

Relative-Rate Tests and Biological Causes of Molecular Evolution in Hummingbirds

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Hummingbirds (Trochilidae) present extreme variation in several factors thought to affect rates of molecular evolution, including generation time, species diversity, body mass, and metabolic rate. A published DNA hybridization phylogeny was used to examine experimental and biological causes of apparent rate variation among 26 species representing the principal lineages in the family. Molecular evolutionary rates (fitted path lengths based on ΔT_m H-C) among the various lineages differed significantly as determined by Felsenstein's *F* ratio test. Parametric and nonparametric correlations between relative rates and various predictor variables were qualitatively similar for outgroup species within and across different lineages except for outgroups that required comparisons among a small set of ingroups. Thus, the relative-rate tests appeared to be less sensitive to outgroup specification than to ingroup sampling. Correlations and analyses of covariance with predictor variables and outgroup species nested within the principal lineage indicated consistently significant associations of relative rates with various measures of body mass (negative) and with some mass-specific measures of basal metabolic rate (positive), but not with generation time or species diversity. These patterns held even if correlations among predictor variables were taken into account. Overall, these results for hummingbirds are consistent with hypotheses that relate metabolic processes associated with oxygen consumption to rates of molecular evolution. The results are incompatible with demographic (generation time, speciation) or body temperature effects on rates of DNA evolution. As DNA hybridization distances index the entire single-copy genome, the results also provide evidence for metabolic effects on evolutionary rates of the nuclear germ line.

Introduction

Hummingbirds (Trochilidae) provide a model system for investigating causes of variation in rates of molecular evolution, because they represent the vertebrate extreme for several demographic and physiologic factors with possible links to rate variation (Rand 1994). However, hummingbirds' suite of exceptional attributes also highlights some general problems in distinguishing among the various factors proposed to govern rates of molecular evolution. Based on their general characteristics, hummingbirds might be predicted to have a faster rate of molecular evolution than related birds (Rand 1994) because of their short generation times (Sibley and Ahlquist 1990), great species richness (Bleiweiss 1991), and high metabolic rates (Suarez et al. 1991). Indeed, some previous evidence based on DNA hybridization distance data suggests that hummingbirds are evolving faster than related nonpasserine birds (Bleiweiss, Kirsch, and Lapointe 1994). However, most of the factors enumerated above are correlated across higher taxonomic groups such that specific causes of the rate signature of a higher taxon are difficult to isolate (Hasegawa and Kishino 1989; Mooers and Harvey 1994; Mindell et al. 1996). Specifically, generation time and species diversity themselves may be correlated through the former's effect on rates of population divergence (Brown and Gibson 1983; Kochmer and Wagner 1988; Reaka-Kudla 1991), and generation time and species diversity may correlate with metabolic rate through the common factor of body mass (Bennett and Harvey

1987; Martin and Palumbi 1993; Mooers and Harvey 1994). Unfortunately, few studies of rate variation have successfully isolated these different effects, and the paucity of available data also makes difficult any quantification of experimental error, which may be a problem, especially for tests that rely on distance measures.

A particular advantage of hummingbirds as regards these confounding influences is that demographic and physiologic factors are not completely covariant within the family. Rather, many life history parameters appear to be constrained by the birds' adaptations to feeding at flowers, whereas the family's overall evolutionary radiation within the nectar-feeding niche has generated surprising physiologic and morphologic diversity. Thus, all hummingbirds appear to lay two eggs, have the same nectar-dependent diets, and breed within their first year of life (Bent 1940; Skutch 1972; Carpenter 1976; Brown, Calder, and Kodric-Brown 1978), whereas species differ by an order of magnitude in body mass (Carpenter 1976; Wolf and Gill 1986).

Here, I use a published DNA hybridization phylogeny for 26 hummingbird species (Bleiweiss, Kirsch, and Matheus 1997) to examine rate variation and its associations with various demographic (generation time, speciation as measured by taxonomic diversity), morphologic, and physiologic variables. Associations are examined through application of relative-rate tests (Sarich and Wilson 1967), which measure genetic distances from an outgroup to members of a monophyletic ingroup with which the outgroup shares a common ancestor; differences in genetic distance along these paths are taken to represent rate variation among ingroup lineages (fig. 1; Sarich and Wilson 1967; Easteal, Collet, and Betty 1995). Relative-rate tests are independent of fossil calibration dates and therefore can be applied to groups with a poor historical record such as is the case

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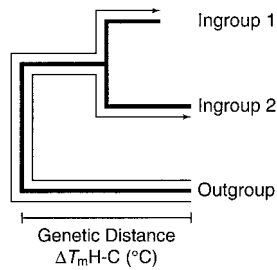


FIG. 1.—Distance-based relative-rate tests from an outgroup to two ingroup taxa. A slower rate is inferred for ingroup 1 based on a shorter path length from outgroup to ingroup 1 than to ingroup 2.

for hummingbirds (Olson and Hilgartner 1982; Bleiweiss 1998a), thus also avoiding errors in rate estimates introduced by uncertainties in the geologic record (Easteal, Collet, and Betty 1995; Springer 1995). However, relative-rate tests may fail to discern rate differences or may give spurious results depending on characteristics of the outgroup chosen to make such tests (Easteal, Collet, and Betty 1995; Springer 1995; Hillis, Mable, and Moritz 1996). As described below, the comprehensive phylogeny lends itself to a rigorous experimental design for isolating biological from various experimental causes of apparent rate variation.

Hummingbird Phylogeny and Design of Relative-Rate Tests

With respect to construction of relative-rate tests, the key well-corroborated features of hummingbird phylogeny (fig. 2) are their distant sister group relationship to swifts (Sibley and Ahlquist 1990; Bleiweiss, Kirsch, and Lapointe 1994); their own basal division into so-called hermit and nonhermit lineages (Bleiweiss, Kirsch, and Matheus 1997; formally recognized as the subfamilies Phaethornithinae and Trochilinae, respectively); and the subdivision of nonhermits into six principal lineages that contain the bulk (over 300 species) of hummingbird taxonomic and phenotypic diversity: mangoes, brilliants, coquettes, emeralds, mountain gems, and bees (fig. 2; for details see Bleiweiss, Kirsch, and Matheus 1997). Along with swifts and hermit hummingbirds, these principal lineages will be referred to as the “named clades” to distinguish them from their member species.

