differentiation in selected avian and other vertebrate genera. Mol. Biol. Evol. **2**:109–125.

KING, J. R., and D. S. FARNER. 1961. Energy metabolism, thermoregulation, and body temperature. Pp. 215–279 *in* A. J. MARSHALL, ed. Biology and comparative physiology of birds. Vol. 2. Academic Press, New York.

KOCHER, T. D., W. K. THOMAS, A. MEYER, S. EDWARDS, S. V. PAABO, F. X. VILLABLANCA, and A. C. WILSON. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA **86**:6169–6200.

KRAJEWSKI, C. 1990. Relative rates of single-copy DNA evolution in cranes. Mol. Biol. Evol. **7**:65–73.

KRAJEWSKI, C., M. BLACKET, L. BUCKLEY, and M. WESTERMAN. 1997*a*. A multigene assessment of phylogenetic relationships within the dasyurid marsupial subfamily Sminthopsinae. Mol. Phylogenet. Evol. **8**:236–248.

KRAJEWSKI, C., A. C. DRISKELL, P. B. BAVERSTOCK, and M. J. BRAUN. 1992. Phylogenetic relationships of the thylacine (Mammalia: Thylacinidae) among dasyuroid marsupials: evidence from cytochrome b DNA sequences. Proc. R. Soc. Lond. B Biol. Sci. **250**:19–27.

KRAJEWSKI, C., J. PAINTER, A. C. DRISKELL, L. BUCKLEY, and M. WESTERMAN. 1993. Molecular systematics of the New Guinean Dasyuridae (Marsupialia: Dasyuridae). Sci. New Guinea **19**:157–166.

KRAJEWSKI, C., J. YOUNG, L. BUCKLEY, and P. A. WOOLLEY. 1997*b*. Reconstructing the evolutionary radiation of dasyurine marsupials with cytochrome b, 12S rRNA, and protamine P1 gene trees. J. Mamm. Evol. **4**:217–236.

LEDJE, C., and U. ARNASON. 1996. Phylogenetic analyses of complete cytochrome b genes of the order Carnivora with particular emphasis on the Caniformia. J. Mol. Evol. **42**: 135–144.

MARGOLIASH, E. 1963. Primary structure and evolution of cytochrome *c*. Proc. Natl. Acad. Sci. USA **50**:672–679.

MARTIN, A. P., G. J. P. NAYLOR, and S. R. PALUMBI. 1992. Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. Nature **357**:153–155.

MARTIN, A. P., and S. R. PALUMBI. 1993. Body size, metabolic rate, generation time, and the molecular clock. Proc. Natl. Acad. Sci. USA **90**:4087–4091.

MINDELL, D. P., A. KNIGHT, C. BAER, and C. J. HUDDLESTON. 1996. Slow rates of molecular evolution in birds and the metabolic rate and body temperature hypotheses. Mol. Biol. Evol. **13**:422–426.

NACHMAN, M. W., W. M. BROWN, M. STONEKING, and C. F. AQUADRO. 1996. Nonneutral mitochondrial DNA variation in humans and chimpanzees. Genetics **142**:953–963.

NEI, M., and T. GOJOBORI. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. **3**:418–426.

NUNN, G. B., J. COOPER, P. JOUVENTIN, C. J. R. ROBERTSON, and G. G. ROBERTSON. 1996. Evolutionary relationships among extant albatrosses (Procellariiformes: Diomedeidae) established from complete cytochrome-*b* gene sequences. Auk **113**:784–801.

NUNN, G. B., and J. CRACRAFT. 1996. Phylogenetic relationships among the major lineages of the birds-of-paradise (Paradisaeidae) using mitochondrial DNA gene sequences. Mol. Phylogenet. Evol. **5**:445–459.

NUNN, G. B., and S. E. STANLEY. 1998. Body size effects and rates of cytochrome *b* evolution in tube-nosed seabirds. Mol. Biol. Evol. **15**:1360–1371.

OHTA, T. 1972. Population size and rate of evolution. J. Mol. Evol. **1**:305–314.

———. 1992. The nearly neutral theory of molecular evolution. Annu. Rev. Ecol. Syst. **23**:263–286.