The one formal requirement for constructing relative-rate tests is that the paths from the outgroup must

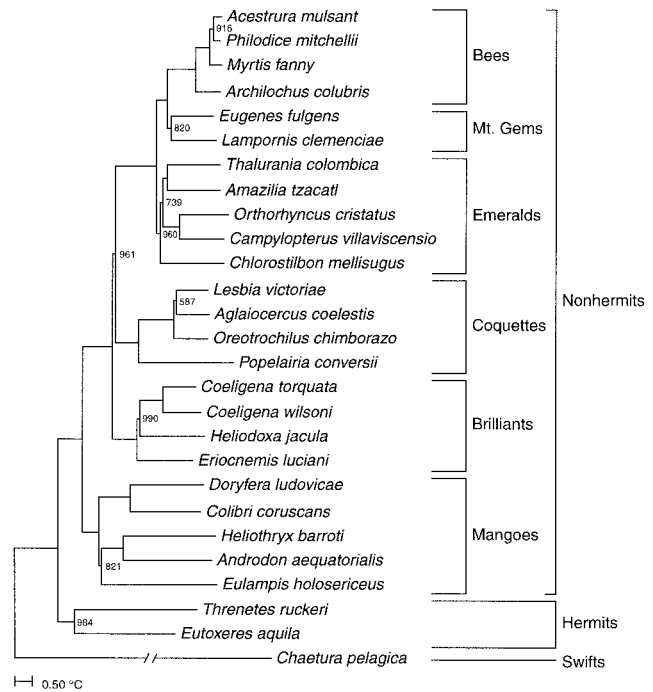


FIG. 2.—Consensus bootstrap FITCH topology based on symmetrized ΔT_{mH-C} s (see text for additional details). Branch lengths are averages over the 1,000 bootstrap pseudoreplicate trees and are drawn to scale for hummingbirds. Numbers indicate trees supporting a node out of the 1,000 bootstrap pseudoreplicate trees (if less than 100%). Named clades are as defined in text.

pass through an internal node shared by all ingroup taxa (fig. 1). In the context of the branching hierarchy of the named hummingbird clades, this constraint implies a natural hierarchy of rate comparisons; each successively more terminal named clade provides a set of outgroups for relative-rate measurements to a reduced subset of ingroups (table 1, fig. 2). This “subset” design facilitates examination of the possible effects of both outgroup mean distance (named clades) and experimental replication (species within named clades), which may impose one or more biases on correlations between relative-rate distances and biologically relevant variables. For instance, relative-rate differences are confined to the unshared portion of the path from outgroup to ingroup, which thereby comprises proportionately less of the total path measured from more distant outgroups. Added to such autocorrelation, more distant outgroups also may

Table 1
Design of Relative-Rate Tests

Outgroup	N	Ingroups	N
Swift	1	Hermits; mangoes; brilliants; coquettes; emeralds; mountain gems and bees	26
Hermits	2	Mangoes; brilliants; coquettes; emeralds; mountain gems and bees	24
Mangoes	5	Brilliants; coquettes; emeralds; mountain gems and bees	19
Brilliants	4	Coquettes; emeralds; mountain gems and bees	15
Coquettes	4	Emeralds; mountain gems and bees	11
Emeralds	5	Mountain gems and bees	6
Mountain gems and bees	6	Emeralds	5

NOTE: See figure 2 for topology and species membership in named clades. Emeralds or mountain gems and bees can be designated as the terminal clade of outgroups.

suffer greater saturation in nucleotide substitution rates caused by multiple hits and reversals in base substitutions (Jukes and Cantor 1969). On the other hand, more terminal outgroups serve to compare relatively fewer ingroups, which may contribute to sampling bias.

The several species within each named clade themselves provide replicate outgroups for an examination of variation (in correlations of relative-rate distances with biologically relevant variables) associated with each outgroup's DNA, which contributes one strand to all duplex DNAs made for that outgroup's set of comparisons (Sheldon and Bledsoe 1989; Bleiweiss and Kirsch 1993a, 1993b). The experimental errors contributed by the outgroup DNA arise from many sources, but principally from the DNA extraction and radioactive-labeling procedures, of which the latter is associated with a markedly high variance in melting temperature (Bleiweiss and Kirsch 1993b). Inconsistent associations between predictor variables and relative-rate measures from different outgroups within named clades would suggest that relative-rate measures simply are not sufficiently accurate or precise for testing hypotheses about evolutionary rates, regardless of any biases evident at the higher level of the named clades.

Materials and Methods

Hypotheses of Rate-Variation and Predictor Variables Generation Time

The original suggestion of Laird, McConaughty, and McCarthy (1969) was that rate variation depends inversely on generation time or its various correlates, including the number of germ line replications per year (Britten 1986; Li, Tanimura, and Sharp 1987) and age at first breeding (Sibley and Ahlquist 1990). Although available evidence indicates that all hummingbirds breed before the end of their first year of life (Bent 1940; Johnsgard 1983), interspecific variation in age at first breeding appears to occur within this narrow time frame. At one extreme, some hummingbirds are seasonal breeders that emigrate from their breeding grounds until the following year. These species include temperate high-latitude forms that are long-distance migrants and many tropical montane species that are elevational migrants. At the other extreme, resident tropical (mostly lowland) species potentially breed at any time of year (continuously), thereby allowing for the possibility that their generation times are different (shorter) than those of migratory species. For example, immature male hermits (*Phaethornis superciliosus*) begin to attend lek display areas within 3–4 months of fledging, with peak recruitment by 6–8 months (Stiles and Wolf 1979). Finally, idiosyncratic patterns occur in species such as the Andean hillstar (*Oreotrochilus estella*), which lives in a very seasonal high-altitude environment (Carpenter 1976). Immature hillstars acquire adult plumage and potential breeding status prior to their first winter, within 7 months of fledging (Carpenter 1976). Thus, even though most hummingbirds appear to mate in their first year of life, important differences in breeding patterns among species may impart different generation times.

I quantified these breeding patterns in three ways. First, a continuous measure of differences in breeding patterns is provided by the duration of the breeding season (in months, estimated from literature records of the earliest and latest dates on which active nests were found for a particular species). Generation time may associate negatively with duration of breeding season, in that longer breeding seasons allow for earlier ages at first reproduction, whereas shorter breeding seasons constrain some or all first-year birds to wait until the next annual cycle to breed. Under the generation-time hypothesis, therefore, rates of molecular evolution may associate positively with duration of breeding season. I also assigned integer rankings of generation time (from long to short) based on periodicity of breeding seasonality (seasonal, accelerated [e.g., *Oreotrochilus*], or continuous breeders) or based on migratory habits (long-distance migrant, pronounced elevational migrant, elevational migrant, resident). These categories also may reflect constraints that set different lower limits on the interval between breeding seasons and thus may correlate with generation time.

Species Diversity

Analogous to generation time, it has also been suggested that rates of genetic change are directly proportional to rates of species formation and the demographic consequences associated with cladogenesis (Avise and Ayala 1976; Avise and Aquadro 1982; Mindell, Sites, and Grauer 1989; Barraclough, Harvey, and Nee 1996). As the phylogeny encompasses less than 10% of all hummingbird species, rates of cladogenesis were estimated indirectly as the number of congeneric species per terminal taxon (numbers obtained from standard literature sources [Peters 1945; Monroe and Sibley 1993]). Given that relative-rate tests discriminate rate-variation toward the tips of the phylogeny, some measure of cladogenesis near the tips is biologically reasonable.

Physiology

The two physiologic mechanisms proposed to govern molecular rate variation make contrasting predictions about both the underlying cause of rate variation and its correlation with proxy variables such as body mass. The body temperature hypothesis proposes that elevated body temperatures limit the number of functional variants of a protein, thereby constraining the number of substitutions permissible in regions of DNA that code for that protein (Prager et al. 1974; Somero 1978; Mindell et al. 1996). Consequently, the hypothesis predicts that the rate of molecular evolution will increase with body mass and the corresponding decrease in mean body temperature. The alternative metabolic-rate hypothesis is based on the observation that rates in some groups decrease with body mass, leading to the explanation that physiological processing of oxygen increases free radical production and/or rates of DNA synthesis and, hence, nucleotide replacement rates in smaller-bodied forms (Kocher et al. 1989; Thomas and Beckenback 1989; Martin, Naylor, and Palumbi 1992; Ada-

chi, Cao, and Hasegawa 1993; Martin and Palumbi 1993).

Literature values of basal metabolic rate (BMR) measured in kilocalories per day were available for eight taxa (Bennett and Harvey 1987) represented in the DNA hybridization study. Although Bennett and Harvey report several different values of BMR for some of these species, I used those values selected for analysis by Bennett and Harvey (1987); they chose the lowest reported value under the supposition that BMR represents a minimum value within the zone of thermoneutrality. Given that BMR and body temperature are highly correlated with body mass among homeotherms (Schmidt-Nielsen 1984), I used body mass both as another predictor variable and as a proxy measure of both metabolic rate and body temperature. To ensure that genetic data were matched to accurate measures of body mass, I obtained mass data on wild-caught birds from the source populations used in the DNA hybridization experiments (locality data presented in Bleiweiss, Kirsch, and Matheus 1997). Birds were weighed with a Pesola scale to the nearest 0.1 g (> 5.0 g) or 0.05 g (<5.0 g) immediately after capture by placing them in preweighed cloth bags. As hummingbirds show significant sexual size dimorphism (Payne 1984), I estimated the mean for each sex as well as a species mean defined as the midpoint between the two sex-specific means; females of *Heliostyris barroti* and *Eugenes fulgens* were not captured, so their values cannot be reported. For similar reasons, mass-specific BMRs were calculated based on the values given for body mass in conjunction with BMR data (in Bennett and Harvey 1987).

Estimation of Genetic Distances

Experimental and Analytical Methods

Procedures for generation of the complete matrix of reciprocal distances are detailed elsewhere (Bleiweiss, Kirsch, and Matheus 1997). Briefly, a series of experimental and algorithmic methods were used to improve the accuracy of measured distances and fitted path lengths, including use of median melting temperature (T_m) to index genetic change across more of the genome; use of different individuals to generate each replicate measure to provide the best assessments of both average distance and replicate variance for bootstrap resampling; corrections for normalized percentage of hybridization (NPH) and homoplasy (Jukes and Cantor 1969) to yield the index T_m H-C (transformation equations and justifications given in Bleiweiss, Kirsch, and Matheus 1997). The complete distance matrix was then constructed by converting the raw distances to delta values (ΔT_m H-C), calculated as the difference from the homologous (the radioactively labeled strand matched to a second strand from the same species) to the heterologous (the labeled strand matched to a different species) hybrids. For the detection of rate differences among taxa, symmetrization of the distance matrix is important, because it eliminates systematic experimental error that could be mistaken for rate variation, such as through compression of distances caused by lower melting temperatures among the ho-

mologous standards used to estimate delta values (Springer and Kirsch 1991).

Rate Variation

Test of Significant Rate Variation

I tested for overall deviations from uniform rates of molecular evolution among lineages (molecular clock) through application of Felsenstein's (1993) F ratio test, for which ΔT_m H-C provides the most appropriate index (Springer and Kirsch 1989). This test evaluates whether the sum of squares (SS) of the tree-fitted distances of the best-fit FITCH tree (no assumption of clock) is significantly smaller than the sum of squares of the tree-fitted distances obtained for the best-fit KITSCH tree (assumption of clock), as given by:

$$\frac{(SS_{\text{KITSCH}} - SS_{\text{FITCH}}/df_{\text{KITSCH}} - df_{\text{FITCH}})}{\div (SS_{\text{FITCH}}/df_{\text{FITCH}})},$$

here using the more conservative assignment of degrees of freedom (no accounting of the number of subreplicates; Felsenstein 1993), in which case $df_{\text{KITSCH}} = [(n^2 - n)/2] - (n - 1)$, $df_{\text{FITCH}} = [(n^2 - n)/2] - (2n - 3)$, and n is equal to the number of taxa (27). The average bootstrapped (1,000 times) path lengths from the designated outgroup to all appropriate ingroup taxa (Bleiweiss, Kirsch, and Matheus 1997) then served to estimate relative rates for testing associations between rates and the predictor variables (table 2 and fig. 2).

Tests of Significant Associations

I \log_e -transformed both dependent and predictor variables to improve normality and/or homogeneity of variance. Male body mass was used to remove mass-dependent effects from other variables (duration of breeding season, species diversity, metabolic rate), because I lacked measures of female body mass for two species. As body mass itself may be associated with environmental variables such as elevation (Mayr 1963), I removed the potential confounding effects of the latter on mass by regressing body mass on the midpoint between the minimum and maximum elevational occurrence of each species. Other measures of elevational occurrence (minimum or maximum) gave qualitatively similar results. Residual analyses excluded the swift, which was used only as an outgroup.