OVENDEN, J. R., A. G. MACKINLAY, and R. H. CROZIER. 1987. Systematics and mitochondrial genome evolution of Australian rosellas (Aves: Platycercidae). Mol. Biol. Evol. **4**: 526–543.

PATTON, J. C., and J. C. AVISE. 1985. Evolutionary genetics of birds IV. Rates of protein divergence in waterfowl (Anatidae). Genetica **68**:129–143.

PRAGER, E. M., A. H. BRUSH, R. A. NOLAN, M. NAKANISHI, and A. C. WILSON. 1974. Slow evolution of transferrin and albumin in birds according to micro-complement fixation analysis. J. Mol. Evol. **3**:243–262.

RAND, D. M. 1994. Thermal habit, metabolic rate and the evolution of mitochondrial DNA. Trends Ecol. Evol. **9**:125–131.

RAND, D. M., and L. M. KANN. 1996. Excess amino acid polymorphism in mitochondrial DNA: contrasts among genes from Drosophila, mice, and humans. Mol. Biol. Evol. **13**: 737–748.

RANDI, E. 1996. A mitochondrial cytochrome *b* phylogeny of the *Alectoris* partridges. Mol. Phylogenet. Evol. **6**:214–227.

SHELDON, F. H., and A. H. BLEDSOE. 1993. Avian molecular systematics, 1970's to 1990's. Annu. Rev. Ecol. Syst. **24**: 243–278.

SHIELDS, D. C., P. M. SHARP, D. G. HIGGINS, and F. WRIGHT. 1988. ''Silent'' sites in Drosophila genes are not neutral: evidence of selection among synonymous codons. Mol. Biol. Evol. **5**:704–716.

SHIELDS, G. F., and A. C. WILSON. 1987. Calibration of mitochondrial DNA evolution in geese. J. Mol. Evol. **24**:212–217.

SHIGENAGA, M. K., C. J. GIMENO, and B. N. AMES. 1989. Urinary 8 hydroxy-2′-deoxyguanosine as a biological marker of in-vivo oxidative DNA damage. Proc. Natl. Acad. Sci. USA **86**:9697–9701.

SOKAL, R. R., and F. J. ROHLF. 1981. Biometry. The principles and practice of statistics in biological research. Freeman, New York.

SOMERO, G. N. 1978. Temperature adaptation of enzymes. Annu. Rev. Ecol. Syst. **9**:1–29.

STANLEY, H. F., M. KADWELL, and J. C. WHEELER. 1994. Molecular evolution of the family Camelidae: a mitochondrial DNA study. Proc. R. Soc. Lond. B Biol. Sci. **256**:1–6.

SYSTAT. 1992. Graphics. Version 5.2. SYSTAT, Evanston, Ill.

TANAKA, T., and M. NEI. 1989. Positive Darwinian selection observed at the variable-region genes of immunoglobins. Mol. Biol. Evol. **6**:447–459.

THOMAS, W. K., and A. T. BECKENBACH. 1989. Variation in salmonid mitochondrial DNA: evolutionary constraints and mechanisms of substitution. J. Mol. Evol. **29**:233–245.

WILSON, A. C., S. S. CARLSON, and T. J. WHITE. 1977. Biochemical evolution. Annu. Rev. Biochem. **46**:573–639.

WRIGHT, F. 1990. The 'effective number of codons' used in a gene. Gene **87**:23–29.

WYLES, J. S., J. G. KUNKLE, and A. C. WILSON. 1983. Birds, behavior, and anatomical evolution. Proc. Natl. Acad. Sci. USA **80**:4394–4397.

YANG, Z. 1998. Phylogenetic analysis by maximum likelihood (PAML). Version 1.4. University College, London.

ZINK, R. M., and J. C. AVISE. 1990. Patterns of mitochondrial DNA and allozyme evolution in the avian genus Ammodramus. Syst. Zool. **39**:148–161.

ZUCKERKANDL, E., and L. PAULING. 1962. Molecular disease, evolution and genic heterogeneity. Pp. 189–225 *in* M. KASH and B. PULLMAN, eds. Horizons in biochemistry. Academic Press, New York.

———. 1965. Evolutionary divergence and convergence in proteins. Pp. 97–166 *in* V. BRYSON and H. J. VOGEL, eds. Evolving genes and proteins. Academic Press, New York.

DAVID RAND, reviewing editor

Accepted August 9, 1999