Statistically based comparative methods to account for nonindependence caused by phylogenetic relatedness are not developed for relative-rate data, as comparative methods also use branch lengths to standardize contrasts so that the usual probability tables can be employed for testing hypotheses (Harvey and Pagel 1991; Garland, Harvey, and Ives 1992). Therefore, I designed statistical analyses of associations between path lengths and the various predictor variables to facilitate comparisons with previous studies of relative-rate variation, in particular, those based on DNA hybridization data (Mooers and Harvey 1994). To assess the effects of outgroup specification, I examined patterns in the occurrence of significant correlations (both parametric and nonparametric) in a matrix of all such comparisons between the 27 outgroups and 15 predictor variables (including raw val-

Table 2
Average Path Length Half-Matrix for Symmetrized ΔT_m H-C Among the 26 Hummingbirds and Swift (*Chaetura pelagica*) Used for Relative-Rate Tests

	LC	TC	AC	CW	EL	EH	CC	EF	AM	AO	MF	PM	AT	CV	AA	LV	DL	OC	HJ	HB	CM	CT	PC	OS	TR	EA	CP	
LC	0.00																											
TC	4.12	0.00																										
AC	6.40	6.55	0.00																									
CW	6.34	6.48	6.08	0.00																								
EL	6.10	6.24	5.84	4.03	0.00																							
EH	8.98	9.13	8.72	8.51	8.27	0.00																						
CC	8.47	8.61	8.20	7.99	7.75	7.40	0.00																					
EF	2.92	4.04	6.33	6.26	6.02	8.91	8.39	0.00																				
AM	3.50	4.34	6.62	6.56	6.32	9.20	8.68	3.39	0.00																			
AQ	3.41	4.25	6.53	6.47	6.23	9.11	8.59	3.30	1.71	0.00																		
MF	3.51	4.34	6.63	6.56	6.32	9.20	8.69	3.39	0.88	1.72	0.00																	
PM	3.42	4.26	6.54	6.47	6.23	9.12	8.60	3.31	0.54	1.63	0.81	0.00																
AT	4.13	3.56	6.56	6.49	6.25	9.13	8.62	4.05	4.35	4.26	4.35	4.26	0.00															
CV	4.25	4.02	6.68	6.62	6.37	9.26	8.74	4.18	4.47	4.38	4.47	4.39	4.03	0.00														
AA	8.80	8.95	8.54	8.33	8.08	7.61	7.21	8.72	9.02	8.93	9.02	8.94	8.95	9.08	0.00													
LV	6.36	6.50	2.12	6.03	5.79	8.67	8.16	6.28	6.58	6.49	6.58	6.50	6.51	6.64	8.49	0.00												
DL	8.53	8.67	8.26	8.05	7.81	7.46	4.81	8.45	8.75	8.66	8.75	8.66	8.68	8.81	7.28	8.22	0.00											
OC	6.37	6.52	2.22	6.04	5.80	8.68	8.17	6.30	6.59	6.50	6.59	6.51	6.52	6.65	8.50	2.12	8.23	0.00										
HJ	6.45	6.59	6.19	4.15	4.14	8.62	8.10	6.37	6.67	6.58	6.67	6.59	6.60	6.73	8.44	6.14	8.17	6.16	0.00									
HB	8.96	9.10	8.69	8.48	8.24	7.77	7.37	8.88	9.17	9.08	9.18	9.09	9.11	9.23	6.09	8.65	7.43	8.66	8.59	0.00								
CM	4.24	4.12	6.67	6.61	6.37	9.25	8.73	4.16	4.46	4.37	4.46	4.37	4.13	4.23	9.07	6.63	8.80	6.64	6.72	9.23	0.00							
CT	6.11	6.25	5.85	2.41	3.79	8.28	7.76	6.03	6.33	6.24	6.33	6.24	6.26	6.38	8.09	5.80	7.82	5.81	3.92	8.25	6.38	0.00						
PC	7.31	7.45	5.50	6.98	6.74	9.62	9.11	7.23	7.53	7.44	7.53	7.45	7.46	7.59	9.44	5.46	9.17	5.47	7.09	9.60	7.58	6.75	0.00					
OS	4.37	4.14	6.80	6.73	6.49	8.38	8.86	4.30	4.59	4.50	4.59	4.51	4.15	3.12	9.20	6.76	8.92	6.77	6.85	9.35	4.35	6.50	7.71	0.00				
TR	10.70	10.84	10.43	10.22	9.98	10.75	10.23	10.62	10.92	10.83	10.92	10.84	10.85	10.98	10.57	10.39	10.30	10.40	10.34	10.72	10.97	9.99	11.34	11.10	0.00			
EA	9.96	10.10	9.69	9.48	9.24	10.01	9.49	9.88	10.18	10.09	10.18	10.10	10.11	10.24	9.83	9.65	9.56	9.66	9.60	9.98	10.23	9.25	10.60	10.36	7.52	0.00		
CP	33.34	33.48	33.07	32.86	32.62	33.38	32.87	33.26	33.55	33.46	33.56	33.47	33.49	33.61	33.20	33.03	32.93	33.04	32.97	33.36	33.63	33.98	33.73	31.90	31.15	0.00		

NOTE.—Zeros along the diagonal indicate homologous comparisons ($\Delta = 0$). Species codes: LC = *Lampornis clemenciae*; TC = *Thalurania colombica*; AC = *Agelaiocercus coelestis*; CW = *Coeligena wilsoni*; EL = *Eriocnemis luciani*; EH = *Eulampis holosericeus*; CC = *Colibris coruscans*; EF = *Eugenes fulgens*; AM = *Acestrura mulsant*; AO = *Archilochus colubris*; MF = *Myiurus fanny*; PM = *Philotice mitchelli*; AT = *Amazilia taczali*; CV = *Campylopterus villaviscensio*; AA = *Androdon aequatorialis*; LV = *Lesbia victoriae*; DL = *Doryfera ludovicianae*; OC = *Oreotrochilus chimborazo*; HJ = *Heliodoxa jacula*; HB = *Heliothryx barroti*; CM = *Chlorostibon mellisugus*; CT = *Coeligena torquata*; PC = *Popelairia conversii*; OS = *Ornithonyx cristatus*; TR = *Threnetes ruckeri*; EA = *Eutoxeres aquila*; CP = *Chaetura pelagica*.

Table 3
Predicted Correlations Between Predictor Variables and Rate of Molecular Evolution for Four Hypotheses

Hypothesis	Genetic Rate (dependent variable)	Body Mass (independent variable)
Generation time	Negative	Positive
Species diversity	Positive	Negative
Body temperature	Negative	Negative
Specific oxygen consumption . . .	Positive	Negative

ues and residuals). To assess overall significance of associations with relative rates (as dependent variable), I constructed separate general linear models analogous to analyses of covariance (ANCOVAs) in turn for each predictor variable (covariate), nesting the variable and outgroup species (main effect) under the corresponding named clades (blocks; mountain gems and bees treated as a single block). To maintain the “subset” design, one or the other terminal sister clade, emeralds or mountain gems and bees, was excluded in turn from the ANCOVA.

All analyses were based on path lengths from the consensus bootstrap FITCH topologies (fitted by unweighted least-squares, specifying $P = 0$ in FITCH; Felsenstein 1993). One-tailed critical values were used as the basis for testing the various hypotheses, which make specific directional predictions about the relationship between the independent and dependent variables (table 3). For the purpose of exploring data structure in the 27×15 table, I report nominal significance to $P < 0.10$. All statistical tests were performed in SAS for UNIX on a SPARCstation 20.

Results

Rate Variation

To implement the F -test, the topology generated by FITCH was forced for the KITSCH option by specifying a user-defined tree. The F -test indicates significant disparities in rates among hummingbird lineages as a whole and among just the more diverse nonhermits (table 4). As shown elsewhere (Bleiweiss 1998b), hermits are significant outliers for rate variation that exists among hummingbirds generally, and they were analyzed separately (as ingroups) from nonhermits.

Data Characteristics

The 24 ingroup species cover most of the intrafamilial variation observed for the 15 predictor variables (appendix) except that my sample omits the 20.0-g giant hummingbird *Patagona gigas* (a distinct outlier at the large end of hummingbird body masses; see Carpenter 1976). The distribution of significant correlations between path lengths and predictor variables in the 27×15 table of all such comparisons reveals several patterns (fig. 3). First, the overall significances of correlations between path lengths and predictor variables are remarkably similar for the suite of outgroup taxa within each named clade. Of the 86 such blocks of taxa in each

Table 4
 F Ratio Test of Rate Variation Among Hummingbird Lineages

Ingroups	n	SS_{KITSCH}	SS_{FITCH}	df_{KITSCH}	df_{FITCH}	F ratio	P
Hermits + nonhermits	27	571.696	268.403	325	300	13.56 ^a	<0.001
Nonhermits	25	453.895	232.297	276	253	10.50 ^b	<0.001

NOTE.— SS_{KITSCH} = sum of squares of the tree-fitted distances of the best-fit KITSCH tree; SS_{FITCH} = sum of squares of the tree-fitted distances of the best-fit FITCH tree.

^a Degrees of freedom for F -test: 26, 300.

^b Degrees of freedom for F -test: 24, 253.

table of correlations (excluding the singletons representing the swift clade and six blocks with insufficient observations to calculate a correlation), results are heterogeneous within only three blocks for Pearson correlations (<3.5%) and within only two blocks for Spearman correlations (<2.4%). Of 172 such blocks across both tables, only one (<0.6%) has both significant and nonsignificant cell P values (by the criteria given above). This remarkable consistency suggests that the significance level of a correlation depends largely on characteristics common to members of the clade. Thus, differing results among named clades presumably result from analytical considerations and not from confounding experimental error such as might arise through factors that determine the melting temperature of a specific outgroup's DNA.

Consistent with this interpretation, the outgroup taxa within each named clade provide very similar path length statistics for the associated set of ingroup taxa, with characteristic levels of path length variation (standard deviation [fig. 4a] and standard error [fig. 4b]) associated with outgroups within each named clade. However, the statistics of variation in path lengths measured from outgroups at different depths in the tree indicate no simple trend (fig. 4), as might otherwise be expected from autocorrelation and/or saturation effects alone.

To quantify the tablewide occurrence of significant correlations, I scored each cell (in fig. 3) as a 1 (significant; one-tailed $P < 0.10$) or 0 (nonsignificant) and then applied tests of linear trends on these categorical scores across each group of predictor variables (demography [generation time, species diversity], body mass, metabolic rate). These tests indicate consistent results (nonsignificant for demographic variables, significant for body mass variables; table 5) for outgroups in more basal, but not more terminal, named clades. The overall inconsistent results that obtain for associations based on relative rates measured from more terminal outgroups (fig. 3) presumably are caused by sampling biases, and these patterns are difficult to interpret.

Effects of Predictor Variables on Rates of Molecular Evolution

For nonhermits, path lengths from most outgroups were significantly and negatively correlated with all measures of body mass. Significance levels for residuals (of mass regressed on elevational occurrence) some-

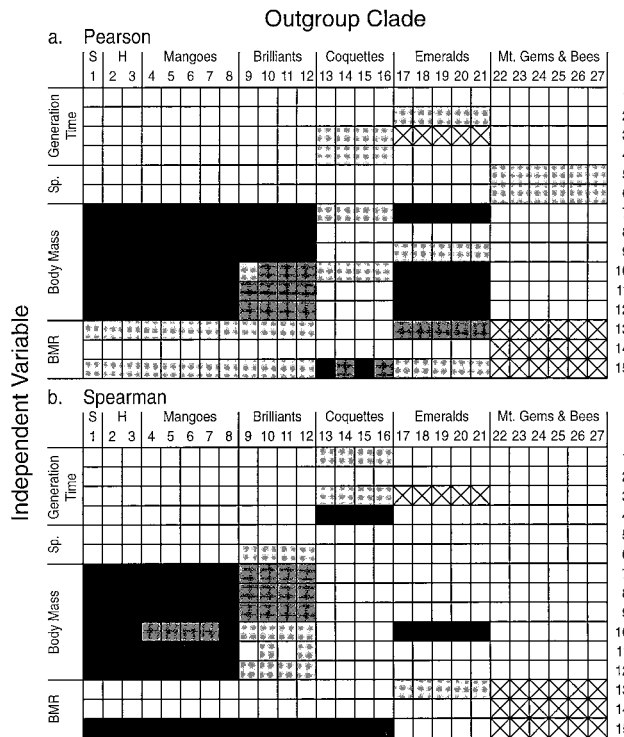


FIG. 3.—Schematic representation of results for Pearson (a) and Spearman (b) correlations (raw correlations, exact two-tailed P values, and sample sizes available from the author) with relative rates. Shading indicates level of significance: black for two-tailed significance at $P < 0.05$, dark gray for one-tailed significance at $P < 0.05$, light gray for one-tailed significance at $P < 0.10$, and white for nonsignificance (see text for further discussion). Hatched cells had too few observations for correlations to be calculated. Horizontal cells correspond to outgroup taxa grouped by outgroup (named) clade: 1 = *Chaetura pelagica*; 2 = *Threnetes ruckeri*; 3 = *Eutoxeres aquila*; 4 = *Eulampis holosericeus*; 5 = *Colibri coruscans*; 6 = *Androdon aequatorialis*; 7 = *Doryfera ludovicae*; 8 = *Heliothryx barroti*; 9 = *Coeligena wilsoni*; 10 = *Eriocnemis luciani*; 11 = *Heliodoxa jacula*; 12 = *Coeligena torquata*; 13 = *Aglaiocercus coelestis*; 14 = *Lesbia victoriae*; 15 = *Oreotrochilus chimborazo*; 16 = *Popelairia conversii*; 17 = *Thalurania colombica*; 18 = *Amazilia tzacatl*; 19 = *Campylopterus villaviscensio*; 20 = *Chlorostilbon mellisugus*; 21 = *Orthorhynchus cristatus*; 22 = *Lampornis clemenciae*; 23 = *Eugenes fulgens*; 24 = *Archilochus colubris*; 25 = *Myrtis fanny*; 26 = *Philodice mitchellii*; 27 = *Acestrura mulsant*. Vertical cells correspond to predictor variables: 1 = duration of breeding season; 2 = residual duration of breeding season (duration of breeding season regressed on male field masses obtained for this study); 3 = breeding seasonality; 4 = migratory behavior; 5 = number of congeneric species; 6 = residual number of congeneric species (number of congeneric species regressed on male field masses obtained for this study); 7 = male body mass; 8 = female body mass; 9 = average body mass; 10 = residual of male body mass (field masses obtained for this study regressed on midpoint of elevational occurrence); 11 = residual of female body mass; 12 = residual of average body mass; 13 = basal metabolic rate; 14 = residual of basal metabolic rate (metabolic rates regressed on corresponding masses from Bennett and Harvey 1987); 15 = mass-specific basal metabolic rate (based on corresponding masses from Bennett and Harvey 1987). Residual of mass-specific basal metabolic rate (mass-specific metabolic rates regressed on corresponding masses from Bennett and Harvey 1987) equivalent to 14 above. Results for number of congeneric species are qualitatively similar if based on analysis of one or both *Coeligena* species.

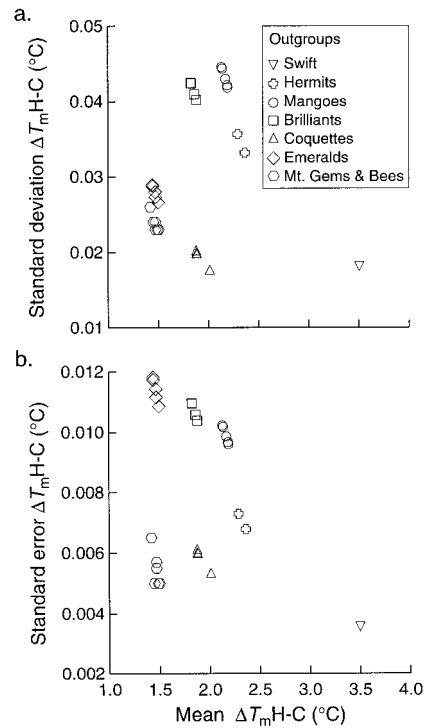


FIG. 4.—Scatter plot of standard deviation (a) and standard error (b) for \log_e genetic distance (ΔT_mH-C) for all outgroups, identified by the named clade to which they belong (see text for discussion).

times were lower than those for raw values, but even these conservative estimates remain highly significant over a broad range of outgroups. With few exceptions (always involving more terminal outgroups), correlations between measures of generation time or species diversity and path lengths were nonsignificant (and inconsistent in sign). For hermits, the much larger *Eutoxeres aquila* was evolving more slowly than *Threnetes ruckeri* (sign tests for ranked path lengths from each of the 24 nonhermits and the swift: $x = 0$, $n = 24$, $P \ll 0.0001$), though *E. aquila* also has a longer generation time and fewer (two as compared to three) congeneric species. Correlations between BMR (available for nonhermits only) and path lengths were inconsistent, bordering on (BMR, mass-specific BMR: one-tailed $P < 0.10$; Pearson) or attaining (mass-specific BMR only: two-tailed $P < 0.05$; Spearman) a significant positive correlation for some measures of metabolic rate (fig. 3).

The separate ANCOVAs subsuming each predictor variable (as covariate) and outgroup (as main effect) within a named clade (fig. 5; using either emeralds or mountain gems and bees as most terminal named clade of outgroups) indicate that path lengths are significantly associated with all measures of body mass (negative) and with mass-specific BMR (positive), but with no measures of generation time, species diversity, or mass-independent BMR (when calculations are possible; see fig. 3).

The significant mean effect of named clades no doubt reflects the appreciable shortening of distances from outgroups to ingroups imposed by the branching

Table 5
Cochran-Mantel-Haenszel Chi-Square Test of Linear Trends in Figure 3 with Mountain Gem and Bee Clade as Ingroup Only

PREDICTOR	N	df	PEARSON		SPEARMAN	
			χ^2	P	χ^2	P
Demography^a						
Linear trend ^b	126	1	10.732^d	0.001	3.099	0.078
Mean row difference ^c		20	23.145	0.282	41.477	0.003
Body mass						
Linear trend	126	1	6.074	0.014	72.877	0.000
Mean row difference		20	77.273	0.000	107.607	0.000
Basal metabolism						
Linear trend	63	1	0.349	0.555	0.000	1.000
Mean row difference		20	4.438	1.000	0.000	1.000

^a Cell values (see text) were combined across each measure of the general class (six for demography [four for generation time, two for species diversity], six for body mass, and three for basal metabolism) of predictor variable.

^b Tests for linear association between the predictor (row) and outgroup (column) variables. Results are similar with mountain gems and bees as terminal outgroups. Cells with too few observations to calculate a correlation are scored as nonsignificant.

^c Test of significant heterogeneity in frequency of nonsignificant and significant correlations.

^d Bold values significant at indicated P value.

hierarchy of named clades within the phylogeny. The significant mean effect of a specific outgroup within a named clade is more difficult to interpret, because differences in mean distance to the same set of ingroups within each named clade can arise in two ways. One way is that different outgroup taxa may be evolving at different rates, which would affect all distances measured from that outgroup to the same set of ingroups. The other way is that experimental errors associated with extracting the DNA and labeling it with radioactive iodine may cause differences in melting temperatures among outgroup DNAs. As there is no replication of labels within outgroup species, such experimental effects cannot be separated by my analytical design. However, the lack of significant interaction between outgroup species and predictor variable indicates that the functional relationships between path lengths and predictor variables are very similar across outgroups within each named clade despite possible differences in label melting temperatures.

Discussion

The results of this study suggest that relative-rate tests based on path length distances give consistent re-

sults as long as outgroups capture a large number of ingroups. Over the range of distances and rates measured here, limitations imposed by autocorrelation and/or saturation effects seem less important, although comparisons more distant than those measured from the swift might reveal such effects. Given that relevant aspects of the biology of hummingbirds differ greatly from those of other vertebrates, I first discuss associations between relative rates and predictor variables in light of what is known about hummingbird biology, and then compare the results with those obtained for other organisms.

Predictor Variables

The close parallel that often exists between generation time, metabolic rates, and body mass has made the task of separating their effects on molecular evolution difficult. Exceptions to their covariation have been noted in sharks, birds, and certain insects, where body mass and molecular evolutionary rate covary in the absence of corresponding differences in generation time (Martin, Naylor, and Palumbi 1992; Martin and Palumbi 1993; Rand 1994; Krajewski and King 1996). It is tempting to dismiss generation time as a cause of variation in rates of molecular evolution among hummingbirds simply because all species studied to date appear to breed in their first year (Bent 1940; Skutch 1972; Johnsgard 1983). However, even when such variation as may be present is considered, differences in generation time appear to be inconsequential.

A consideration of the extremes for generation time in the family also fails to support the hypothesis. For example, the tiny thornail (*Popelairia conversii*) and bee (*Archilochus colubris* and relatives) hummingbirds (table 2 and fig. 2) probably have the longest generation times among trochilids as a consequence of their long-distance or elevational migrations, which absent them from the breeding grounds for most of the year (Johnsgard 1983; Hilty and Brown 1986). Indeed, males of these species adopt eclipse plumages that lack iridescent

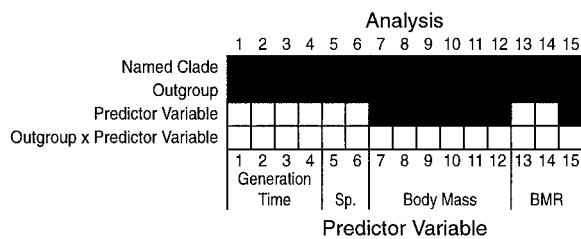


FIG. 5.—Schematic representation of results of nested ANCOVA for each row in figure 3. Notation is as in figure 3 (with black indicating two-tailed significance at $P < 0.05$), with emeralds as the terminal outgroup. Results are similar with mountain gems and bees as terminal outgroups except that small ingroup sample size prevented estimation of associations with metabolic rate.

patches and ornamental plumes for much of the year (Johnsgard 1983; personal observations), again suggesting that they breed only for a few months over the annual cycle. Contrary to the generation-time hypothesis, however, these species express some of the fastest recorded rates of molecular evolution among the hummingbirds examined (table 2 and fig. 2). In *Oreotrochilus*, by contrast, accelerated development of adult plumage, and possibly of breeding, is associated with one of the slowest rates of molecular evolution (table 2 and fig. 2). Finally, the young of lek-mating hermit hummingbirds may begin to attend mating aggregations (and potentially breed) as soon as 3 months after fledging (Stiles and Wolf 1979), but such hermits do not demonstrate an accelerated rate of molecular evolution (table 2; Bleiweiss 1998b, 1998c).

Although I failed to detect any relationship between species diversity and rates of evolution, the actual limits of monophyletic clades comprising taxa used in this study are still largely unresolved (Bleiweiss, Kirsch, and Matheus 1997). Possibly, then, the effects of species diversity on rates of molecular evolution in hummingbirds are not adequately tested by correlating rates with numbers of congeneric species, or at least not until more realistic generic limits for hummingbirds are determined. Additionally, hermit hummingbirds are almost an order of magnitude less diverse than nonhermits (Bleiweiss 1991), and the hybridization data suggest that the hermit rate is indeed significantly slower than that of nonhermits (table 2; Bleiweiss 1998b). Nevertheless, thorntails (fig. 2; *Popelairia*) are evolving more rapidly than any other clade in the phylogeny despite a species-diversity less than half that of brilliants or bees (table 2 and fig. 2). Conversely, the brilliant clade is as diverse or more so than the bees (Bleiweiss, Kirsch, and Matheus 1997), yet its molecular rate is much slower in comparison (table 2 and fig. 2). Thus, species diversity is unlikely to explain much of the variation in rates of molecular evolution among principal hummingbird clades, even given possible changes to generic limits.

The significant negative association between rate of DNA evolution and body mass documented here is inconsistent with the body temperature hypothesis, which predicts a positive association. On the other hand, the negative association between rate and body mass and the positive association between rate and mass-specific metabolism agree with the predictions of the metabolic-rate hypothesis (table 2 and figs. 2 and 3). However, evidence for a direct association between molecular rates and mass-independent metabolic rates is weaker, perhaps reflecting the small sample size and high levels of experimental error for the metabolic data (Bennett and Harvey 1987). Given these considerations and the close relationship between metabolism and body mass, the latter may be a more accurate predictor of the effect of metabolic rate on genomic evolution than are currently available experimental values of basal metabolic rate (Mooers and Harvey 1994).

Implications for Comparative Studies

Taken together, my results suggest that metabolic factors are important correlates, if not actual determinants of variation, in rates of molecular evolution in hummingbirds. These results differ from those obtained in several previous studies, including studies of birds, which have failed to support the metabolic-rate hypothesis (Adachi, Cao, and Hasegawa 1993; Mindell et al. 1996) or have supported body temperature (Mindell et al. 1996), generation time (Prager et al. 1974; Britten 1986; Sibley and Ahlquist 1990; Mooers and Harvey 1994), speciation (Mindell, Sites, and Grauer 1989; Barraclough, Harvey, and Nee 1996), or some combination of these other factors. Moreover, evidence for associations between metabolic and molecular evolutionary rates has been obtained largely for the DNA of mitochondria, whose rates of genomic evolution can be linked directly to their function in oxidative metabolism (Martin and Palumbi 1993). By contrast, DNA hybridization distances reflect mostly change in the single-copy nuclear DNA fraction.

The results for hummingbirds may reflect the exceptional biologies of these birds. Thus, hummingbird metabolism may be of sufficient magnitude (Suarez et al. 1991) to alter substitution rates even in the nuclear germ line. Moreover, hummingbirds appear to be exceptions to the general pattern of smaller-bodied species having shorter generation times; the relatively long generation times in small-bodied hummingbirds are probably caused by interspecific dominance patterns that force small-bodied species to emigrate to or from breeding habitat when nectar-based competition with larger hummingbirds increases. Finally, general constraints on hummingbird life history also may reduce variation in generation time and other demographic factors such that their effects on molecular evolution are obscured by the much larger effects of body mass. An appreciation that different mechanisms control rates of molecular evolution at different steps in the process of transcribing DNA into a functional protein also may help reconcile otherwise contradictory results across studies. For example, the body temperature hypothesis concerns functional constraints governing the effects of nonsynonymous substitutions, whereas the majority of substitutions measured by DNA hybridization data are probably synonymous.

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APPENDIX

Data for Predictor Variables Used in Analyses

GENUS AND SPECIES	GENERATION TIMES ^a		SPECIES		ELEVATION ^b		FIELD MASS ^c		METABOLISM ^d	
	dbs	bs	mig	congen	min	max	M (n)	F (n)	B&H	BMR
<i>Lampornis clemenciae</i>	4	3	2	6	300	3,900	7.56 (8)	6.00 (1)	7.9	2.1
<i>Thalurania colombica</i>	5	3	2	6	0	1,900	4.68 (8)	3.83 (6)		
<i>Agelaiocercus coelestis</i>	12	1	1	2	300	2,100	5.94 (6)	4.33 (3)		
<i>Coeligena wilsoni</i>				10	700	2,000	7.18 (4)	6.38 (4)		
<i>Eriocnemis luciani</i>			2	10	2,800	4,800	6.30 (3)	5.90 (4)		
<i>Eulampis holosericeus</i>	5	3	1	2	0	600	6.01 (7)	5.30 (2)	8.4	3.2
<i>Colibri coruscans</i>	12	1	2	4	600	3,600	8.83 (10)	7.03 (7)		
<i>Eugenes fulgens</i>	9	3	4	1	900	3,300	7.10 (6)		6.6	2.1
<i>Acestrura mulsant</i>				5	1,500	2,800	3.75 (11)	4.05 (8)	3.3	1.6
<i>Archilochus colubris</i>	4	3	4	2	0	2,400	2.64 (8)	3.12 (2)	3.2	1.6
<i>Myrtis fanny</i>	12			1	1,200	2,800	3.18 (5)	3.40 (1)		
<i>Philodice mitchellii</i>				3	0	1,900	2.98 (2)	3.15 (1)		
<i>Amazilia tzacatl</i>	12	1	1	30	0	1,850	5.64 (8)	4.80 (4)		
<i>Campylopterus villaciscencio</i>				11	400	1,500	8.40 (1)	6.10 (2)		
<i>Androdon aequatorialis</i>				1	0	1,590	7.60 (3)	6.68 (6)		
<i>Lesbia victoriae</i>	9	3		2	2,600	4,000	4.99 (4)	4.25 (2)		
<i>Doryfera ludovicae</i>	7	3	1	2	900	2,700	6.81 (7)	6.70 (3)		
<i>Oreotrochilus chimborazo</i>	7	2	1	5	3,500	5,300	8.58 (10)	7.42 (4)	8.4	4.0
<i>Heliodoxa jacula</i>	7	3	1	9	500	2,300	8.83 (3)	8.20 (2)		
<i>Heliophryx barotti</i>	10	3	1	2	0	1,830	5.13 (3)			
<i>Chlorostilbon mellisugus</i>	8	3		13	0	2,200	3.05 (14)	3.18 (3)	2.9	3.0
<i>Coeligena torquata</i>	9	3	2	10	1,500	3,000	8.14 (8)	7.20 (2)		
<i>Popelairia conversii</i>	6	3	3	4	0	1,400	2.79 (7)	2.68 (6)		
<i>Orthorhyncus cristatus</i>	12	1	1	1	0	650	2.77 (3)	3.00 (1)	2.9	1.9
<i>Threnetes ruckeri</i>	5	3	1	3	0	1,050	6.56 (7)	6.39 (9)		
<i>Eutoxeres aquila</i>	6	3	2	2	0	2,100	10.55 (4)	9.36 (5)		

NOTE.—In two instances, data on predictor variables were available only for a congener of the species included in my study (generation time for *Oreotrochilus [estella]*, not *chimborazo*) and BMR for *Eulampis [jugularis]*, not *holosericeus*). As those species are close relatives to the species in my study, any inaccuracies in these data may be minimal, and results of the analyses remained qualitatively similar whether or not the particular species was excluded from an analysis. I present the more inclusive analyses.

ABBREVIATIONS: dbs = duration of breeding season (in months; see text); bs = breeding seasonality (1 = continuous; 2 = accelerated [*Oreotrochilus*]; see text); 3 = seasonal); mig = migratory behavior (1 = sedentary; 2 = elevational migrant; 3 = pronounced elevational migrant; 4 = long-distance migrant); congen = number of congeneric species; min = minimum elevation (m); max = maximum elevation (m); field mass (g): M = average male mass; F = average female mass; B&H = mass (g) from Bennett and Harvey (1987); BMR = basal metabolic rate (resting metabolic rate; from Bennett and Harvey 1987).

^a Literature sources: Carpenter (1976), Hilty and Brown (1986), Johnsgard (1993).

^b Literature sources as footnote a and data compiled in Bleiweiss (1991).

^c Unpublished field weights.

^d Literature source: Bennett and Harvey (1987).

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